Isolation and sequencing of the cDNA encoding α-actinin 3 in rat skeletal muscle

Thesis

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ABSTRACT

Alpha-actinins are a family of proteins that have been characterized as cross-linkers of filamentous actin in eukaryotes. Their structure is marked by an actin-binding region followed by four spectrin-like repeats which in turn is followed by two EF-hand regions that may or may not bind calcium depending on the isoform. Alpha-actinin 3 (AA3) is a calcium insensitive isoform that is found only in skeletal muscle. Isolation of the cDNA that potentially encodes this protein in rat skeletal muscle was carried out using the Uni-ZAP XR cDNA library and immunoscreening with an anti-sarcomeric-α-actinin antibody. A phagemid containing this cDNA was transfected into *E. coli* and isolated. Primers designed against the multiple cloning site allowed for partial sequencing of the 5' and 3' ends of the insert. Partial sequences facilitated the designing of new primers to eventually bridge the two ends of the cDNA, and to determine a contiguous sequence of the insert using Sequencher software. The cDNA sequence shows strong identity to the AA3s found in mouse and human. The inferred protein sequence also showed 99.32% identity to AA3 in mouse and 96.00% to that of human. Each of the regions contained within the protein individually show strong identity with AA3 in mouse and human implying similar function and structure.

INTRODUCTION

The objective of this project was to isolate and sequence the cDNA encoding the protein (sarcomeric) α-actinin 3 from rat skeletal muscle. The α-actinins (AA) are a family of proteins all of whose isoforms have been shown to cross-link filamentous actin (Beggs *et al.*, 1992). Four distinct isoforms of this protein have been characterized (α-actinins 1-4) in mammals and many more isoforms may potentially exist (Beggs *et al.*, 1992; El-Husseini *et al.*, 2000). AA3 has been shown to be contained within the skeletal muscle while AA2 has been found in cardiac as well as skeletal muscle (Beggs *et al*, 1992). AA1 and 4 are primarily non-muscle isoforms (Beggs *et al.*, 1992; El-Husseini *et al.*, 2000). In muscle, α-actinins have been found along the Z-line and in cardiac muscle along the intercalated disks. Non-muscle isoforms have α-actinin located near adherens junctions (Beggs *et al.*, 1992; Endo and Masaki, 1984).

The structure of these proteins is that of a homodimer with each of its composite monomers oriented in opposing directions. The composition of each of the monomers is marked by an actin-binding domain containing two calponin homology repeats near the amino-terminal end followed by several spectrin-like repeats and terminating with two EF-hand domains near the carboxyl-terminal end (Vancompernolle *et al.*, 1990; Viel, 1999; Beggs *et al.*, 1992). This basic structure is conserved within this family of proteins. Although AA3 has been described and catalogued for mouse and human (Birkenmeier *et al.*, 1998; Beggs *et al.*, 1992), rat AA3 has not been described Nevertheless, we hypothesized rat would express this isoform and that its isolation and

characterization would facilitate the characterization of another possible α-actinin isoform called the G3.5 antigen. The G3.5 antigen is an actin-binding protein that is similar in size to known α-actinins and contains sequence homologies with one of the calponin homology regions and two of the spectrin-like repeats (Bolanos *et al.*, 1998).

The actin-binding region of α-actinin is approximately 250 amino acid residues in length and is marked by two calponin homology tandem repeats. These repeats are each similar in sequence to portions of the protein calponin, but each align with differing sections of calponin and thus contain different sequences (Vancompernolle *et al.*, 1990) Each of these repeats are capable of binding weakly to filamentous actin; however, full functionality of this region is only accomplished with the presence of both calponin homology repeats (Gimona and Mital, 1998; Winder *et al.*, 1995). The EF-hand regions are traditionally known for their ability to bind calcium (Kretsinger, 1980). These regions have been shown to actively bind calcium in the non-muscle isoform α-actinin 1, but to not bind calcium in the non-muscle isoform α-actinin 4 or the muscle isoforms α-actinins 2 and 3 (Beggs *et al.*, 1992; El-Husseini *et al.*, 2000). This is most practical to desensitize the cytoskeleton to changing concentrations of calcium (El-Husseini *et al.*, 2000).

The four spectrin-like repeats are similar to the calponin homology repeats in that they each contain differing sequences The differences in these sequences allow the formation of the homodimer in α-actinins (Beggs *et al.*, 1992; Viel, 1999). When the monomers are oriented 180° with respect to each other, the helix formed by the first spectrin-like repeat associates with the helix of the fourth spectrin-like repeat on the opposing monomer. The second and third repeats will likewise align in a similar fashion

(Viel, 1999). While the presence of these spectrin-like repeats is conserved among the isoforms of α-actinin, the actual sequences are not conserved among all of the isoforms. It is possible that this is to prevent the formation of heterodimers arising from two separate isoforms.

The isolation of the cDNA encoding rat AA3 was attempted using a cDNA library. A cDNA library uses DNA that has been transcribed using messenger RNA (mRNA) as a template. A cDNA library should theoretically contain the coding sequences for all of the proteins being produced in the tissue of origin. Therefore, a cDNA library derived from skeletal muscle should theoretically contain the coding sequence for AA3. The cDNA library used in this experiment originated from 12-week old Wistar rat skeletal muscle that has been cloned unidirectionally into the λ phage Uni-ZAP® XR vector from Stratagene (La Jolla, CA). This library contains cDNA of an average size of 1.0 kilobase inserted into a pBluescript® SK phagemid that has been double digested with the restriction enzymes *Eco*R1 and *Xho*1 A phagemid is a vector that contains replication origins of both bacteriophage and plasmid, which allow it to be replicated during bacterial replication as well as during the lytic cycle of the phage. The cDNA inserts are cloned into the multiple cloning site. Double digestion of the phagemid allows for the introduction of the cDNA with a lower probability of re-circularization of the phagemid without the insert and increases the rate of recombination. An f1 helper phage and two bacterial hosts are included with the cDNA library from Stratagene (La Jolla, CA). The helper phage is called the ExAssist phage. The Uni-ZAP® XR vector requires co-infection with an f1 helper phage to supply the proteins needed for excision

of the phagemid. The two *Escherichia coli* hosts are designated as SOLR and XL1-Blue MRF'.

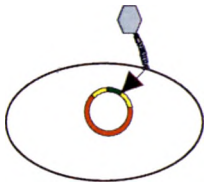
The pBluescript® phagemid contains a coding sequence that will confer resistance to ampicillin to recombinant hosts containing the excised phagemid to aid in their isolation. Also contained within the phagemid is the *lacZ* gene that in the presence of isopropyl-1-thio-β-D-galactopryanoside (IPTG) will encode for the enzyme β-galactosidase. However, if cDNA has been inserted into the multiple cloning site just upstream of the *lacZ* gene but within the *lac* operon, the cDNA insert will be transcribed instead and the product translated into protein (see Stratagene product documentation). When culture plates are overlaid with nitrocellulose containing IPTG all proteins represented in the cDNA library, including the target protein, will be produced and deposited on the membrane upon lysis of the bacterial cell. This membrane may then be subjected to a western blot using a sarcomeric α-actinin antibody as a probe. The phages contained within the plaque that produces the target protein can be used to transfect the XL1-BLUE MRF' strain in the presence of the Ex-Assist phage This will allow the phagemid to be excised and packaged into an f1 phage capsid. This new phage can then be used to transfect the SOLR strain. The phagemid may then be isolated from this strain and sequenced. Specific primers for the inserted cDNA regions are not needed since it is still contained within the pBluescript® phagemid Because the cDNA is inserted into the center of the multiple cloning site, it retains portions of the polylinker of the multiple cloning site at both ends of the insert. In this case, after double digestion with *Eco*R1 and *Xho* 1, the sequences to either side of the insert are known and can thus be used to design primers.

