Biochemical and Biophysical Research Communications **265,** 256–259 (1999)
Article ID bbrc.1999.1661, available online at http://www.idealibrary.com on IDEAL®

Fine Mapping and Genomic Structure of ACTN2, the Human Gene Coding for the Sarcomeric Isoform of α-Actinin-2, Expressed in Skeletal and Cardiac Muscle

N. Tiso,* M. Majetti,* F. Stanchi,† A. Rampazzo,* R. Zimbello,† A. Nava,‡ and G. A. Danieli*,[1]
Biology Department, ‡Cardiology Department, and †CRIBI Biotechnology Centre, University of Padova, Padua, Italy

Received September 27, 1999

The present paper reports on the fine mapping of the ACTN2 gene and on the reconstruction of its genomic structure. By radiation hybrid mapping, the gene was located about 912 cR from the 1p-telomere. ACTN2 was placed between the marker WI-9317 (alias D1S2421) and the marker AFMA045ZC5, within the chromosomal band 1q43. The gene was detected in YAC 955_c_12. This YAC was used as template DNA for long-distance and Alu-PCR, using a set of putative exonic primers, designed on the cDNA sequence of α-actinin-2, in order to characterize the ACTN2 intron–exon boundaries. The entire genomic structure of the gene was reconstructed. The ACTN2 gene contained 21 exons, in a segment spanning about 40 kb of genomic DNA. Only the proximal part of the gene shows a high conservation through evolution, whereas in the remaining part a divergence from the genomic organization of *C. elegans* and *D. melanogaster* was noticed. A series of intronic primers was specifically designed and produced, to amplify all the exons of ACTN2, directly from genomic DNA. This will enable mutation screening in patients affected with hereditary diseases linked to the marker CA4F/R, a polymorphism in the last intron of the α-actinin-2 gene.

Alpha actinins are members of the spectrin gene superfamily, characterized by a N-terminal actin-binding domain, a central rod composed of 4 (α-actinin) to 24 (dystrophin) repeated units, and a C-terminal domain with EF hand structures (1). In humans, four α-actinin genes were reported so far: two sarcomeric isoforms (ACTN2 and ACTN3), one non-muscle (ACTN1) and one smooth-muscle isoform (ACTN4) (1–3). It was shown that all the corresponding proteins share extensive homology and have similar molecular weights, approximately 100 kDa (4). The α-actinin-2 protein is organized as an anti-parallel dimer and localizes at the Z discs and intercalated discs, where it anchors F-actin (5). More recently, two-hybrid studies have demonstrated that α-actinin-2 possesses a titin-binding site (6) and it is able to bind the carboxyl-terminus of dystrophin (7). Moreover, α-actinin-2 is co-expressed with ACTN3 in the skeletal muscle, but it is specifically expressed in cardiac muscle. No detectable expression of ACTN2 was observed in brain, liver, kidney, and small intestine (1). The ACTN2 gene has been mapped to 1q42-q43 by FISH and the full-length cDNA sequence has been obtained (1).

The present paper reports on the fine mapping of ACTN2 gene and on the reconstruction of its genomic structure.

MATERIALS AND METHODS

RH mapping. The radiation hybrid mapping of α-actinin-2 was performed by taking advantage of a published ACTN2 intronic marker (CA4F/R, GDB: 9845346) (8). This primer pair was used to PCR amplify the Genebridge 4 Radiation Hybrid Panel (Research Genetics, Huntsville, AL) (9). Twenty-five nanograms of template DNA was used for amplification in 12.5 μl of PCR buffer (16.6 mM $(NH_4)_2SO_4$; 67 mM Tris–HCl, pH 8.3; 0.01% Tween 20; 1.5 mM MgCl2) containing 1 μM of each of the forward and reverse primers, 0.2 units of DNA polymerase (ExperTaq; Experteam, Italy) and 25 μM of each of the four dNTPs. Cycling conditions were: 1 min and 15 s at 94°C, followed by 33 cycles of 15 s at 94°C, 25 s at 60°C, 30 s at 72°C, and a final extension step for 1 min and 30 s at 72°C (PTC-225 Peltier Thermal Cycler, M. J. Research). The retention profile was processed by the RHMAPPER software program (10) at the Whitehead Institute/MIT Centre for Genome Research (Cambridge, MA) (http://www-genome.wi.mit.edu/). The cytogenetic localization of the gene was then defined according to the markers listed in the GMAP option of the Location DataBase (LDB) (11) (http://cedar.genetics.soton.ac.uk/public_html).