For my thesis research, I have successfully applied the approach described above to isolate and sequence the cDNA for rat α-actinin 3. This research shows that α-actinin 3 is expressed in rat skeletal muscle as well as how it compares with other known isoforms. The features of the inferred amino acid sequence and its relationship to other α-actinins are discussed.

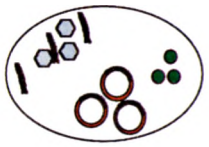
METHODS

A. Immunoscreening and Plaque Lifts

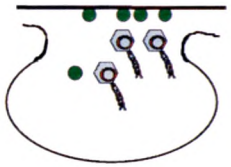
An diagrammatic overview of the methods can be viewed in Figure 1. The Uni-ZAP® XR cDNA library (Stratagene, La Jolla, CA) was used to transfect the XL1-Blue MRF' strain for immunoscreening as per the accompanying instructions. The XL1-Blue MRF' strain was used to inoculate 50ml of Luria-Bertani (LB) broth (1% NaCl, 1% tryptone, 0.5% yeast extract at pH = 7.0) supplemented with 10mM $MgSO_4$ and 0.2% maltose. The culture was grown overnight at 30°C in a shaker bath to late log phase. A lower temperature of 30°C versus 37°C prevents overgrowth during overnight incubation. The culture was transferred to a 50ml centrifuge tube placed in a Sorvall F28/50 rotor and spun at 2,000rpm for 10 minutes in a Sorvall RC28S centrifuge. The supernatant was discarded and the pellet was gently resuspended in 10mM $MgSO_4$ to an OD_{600} = 0.5. XL1-Blue MRF' (600µl, OD_{600} = 0.5) and 0.5µl of Uni-ZAP® XR (diluted 1:100 in 10mM $MgSO_4$) were mixed in each of six 50ml centrifuge tubes. This mixture was allowed to incubate for 15 min at 37°C to allow the phage to attach to the host. NZY top agar (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_20$, 0.5% yeast extract, 1% NZ amine, 1.5% agarose at pH = 7.5) was autoclaved and cooled to ~ 48°C and 6.5ml was added to the 50ml centrifuge tube containing the bacteria/phage mixture. This mixture was then layered over the surface of warm (37°C) NZY agar (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_20$, 0.5% yeast extract, 1% NZ amine, 1.5% agar at pH = 7.5) in numbered 15cm glass petri dishes



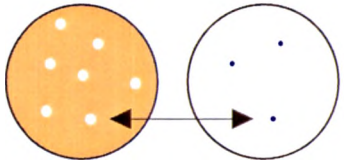
1. Infection of the XL1-Blue MRF' strain with the Uni-ZAP XR λ-phage



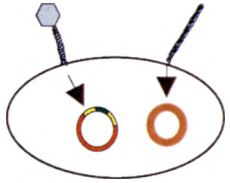
2. Translation of the target protein during lytic phase



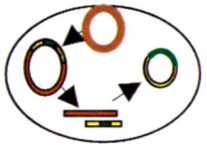
3. Protein adheres to nitrocellulose membrane upon cell lysis.



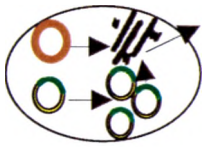
4. Identification of plaques containing protein via western blot



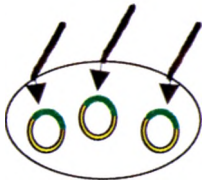
5. Co-infection of XL1-Blue MRF' with Uni-ZAP XR and Ex-Assist



6. Ex-Assist produces restriction enzymes to excise the phagemid from the λ-phage genome.

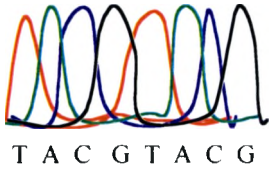


7. Phagemid is packaged into filamentous phage capsid.



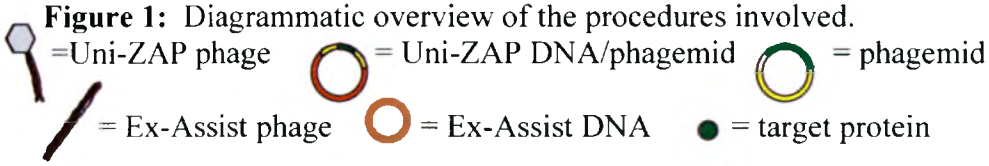
8. New filamentous phage used to transfect SOLR strain

9. Transfected SOLR cultured and phagemid harvested.



10. Use primers designed against the MCS to initiate sequencing. Use returned sequences to design more primers to complete sequence.

Figure 1: Diagrammatic overview of the procedures involved.



and allowed to harden for 15 minutes at room temperature. The plates were then inverted and allowed to incubate at 42°C (optimal temperature for accelerated plaque formation) for 8 hours until plaques began to form. Six nitrocellulose (Bio-Rad, Hercules, CA) membranes were prepared by immersion in 10mM IPTG and air-dried. These membranes were numbered 1-6 at the edge with waterproof ink and overlaid onto the corresponding agar plate. Air bubbles were removed with a sterile glass rod. The plates were then allowed to incubate at 37°C for an additional 3.5 hours. The plates were then sealed with parafilm and stored at 4°C for 3 hours.

The membranes were then marked for orientation by coating a needle with waterproof ink and puncturing the membrane and agar once on the left side and twice on the right side. The membranes were carefully removed and washed 2x15 minutes in TBST [TBS (20mM Tris-HCl pH=7.5, 150mM NaCl) plus 0.05% Tween 20]. Residual top agar was removed carefully with a gloved hand to avoid introducing additional proteins to the membrane. Membranes were placed in blocking solution (1% BSA, 0.02% sodium azide, in TBS) for 1 hour at room temperature on a single speed rocker plate. Membranes were washed 2x15 minutes in TBST. Membranes were then incubated for 90 minutes at room temperature on a rocking plate in mouse anti-rabbit-α-actinin (sarcomeric) IgG antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:1,300 in blocking solution. Membranes were washed 3x15minutes in TBST. Membranes were then allowed to incubate for 90 min at room temperature on a rocking plate in goat anti-mouse polyvalent antibody conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) diluted 1:4,000 in blocking solution. Membranes were washed 2x15 min in TBST and then 2x15 min in TBS. Each membrane was developed in 10ml color

development solution (100mM Tris-HCl pH=9.5, 100mM NaCl, 5mM $MgCl_2$) containing 33µl 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St Louis, MO; 50 mg/ml in dimethyl formamide) and 66µl nitroblue tetrazolium (Sigma, St Louis, MO; 50 mg/ml in 70% dimethyl formamide). Once development was complete, typically in less than 5 minutes, the membranes were washed in TBS for at least 30 seconds and then stop solution (20mM Tris-HCl pH=2.9, 1mM EDTA) for at least 30 seconds.

Ten positive locations seen on a nitrocellulose membrane labeled and the membrane was aligned with the agar plate. Eight of the locations were aligned with their corresponding plaque. These eight positive plaques were cored using a sterile 1ml disposable pipette tip. The tip was used to penetrate the agar and light suction was used to remove the agar and plaque into the pipet tip. Loaded into the pipette tip above the agar core was 500µl of SM buffer (100mM NaCl, 50mM Tris-HCl pH=7 5, 10mM $MgSO_4$). The agar containing the plaque was flushed from the pipet tip into a 1.5ml microcentrifuge tube and 20µl of chloroform was added to each tube. The tubes were vortexed for ten seconds and allowed to incubate overnight at 4°C.

B. Secondary Screen and Phagemid Excision

The XL1-Blue MRF' bacteria were grown and prepared as described previously to a dilution of $OD_{600} = 0.5$. Serial dilutions of 10^{-2}, 10^{-3}, and 10^{-4} were prepared in 1.5ml centrifuge tubes using phage stock from cored plaques 2-6 and 8-10. One microliter of each of the dilutions was added to 200µl of the prepared bacterial stock in a 15ml centrifuge tube. This mixture was allowed to incubate for 15 minutes at 37°C to allow the phage to attach to the host. NZY top agar, melted and cooled to ~48°C, was added to bring the mixture to a total volume of 3ml. The mixture was poured onto 10cm NZY

agar plates, swirled to allow homogenous distribution, and allowed to harden. The plates were inverted and allowed to incubate overnight at 37°C. The number of plaques per plate was counted, and the phage titer was calculated. This calculation dictated the amounts of phage stock to be used in subsequent infections.

The XL1-Blue MRF' bacteria were grown and prepared as described previously to a dilution of $OD_{600} = 0.5$. In each of four 15ml centrifuge tubes was added 200µl of the prepared bacterial stock. The tubes were numbered 3, 4, 6, and 9. To its respective tube was added 0.18µl from phage stock 3, 0.12µl from phage stock 4, 0.12µl from phage stock 6, and 0.22µl from phage stock 9. The samples were plated, overlaid with nitrocellulose and immunoscreened as described above. Four positive plaques were cored and numbered 3, 4, 6-1 and 6-2.

The XL1-Blue MRF' bacteria were grown and prepared as described above to a dilution of $OD_{600} = 1.0$. To each of four 50ml centrifuge tube was added 200µl of the prepared bacterial stock. The tubes were numbered 3, 4, 6-1 and 6-2. To its corresponding tube was added 250µl of the phage stock. To each tube was also added 1µl of ExAssist phage. This mixture was allowed to incubate at 37°C for 15 minutes. To each tube was added 3ml of LB broth. This was allowed to incubate for 3 hours at 37°C. The tubes were then heated to 70°C for 15 minutes and subsequently centrifuged at 1000x*g* for 15 minutes. The supernatants were then decanted into 15ml centrifuge tubes and stored at 4°C.

The SOLR bacteria was grown in 50ml LB broth supplemented with 0.2% maltose and 10mM $MgSO_4$ for four hours at 37°C in a shaker bath. The cultures were then spun at 1000x*g* and the supernatant was discarded. The pellet was resuspended in

$MgSO_4$ to an OD_{600} = 1.0. Eight 1.5ml centrifuge tubes were labeled 3, 3', 4, 4', 6-1, 6-1', 6-2, and 6-2'. Into each tube was added 200µl of prepared SOLR bacteria. Into tubes 3 and 3' were added 100µl of phage stock 3. Phage stock was added to the remainder of tubes in a similar fashion. The tubes were allowed to incubate for 15 minutes at 37°C. Eight warm LB agar (1% NaCl, 1% tryptone, 0.5% yeast extract, 2.0% agar at pH = 7.0) plates were prepared with 10µl X-gal (250mg/ml in dimethyl formamide), 40µl ampicillin (50mg/ml in nanopure H_2O), and 4µl of 0.5M IPTG (in nanopure H_2O) spread across the surface with a sterile glass rod. Plates were numbered in a similar fashion as the samples, and 200µl of each sample was spread atop the corresponding plate with a glass rod. Plates were allowed to incubate overnight at 37°C Transfected cells were able to grow within the zone of inhibition created by the ampicillin, and recombinants produced white colonies.

C. Phagemid Recovery

The phagemid was isolated from the cells using a Quantum Prep™ Plasmid Midiprep Kit (Bio-Rad, Hercules, CA) as per the accompanying instructions. A colony from plate 6-2' was used to inoculate 50ml LB broth supplemented with 100µl of ampicillin (50 mg/ml) and grown for 15 hours at 37°C in a shaker bath. The culture was transferred to a 50ml centrifuge tube and spun at 3000x*g* for 5 minutes. The supernatant was discarded. The pellet was resuspended in 5ml of Cell Resuspension Solution and vortexed. Cells were lysed by adding 5ml of Cell Lysis Solution followed by inversion of the tubes 6-8 times. Neutralization Solution (5ml) was added followed by inversion of the tubes 6-8 times. The tube was then centrifuged at 7,500x*g* for 10 minutes. The supernatant was decanted into a fresh 50ml tube and was carefully mixed with 1ml of

Quantum Prep matrix. The tube was then centrifuged at 7,500x*g* for 2 minutes and the supernatant was discarded. The pellet was resuspended in 10ml wash buffer with shaking. The tube was centrifuged at 7,500x*g* for 2 minutes and the supernatant discarded. The pellet was resuspended in 600µl wash buffer and transferred into a spin column in a collection tube. The column was centrifuged for 30 seconds at 14,000x*g* and its flow-through discarded. Another 500µl wash buffer was added to the spin column followed by centrifugation at 14,000x*g* for 30 seconds. The flow-through was discarded and the spin column was spun for 2 minutes at 16,000x*g* to remove any residual buffer. The spin column was placed in a fresh collection tube. The column was filled with 600µl of H_2O and centrifuged at 14,000x*g* for 2 minutes. The collection tube containing the phagemid was labeled and stored at -20°C.

D. PCR and Sequencing

The phagemid stock was run on a 0.8% agarose gel at 120V for 1 hour at 5µl of sample at full concentration and also diluted 1:1000 (in nanopure, sterile H_2O). The gel was then stained in ethidium bromide (1.5mg/ml diluted 1:200 in H_2O) and viewed under ultraviolet light. A picture was obtained (Figure 2) using a Nikon COOLPIX 990 digital camera with a UV filter and was processed with Adobe Photoshop version 5.5 software (Adobe Systems Inc., San Jose, CA). A polymerization chain reaction (PCR) was run using the parameters shown in Table 1. The samples were initially denatured at 94°C for 3 minutes. The samples were then cycled 40 times through denaturing at 94°C, primer annealing at 50°C, and polymerization at 72°C for 1 minute, 1 minute, and 4 minutes respectively. The samples were subjected to a final 10 minutes at 72°C and stored at 4°C. The samples were then run on a 0.8% agarose gel for 1 hour at 120V. The gel was

stained with ethidium bromide, viewed under ultraviolet light, and a picture was obtained (Figure 3) as described above.

Cycle sequencing of sample 6-2' was carried out with Applied Biosystems Inc. (ABI) cycle sequencing kits with either Big Dye or FS terminators and also using the M13-20 and T3 primers. Sequences were run on an ABI model 377 autosequencer. Sequence fragments obtained from the 5' and 3' ends of the cDNA insert were compared to known sequences in the NCBI database using a BLAST search (Altschul *et al.*, 1990). Further sequencing was carried out using ABI cycle sequencing kits with either Big-Dye or FS terminators in the amounts indicated in Table 3. Primers used for cycle sequencing are listed in Table 2 and shown aligned with the cDNA in Figure 5.

Sequences obtained were analyzed using Sequencher 4.1 software (Gene Codes Corp., Ann Arbor, MI). Sequences were aligned using *Mus musculus* α-actinin 3 as a template to view orientation of sequences. Ambiguous bases were determined once all sequences were obtained, and *Mus musculus* α-actinin 3 was removed from the alignment. The amino acid sequence was inferred using Sequencher software.