YAC screening. According to the RH mapping results, a subset of Whitehead Institute YAC contigs, from the chromosomal region containing the gene, were selected. YAC screening, in order to detect clones positive for the α-actinin-2 gene, was performed by PCR. The CEPH YAC clones were provided by the DIBIT Centre, S. Raffaele Hospital, Milan, Italy. Single colonies were grown for three days at 30°C in liquid YPD medium (1% yeast extract, 2% bactopeptone, 2% dextrose). The YAC DNA was prepared following standard protocols

[1] To whom correspondence should be addressed. Fax: ++39 (0) 49 827 6209. E-mail: danieli@civ.bio.unipd.it.

0006-291X/99 $30.00

TABLE 1

Radiation Hybrid Mapping of ACTN2

Locus	Retention profile	Map position
WI-9317 (D1S2421)	0000100010001211001001001100010000010000110011100010001000000100000001001 01000000102000010201	4.3 cR from WI-6955 1.5 cR from ACTN2 910.43 cR-252 Mb-1q43
ACTN2	0000100000001011101001001100010000020000110021100010002000000000000001001 01000000101000010001	1.5 cR from WI-9137 1.5 cR from AFMA045ZC5
AFMA045ZC5	0000100010001011101001001100010000010000110011100010001000000100000001001 12000000102000010201	1.5 cR from ACTN2 4.6 cR from WI-4866 913.47 cR-1q43

Note. The retention profile of ACTN2, obtained on the Genebridge4 Radiation Hybrid Panel, is placed by RHMAPPER program among the framework markers of the WI Chromosome 1 RH map. The screening results are represented as a vector of 0's and 1's. Unknown/uncertain data are indicated with 2's.

(12) and screened by using the same marker CA4F/R on PCR conditions previously described.

Bio-informatic analysis and primer designing. The ACTN2 full-length cDNA (GenBank M86406) was analysed by using the RNASPL program, which predicts potential exon-exon junction positions in a human cDNA sequence (13) (http://dot.imgen.bcm.tmc.edu:9331/). Putative exonic primers were designed by OLIGO software (14) along the coding regions at low probability of interruption. The oligonucleotides were selected according to the following parameters: average size of 20 bp, annealing temperature between 52 and 62°C, GC content around 50%. Intronic primer pairs for mutation screening were designed by PRIMER3 software, following the same criteria (15) (http://www-genome.wi.mit.edu).

Long-distance and Alu-PCR. Amplification conditions were optimized in order to obtain long PCR fragments with exonic primer pairs that could trap each intron of the gene. In case of large introns, exonic oligonucleotides were combined with Alu primers (Alu: 5′-CCCAAATTGCTGGGTTAC-3′, AluOpp: 5′-GTAACCCAGCAATTTGGG-3′).

Twenty nanograms of YAC DNA was amplified in 12.5 μl of PCR buffer containing 1 μM of each of the forward and reverse primers, 0.5 units of ExperTaq polymerase and 25 μM of each of the four dNTPs. Cycling conditions were: 5 min at 94°C, followed by 33 cycles of 1 min at 94°C, 1 min at the working annealing temperature, 2–3 min at 72°C, and a final extension step for 5 min at 72°C (PTC Programmable Thermal Cycler, M. J. Research).

PCR conditions for the amplification of the exons with intronic primers were modified, by reducing the amount of DNA polymerase and the extension time, according to the size of the expected products.

DNA cloning and sequencing. ACTN2 genomic portions were recovered by gel-extraction (QIAquick Gel Extraction Kit, QUIAGEN) or directly purified from the PCR products (StrataPrep PCR Purification Kit, Stratagene). The fragments were polished and ligated into pPCR-Script Amp SK (+) vector and then used to transform *Epicurian coli* XL10-Gold Kan cells, following the manufacturer's protocols (PCR-Script Amp Cloning Kit, Stratagene). Plasmid DNA was purified from 5 ml O/N bacterial culture (QIAprep Miniprep, QUIAGEN) and sequenced using fluorescent dideoxy terminators, *Taq* Gold (Perkin–Elmer) and T3/T7 primers, on a ABI 377 automated DNA sequencer, following ABI protocols (Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin–Elmer/Applied Biosystems).