E. Comparison with Known Sequences

Comparisons with known sequences were carried out using a BLAST search with the complete nucleotide and protein sequences. The protein sequences for two calponin homology domains, four spectrin repeats, and two EF-hand calcium binding domains for mouse AA2 (Yang and North, 2000), mouse AA3 (Birkenmeier *et al.*, 1998), mouse AA4 (Dear *et al.*, 2000), rat AA1 (Schulz and Seeburg, 1998), rat AA4 (El-Husseini *et al.*, 2000), and human AA3 (Beggs *et al.*, 1992) were compared with the inferred protein sequence for the rat cDNA insert. The comparisons were calculated as percent identity

by dividing the number of common residues by the overall length of the region The inferred protein sequence was also compared to the entire protein sequence of human and mouse AA3.

	Tube 1	Tube 2	Tube 3	Tube 4
Phagemid (undilute)	0.5	0	0.5	0
Phagemid (dil 1:1000)	0	0 5	0	0.5
T3 primer	1	1	1	1
M13-20 primer	1	1	1	1
dNTPs (10mM)	1	1	1	1
10X Herculase Buffer	5	5	5	5
Herculase	0.5	0 5	0.5	0.5
Dimethylsulfoxide	0	0	0.5	0.5
H_2O	41	41	40.5	40.5

Table 1: PCR reactions for sample 6-2'. Volumes are in µl.

Primer	5'-3' sequence
T3*	AATTA ACCCT CACTA AAGGG
M13-20*	GTAAA ACGAC GGCCA GT
FWDAA	CAAGA GCCTG AGCAA GATGA T
FAA2	AAGCT AGTGT CCATC GGTGC
FAA3	CCTAT GTTTC CTGCT TCTAC C
FAA3-1	GGCAG AGAAA TACCT GGACA TCC
FAA4	CTGGT TTCGG ACATA GCCAA TGC
FAA5	CAACC CCTAC ATCAC CCTCA GTTCG C
FAA6	CAACC TCCAT TGCCC GCACC ATC
FAA7	CTACA TTACC CCAGA AGAGC TGCGG AG
RVSAA	GCCCA CAGCC TGATC TTTAT T
RAA2	AGGCT ACCAC TTGTT CAGCC
RAA2-1*	GAGGC TACAA CTTGT TCAGC
RAA4	CTGCT GCCGG GCCAG CTCCT CC
RAA5	GGCTC AGGCG CAGCT TGGTC TGC
RAA6	GCGAA GCTTG CATAG TCAAT G
RAA7	GTCCT CTTCA ATGTT CTCGA TC
RAA8	CAGCT AAGAC CCGGT TGTCC TAGAC GTT
RAA9	CCATT CTCTC TAGTG CATCC CTCCT CTTC
RAA10*	GGTGA GCAGC TGCTC CCAG

Table 2: Primers used for cycle sequencing of sample 6-2'. Asterisks denote primers designed by the author. All others designed by Jamie Dixson. Primers were obtained from Bio-Synthesis, Lewisville, TX.

Tube #	Primer	Primer amt.	Template	FS	Big-Dye	H_2O
JN17	T3	0.5	0 5	4	-	5
JN17-2	T3	0.5	0.5	4	-	5
JN18	M13-20	0.5	0.5	4	-	5
JN18-2	M13-20	0.5	0 5	4	-	5
JN19	FWDAA	0.5	0.5	4	-	5
JN20	FAA2	0.5	0.5	4	-	5
JN21	FAA3-1	0.5	0.5	4	-	5
JN22	RVSAA	0.5	0.5	4	-	5
JN23	RAA2	0.5	0.5	4	-	5
JN24	FAA4	0.5	0.5	4	-	5
JN25	FAA5	0.5	0.5	4	-	5
JN26	FAA6	0.5	0.5	4	-	5
JN27	FAA7	0.5	0.5	4	-	5
JN28	RAA4	0.5	0.5	4	-	5
JN29	RAA5	0.5	0.5	4	-	5
JN30	RAA6	0.5	0.5	4	-	5
JN31	RAA7	0.5	0.5	4	-	5
JN32	FAA3	0.5	0.5	-	3	5
JN33	FAA4	0.5	0.5	-	3	5
JN34	RAA2	0.5	0.5	-	3	5
JN35	RAA8	0.5	0.5	-	3	5
JN36	RAA9	0.5	0 5	-	3	5
JN46	RAA2-1	0.5	1	-	3	4.5
JN47	RAA10	0.5	1	-	3	4 5

Table 3: Sequencing reactions for sample 6-2'. Volumes are in µl

RESULTS

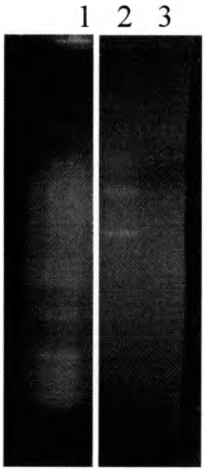
The primary screening of the cDNA library yielded ten positive locations that were labeled 1-10. Eight of these were aligned with their plaque locations. Four of these (3, 4, 6, and 9) were carried through a secondary screen. Two plaques from plate 6 and one plaque from each of plates 3 and 4 were isolated and numbered 3, 4, 6-1, and 6-2. The phagemids from these locations were excised and used to transfect the SOLR strain. A colony from plate 6-2 was isolated and labeled 6-2'. Bacteria from this colony were grown and the phagemid isolated. This phagemid can be viewed in Figure 2. This gel indicates that there was only one phagemid present in sample 6-2'. The three bands shown are characteristic of a single plasmid in linear, circular, and supercoiled configurations (Sambrook *et al.*, 1989); however, the size of the cDNA insert cannot be determined from this gel.

PCR carried out either in the presence or absence of dimethylsulfoxide yielded similar results (see Figure 3) and thus subsequent sequencing was carried out in the absence of dimethylsulfoxide. The gel in Figure 3 shows that the PCR product, including the coding and non-coding regions of the cDNA insert flanked by the multiple cloning site, has an approximate size of 2.8-3.0 kilobase. This size is roughly the same as the cDNAs for many of the α-actinin isoforms which are on the range of 2676 base pairs for the coding region of rat AA1 (Schulz and Seeburg, 1998) to 2736 base pairs for the coding region of mouse AA4 (Dear *et al.*, 2000).

Cycle sequencing of this cDNA using the T3 and M13-20 primers yielded readable fragments of approximately 450 base pairs in length. From these sequences new primers were designed. Ultimately seventeen primers were used and eighteen sequence fragments were obtained. The positions of the sequence fragments and primers relative to the consensus sequence obtained are shown in Figures 4 and 5 respectively.

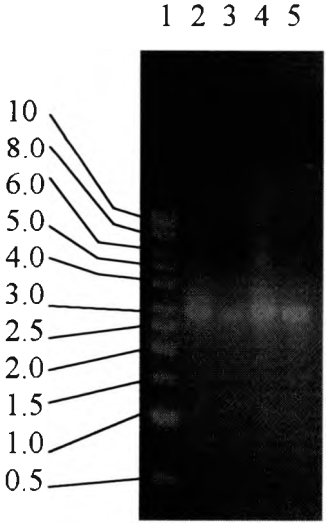
The consensus nucleotide sequence of the rat cDNA insert revealed by alignment of the contiguous fragments is shown in Figure 6. Analysis of the cDNA sequence by BLAST indicated that it was most similar to α-actinins, especially AA3 for mouse The overall sequence identity between the sequence we derived and the mouse cDNA sequence was >95%. The consensus sequence yielded an inferred amino acid sequence highly identical to α-actinin 3 for mouse and human (Birkenmeier *et al.*, 1998; Beggs *et al.*, 1992). This inferred amino acid sequence can be viewed in alignment with the entire protein sequence of mouse AA3 in Figure 7 Comparison of the inferred amino acid sequence of the cDNA insert with various mouse, rat, human isoforms can be viewed in Table 4. The overall amino acid identities are >99% for mouse AA3 and 96% for human AA3. These values are much greater than the overall amino acid identities with other rat and mouse isoforms, which are within the range of 70 to 79%. Comparisons of the inferred amino acid sequence of the cDNA insert with the domains characteristic of α-actinins for various mouse, rat, human isoforms can also be viewed in Table 4. These domains yielded identities ranging from ~99 to 100% for mouse AA3 and ~92 to 100% for human AA3. In contrast, comparison of the same regions with other rat isoforms indicated a range of ~53 to 92%, and comparisons with other mouse isoforms indicated a

range of ~53 to 96%. We therefore conclude that we have isolated the cDNA for rat AA3.



Lane 1: 1kilobase DNA Standard Ladder
Lane 2: phagemid 6-2'
Lane 3: phagemid 6-2' (diluted 1:1,000)

Figure 2: Phagemid isolated from sample 6-2'.



Lane 1: 1kilobase DNA Standard Ladder
Lane 2: 6-2'
Lane 3: 6-2' (dil 1:1,000)
Lane 4: 6-2' w/dimethylsulfoxide
Lane 5: 6-2' (dil 1:1,000) w/dimethylsulfoxide

Figure 3: PCR of Sample 6-2'. Sizes of the standard are in kilobases.

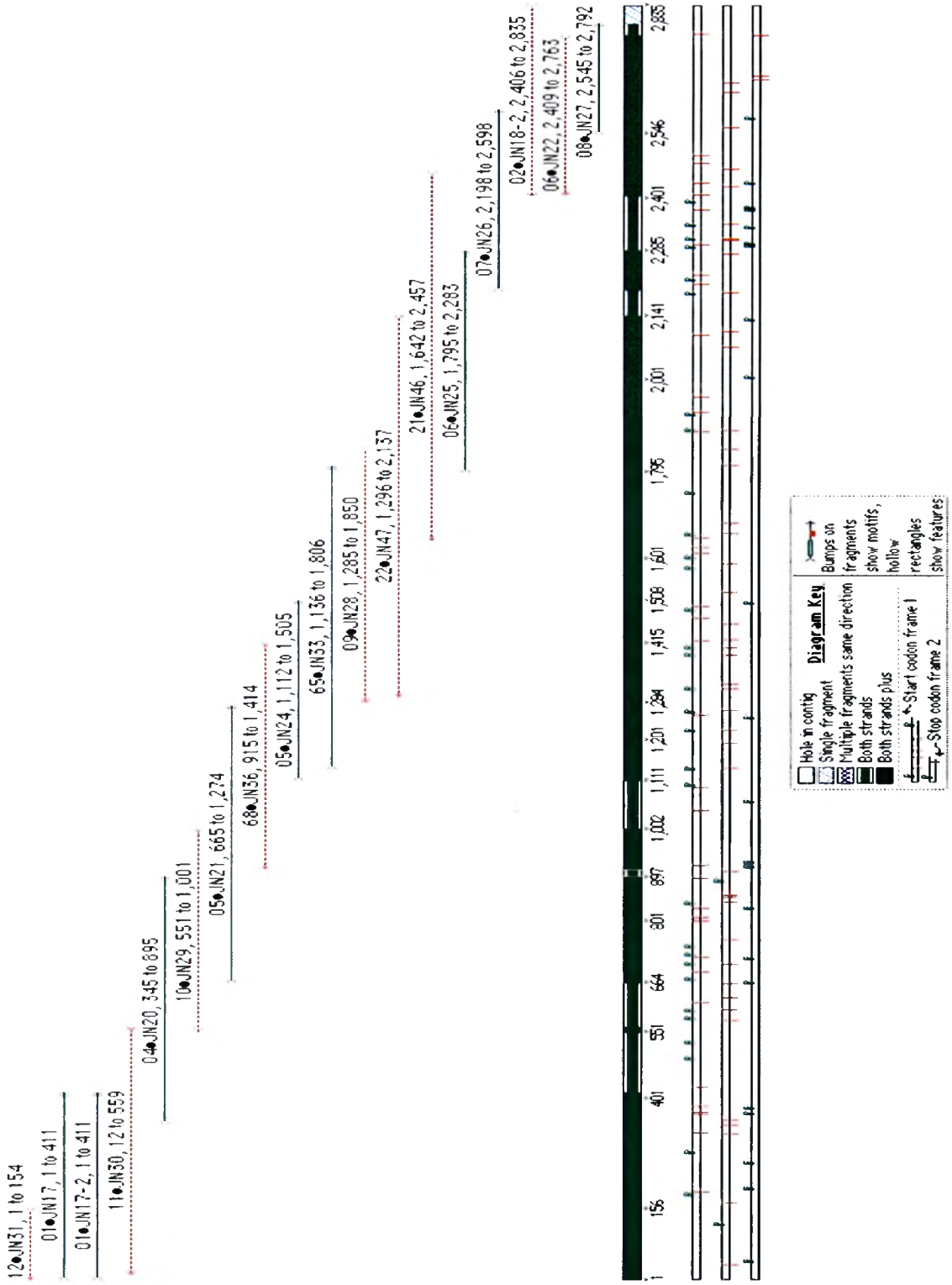


Figure 4: Contiguous sequence constructed from individual sequences of portions of sample 6-2'.

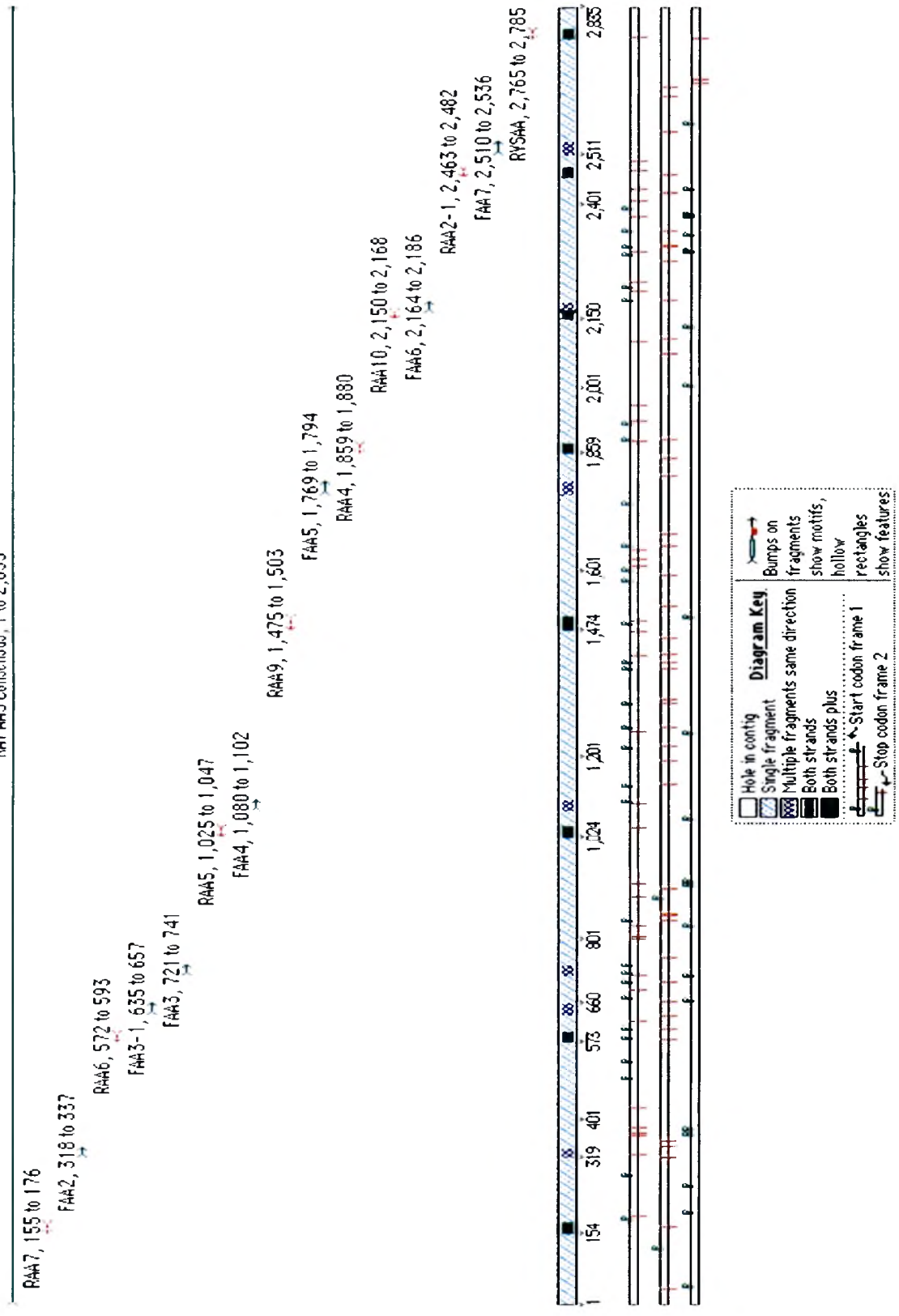


Figure 5: Positions of primers along consensus sequence. T3 and M13-20 primers are not shown as they lie outside the coding sequence.

```
1       CGGCACGAGC CTTCTCTGGT GGCGGGGGCG GTGAGTACAT GGAACAGGAG GAAGACTGGG ACCGCGACTT GCTGTTGGAT CCTGCCTGGG
        AGAAACAGCA GCGGAAAACC TTCACTGCAT GGTGCAACTC CCATCTGCGC AAGGCAGGCA CACAGATCGA GAACATTGAA GAGGACTTCC
181     GCAATGGCCT GAAGCTCATG CTGCTCCTGG AGGTCATTTC TGGAGAGAGG CTGCCCAGGC CAGACAAAGG CAAGATGCGC TTCCACAAAA
        TCGCCAATGT CAACAAAGCC CTGGACTTCA TTGCCAGCAA AGGAGTTAAG CTAGTGTCCA TCGGTGCTGA AGAAATTGTT GACGGGAACC
361     TGAAGATGAC CCTGGGCATG ATCTGGACCA TCATCCTCCG CTTTGCCATT CAGGACATCT CTGTAGAAGA GACCTCAGCC AAAGAAGGCT
        TGCTTCTCTG GTGTCAGCGG AAAACAGCAC CATACCGCAA TGTCAACGTA CAGAACTTCC ATACCAGCTG GAAGGATGGC CTGGCCCTCT
541     GTGCTCTCAT CCACCGCCAC CGGCCAGATC TCATTGACTA TGCCAAGCTT CGCAAGGATG ACCCCATTGG AAACCTGAAC ACTGCCTTTG
        AGGTGGCAGA GAAATACCTG GACATCCCTA AGATGCTGGA TGCAGAAGAT ATTGTGAACA CCCCCAAACC AGATGAGAAA GCCATCATGA
721     CCTATGTTTC CTGCTTCTAC CATGCATTTG CTGGGGCTGA GCAGGCAGAG ACTGCTGCCA ACAGGATCTG CAAGGTCTTA GCTGTGAACC
        AGGAAAACGA GAAGCTGATG GAGGAGTATG AGAAACTGGC TAGTGAGCTG TTGGAGTGGA TCCGCCGCAC TGTCCCATGG CTAGAGAACC
901     GAGTGGGTGA ACCCAGCATG AGTGCCATGC AGCGCAAGCT GGAGGACTTC CGCGACTACA GGCGCCTGCA CAAGCCTCCC CGTGTGCAGG
        AGAAGTGCCA GCTGGAGATC AACTTCAACA CGCTGCAGAC CAAGCTGCGC CTGAGCCACC GACCTGCTTT CATGCCCTCA GAGGGCAAGC
1081    TGGTTTCGGA CATAGCCAAT GCGTGGCGGG GACTGGAGCA GGTGGAGAAG GGCTATGAGG ACTGGCTGCT TTCAGAGATC AGGCGTCTGC
        AGAGGCTTCA GCACCTGGCT GAGAAGTTCC AGCAGAAGGC TTCCCTGCAT GAAGCTTGGA CCCGGGGCAA AGAGGAAATG TTAAACCAGC
1261    ATGACTACGA GTCAGCTTCG CTGCAGGAGG TGCGTGCGCT CTTGCGACGT CATGAGGCCT TTGAGAGCGA CTTGGCTGCA CATCAAGACC
        GGGTGGAACA CATTGCAGCC CTGGCCCAGG AACTCAATGA GCTGGACTAC CATGAGGCAG CCTCGGTGAA TAGCCGCTGC CAAGCCATCT
1441    GCGACCAGTG GGATAACTTG GGTACACTGA CCCAGAAGAG GAGGGATGCA CTAGAGAGAA TGGAGAAGCT CCTGGAAACC ATTGACCAGC
        TGCAGCTGGA GTTTGCTCGG CGGGCAGCGC CCTTCAACAA CTGGCTGGAT GGGGCTATTG AGGACCTGCA GGATGTGTGG CTAGTGCACT
1621    CTGTAGAAGA GACGCAGAGC CTGCTAACAG CACATGAACA GTTCAAGGCA ACGTTGCCTG AGGTGGATCG AGAGCGAGGT GCCATCCTGG
        GCATTCAAGG AGAGATTCAG AAGATCTGTC AGACGTATGG ACTGCGGCCA AAGTCTGGCA ACCCCTACAT CACCCTCAGC TCGCAGGACA
1801    TCAACAATAA GTGGGACACG GTCAGAAAGC TGGTACCCAG CCGTGACCAG ACACTGCAGG AGGAGCTGGC CTGGCAGCAG GTGAATGAGA
        GGCTCCGGCG ACAGTTTGCA GCCCAGGCCA ATGCCATAGG ACCCTGGATC CAGGGAAAGG TGGAGGAAGT AGGGCGGCTG GCAGCTGGGC
1981    TGGCTGGCTC TCTGGAGGAG CAGATGGCAG GTCTGCGACA GCAGGAGCAG AACATCATCA ATTACAAGAG CAACATCGAC CGGCTGGAGG
        GTGACCACCA GCTGCTGCAG GAGGGCCTAG TCTTTGACAA CAAGCACACG GTCTACAGCA TGGAGCACAT CCGTGTGGGC TGGGAGCAGC
2161    TGCTCACCTC CATTGCCCGC ACCATCAATG AGGTGGAGAA CCAGGTACTG ACCCGAGATG CCAAGGGCCT GAGCCAGGAA CAGCTCAACG
        AGTTCCGGGC ATCTTTCAAC CACTTTGACC GGAAGCGGAA TGGGATGATG GAACCTGATG ATTTCCGAGC TTGCCTCATC TCCATGGGCT
2341    ATGATCTGGG AGAGGTGGAG TTTGCTCGGA TCATGACCAT GGTGGACCCC AATGCAGCTG GGGTCGTGAC CTTCCAAGCC TTCATTGACT
        TCATGACCCG AGAGACTGCA GAGACAGACA CAGCTGAACA AGTTGTAGCC TCCTTCAAAA TCCTAGCAGG AGACAAGAAC TACATTACCC
2521    CAGAAGAGCT GCGGAGGGAG CTCCCAGCCG AGCAGGCTGA GTACTGCATC CGTCGGATGG CACCCTACAA AGGATCGGGG GCTCCGTCCG
        GGGCCCTGGA CTACGTGGCT TTCTCTAGTG CCCTCTACGG AGAGAGTGAC CTCTGACATT CTTAGTGGAG AAGAGGAGAG GGCACTTCCT
2701    GTGGTGGATT CCACTGTCCC TCAGAGCAAG GGCTTCAAAT CATCCAGCAC CAACCACATT TCTGAATAAA GATCGGGTTG TGGGCCTCTC
        CCAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA
```

Figure 6: Rat α-actinin 3 cDNA sequence shown 5' to 3'. Includes the poly-A tail.

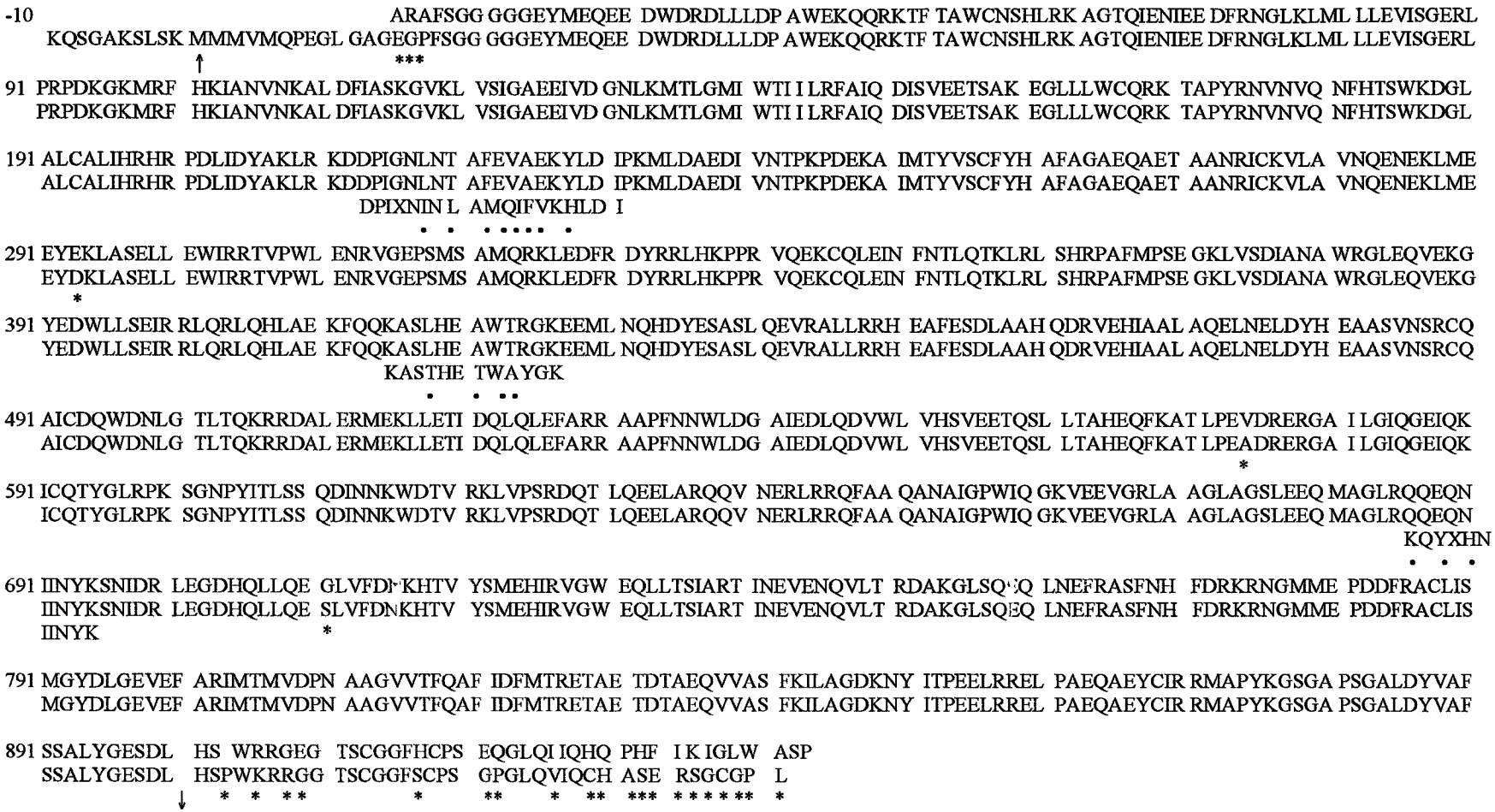


Figure 7: Comparison of the inferred amino acid sequences of rat (top) α-actinin 3, mouse (middle) α-actinin 3, and G3.5 proteolytic fragments (bottom). Asterisks are used to denote differences between rat and mouse α-actinin 3. Bullets are used to denote differences between G3.5 and rat α-actinin 3 Arrows are used to denote the position of the start and stop codons.

	Mouse AA2	Mouse AA3	Mouse AA4	Rat AA1	Rat AA4	*Homo* AA3
Calp Homol 1	96.19	100.00	92.38	92.38	90.48	100 00
Calp Homol 2	90.38	100.00	87.50	85.58	87.50	100.00
Spectrin 1	80.18	99.10	76.58	82.88	76.58	99 10
Spectrin 2	81.13	100.00	73.58	78.30	72.64	95.28
Spectrin 3	63.39	99.10	53.57	62.50	52.68	91.96
Spectrin 4	75.00	99.04	63.46	71.15	65.38	97.12
EF-hand 1	80.00	100.00	63.30	66.67	60.00	100.00
EF-hand 2	82.76	100.00	75.86	82.76	72.41	100.00
Overall	79.00*	99.32	73.00*	77 00*	70.00*	96.00*

Table 4: Comparison of α-actinin regions from differing isoforms in mouse, rat, and human to the inferred amino acid sequence of the rat cDNA. Numbers represent percent identity. *compared using BLAST search.

DISCUSSION

The purpose of this project was to determine whether α-actinin 3 is expressed in rat skeletal muscle and, if so, how its sequence compares with other α-actinins. Primary and secondary screening of the Uni-ZAP® XR yielded a phagemid with a cDNA insert that produced a protein that was labeled with an anti-α-actinin antibody. This phagemid was isolated and the cDNA sequenced. Sequencing of the cDNA insert revealed a fragment that is 2,792 base pairs in length excluding the poly-A tail and 2,662 base pairs from the beginning to the stop codon. The notable lack of start codons (ATGs) near the 5' end of the sequence implies that the entire cDNA sequence is not present. Comparison of the experimental sequence to known sequences in the NCBI database using a BLAST search showed the strongest similarity to mouse α-actinin 3 (Birkenmeier *et al.*, 1998). The mouse α-actinin 3 cDNA is 2,855 base pairs in overall length and 2,700 base pairs in length from the first start to the first stop codon. A potential size difference of 38 base pairs exists between the coding regions of these two cDNAs. The compared sequences of these two coding regions show that of 2,662 base pairs the two have 2,540 bases in common, an identity of 95.40%.

The alignment of the inferred protein sequence of the cDNA insert and mouse α-actinin 3 shows them to be strongly homologous. Of the 887 amino acid residues compared within the coding region only 6 are different. Thus the compared protein sequences for this region are 99.32% identical. Comparison of rat AA3 with human α-actinin 3 (Beggs *et al.*, 1992) shows them to have 96.00% identity. Comparisons of the

eight specific regions of the rat cDNA inferred amino acid sequence to that of various isoforms can be viewed in Table 4. The comparisons of the regions of rat AA1 (Schulz and Seeburg, 1998), rat AA4 (El-Husseini *et al.*, 2000), mouse AA2 (Yang and North, 2000), mouse AA4 (Dear *et al.*, 2000), mouse AA3, and human AA3 to the sample sequence shows that, with the exception of the two calponin homology regions, the highest identity shared for these regions is 82.88%. The calponin homology regions for these proteins range in identity from 85.58 to 96.19% The identity with mouse AA3 being greater than 99% for all regions and the identity with human AA3 being greater than 91% for all regions, with both calponin and EF-hand regions for both proteins at 100%, strongly suggests that the protein is rat AA3. I shall therefore refer to this cDNA and its associated peptide as AA3

Functionally, this protein is expected to behave in a similar fashion to that of the AA3s found in mouse and human. The 100% identities of the calponin homology regions of the rat AA3 to mouse and human AA3 suggest that this protein will bind actin in the same manner. The differences of the spectrin-like repeats of the rat AA3 from that of mouse AA3 are a single amino acid in each of the first, third, and fourth spectrin-like repeats. The substitutions made at these locations are conservative and would not be predicted to have a negative impact on the formation of an α-helix in the protein's secondary structure.

The EF-hand region of the rat AA3 is also predicted to be insensitive to calcium, not only because it is 100% identical to mouse and human AA3 but also because of its alignment to the standard-domain numbering scheme proposed by R H. Kretsinger (1980). Shown in Figure 8 is an alignment of the EF-hand regions of rat AA3 in the

numbering scheme. Positions denoted with an "n" must contain a hydrophobic amino acid. Amino acids in the X, Y, Z, or -X positions must have an oxygen on its sidechain and position –Z is usually glutamate, which can bind with two oxygens. Position 15 must be glycine and position 17 is usually isoleucine. Twelve of the sixteen positions shown in the Figure 8 must match in order for identification of the EF-hand region. Twelve positions in each of the rat AA3 sequences match the proposed scheme. A loss of one of the oxygen donators at positions X, Y, Z, -X, or –Z may cease calcium binding ability. The first EF-hand shows a loss at two of these positions and the second EF-hand shows a loss at three. These differences suggest that this protein will be calcium insensitive, which is expected of a muscle isoform of α-actinin (Noegel *et al.*, 1987, Beggs *et al.*, 1992; El-Husseini *et al.*, 2000).

Position	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
		E	n			n	n			n	X		Y		Z	G		I	–X			-Z	n				n	n			n
EF 1	N	E	F	R	A	S	F	N	H	F	D	R	K	R	N	G	M	M	E	P	D	D	F	R	A	C	L	I	S	M	G
						*							*					*									*				
EF 2	V	E	F	A	R	I	M	T	M	V	D	P	N	A	A	G	V	V	T	F	Q	A	F	I	D	F	M	T	R	E	T
															*			*				*								*	

Figure 8: Alignment of the rat AA3 EF-hand regions 1 and 2 to the standard domain numbering scheme. Asterisks indicate differences.

The difference of three amino acid residues in the N-terminus of the inferred amino acid sequence from that of AA3 in mouse and human would seem to potentially show a divergence in the AA3 across species. However, the notable absence of the start codon in the inferred amino acid sequence compromises the predictability of these residues. The cDNA codons corresponding to these three amino acid residues show little similarity to the codons encoding the amino acids at the same site in other AA3s Unpublished results by Jamie Dixson (Southwest Texas State University) of another

putative rat AA3 sequence also showed little identity with these three amino acid residues although the rest of the protein was identical save one amino acid. These differences may have potentially occurred during the construction of the library and are most likely not authentic to the sequence for rat AA3.

Our original goal in isolating the cDNA for rat AA3 was as a precursor for identifying a possible α-actinin isoform, the G3.5 antigen. Three proteolytic peptide fragments of the G3.5 antigen have been sequenced (Price *et al.*, 1991; Bolanos *et al.*, 1998). When compared to the inferred amino acid sequence of rat AA3, the three sequences align with one of the calponin homology repeats and with two of the spectrin-like repeats. The fragment that aligns with the calponin homology repeat has the amino acid sequence "DPIXNINLAMQIFVKHLDI" (Price *et al.*, 1991). This fragment aligns with "DPIGNLNTAFEVAEKYLDI" in mouse , human, and rat AA3 sequences. The G3.5 peptide fragment is a 19-mer that has 18 identified amino acids of which ten align with AA3, giving an identity of 56%. In contrast, the G3.5 fragment has a much higher identity of 80% when compared to human AA2 (Price *et al.*, 1991). The two fragments, an 11- and 12-mer, that align with the spectrin-like repeats of human AA2 show 100% identity with these regions (Bolanos *et al.*, 1998). When aligned with rat AA3, these regions show 70% and 67% respectively. This indicates that the rest of the G3.5 antigen may display more similarities to AA2. The G3.5 antigen is thought to have the ability to cross-link filamentous actin to intermediate filaments *in vivo* (Bolanos *et al.*, 1998). If this is true, then the G3.5 antigen may have the potential for existing as a heterodimer instead of a homodimer. The identity that these fragments share with AA2 might indicate

that if the G3.5 antigen is a heterodimer that one of the monomers may be similar to AA2.

With the similarities in mind, future attempts to isolate the G3.5 antigen may benefit from an oligonucleotide designed for the cDNA encoding AA2. This may allow the isolation of the cDNAs encoding both proteins. The cDNA isolated could be separated and individually sequenced. Isolation of cDNA encoding the G3.5 antigen would allow for expression of this protein to see if monomers self-associate. If not, it would be interesting to see if they would associate with AA2. Such an experiment should reveal if the G3.5 antigen is indeed a homodimer. Similar experiments to see if there is association of monomers of α-actinin isoforms of different species may also shed light on the evolution of this family of proteins.

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APPENDIX A

Supplies and Suppliers:

Item	Supplier	Category #
Uni-ZAP® XR cDNA Library	Stratagene, La Jolla, CA	937510
Trans Blot Nitrocellulose Membranes	Bio-Rad, Hercules,CA	162-0115
Quantum Prep™ Plasmid Midiprep Kit	Bio-Rad, Hercules, CA	732-6120
Mouse anti-rabbit-α-actinin IgG mAB	Sigma Chemical Co., St. Louis, MO	A-7811
Goat anti-mouse polyvalent alkaline phosphatase conjugated antibody	Sigma Chemical Co., St. Louis, MO	A-0162

APPENDIX B

Terms:

AA - α-actinin

BLAST – Basic Local Alignment Search Tool

cDNA – complimentary DNA

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

IPTG - isopropyl-1-thio-β-D-galactopryanoside

mRNA – messenger RNA

NCBI – National Center for Biotechnology Information

PCR – polymerase chain reaction

RNA - ribonucleic acid

TBS – Tris buffered saline

TBST – Tris buffered saline with Tween-20