Sequence analysis. Sequence data were analyzed using the Chromas 1.5 software (Technelysium Pty. Ltd.) and the DNASTAR package (Dnastar, Inc.). Database homology searches were performed at the NCBI using the BLAST network service (16).

RESULTS AND DISCUSSION

The ACTN2 intronic marker CA4F/R was used to PCR amplify the human/hamster somatic cell hybrid DNAs. The retention profile obtained for ACTN2 gene on the Genebridge 4 Radiation Hybrid Panel is shown in Table 1. By RHMAPPER multipoint linkage, the gene was mapped on Whitehead Chromosome 1 RH Map, at about 912 cR from the p-telomere, with a lod score >15. Thus, ACTN2 was placed 1.5 cR from WI-9317 (alias D1S2421, at 251.820 Mb in the physical map) and 1.5 cR from AFMA045ZC5. Flanking markers map to the cytogenetic band 1q43, as reported in the Location Database.

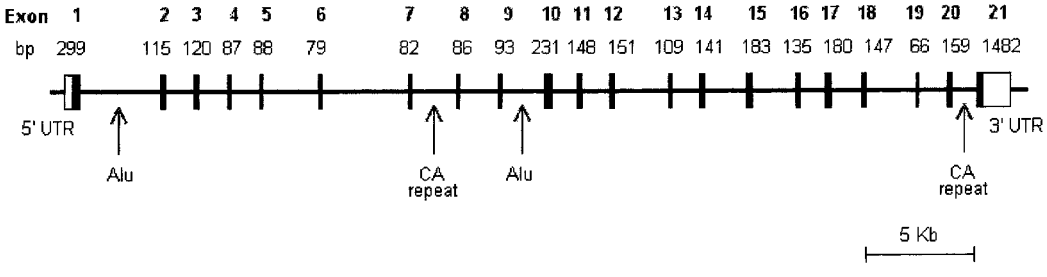

FIG. 1. Genomic structure of the ACTN2 gene. The linear map of the exon–intron structure of ACTN2 is schematically shown. Exons are represented as numbered boxes, introns are represented by solid lines in-between. The 5′ and 3′ UTR are represented by open boxes. Arrows indicate repetitive sequences. The intron sizes are to scale.

According to the RH mapping results, three 1q42-q44 Whitehead YAC contigs were selected for physical mapping of the gene: WC-429, WC-1428 and WC-239. After PCR screening with CA4F/R marker, a positive YAC was found, 955_c_12, belonging to WC-1428 (1q43). To characterize the ACTN2 intron–exon boundaries, this YAC was selected as template DNA for long-distance PCR. A set of putative exonic primers was specifically designed, from the cDNA sequence of α-actinin-2. After PCR amplification with these primers, eighteen heavy products were obtained whose size was larger than expected from the cDNA sequence. Their ends were sequenced, revealing the presence of an intron in each fragment. On the other hand, Alu-PCR was used to detect the edges of two large introns.

The ACTN2 gene resulted to contain 21 exons, in a segment spanning about 35–40 kb of genomic DNA. Nucleotide sequence data of all the exons have been submitted to DDBJ/EMBL/GenBank under Accession Nos. AJ249756 to AJ249776.

The 20 introns are at codon positions 42/43, 81, 121, 150, 179, 205/206, 232/233, 261/262, 292/293, 369/370, 419, 469, 505/506, 552/553, 613/614, 658/659, 718/719, 767/768, 789/790, and 842/843. All the splice sites follow the GT/AG rule (17). The exons have an average size of 150 bp. The genomic structure of the gene is shown in Fig. 1.

Exon 1 includes the 5′ UTR of the gene and the beginning of the actin-binding domain of the protein, which roughly corresponds to the first eight exons of the gene. In fact, according to sequence comparisons among α-actinin genes in Vertebrates and Invertebrates, the boundary of this domain should be placed around the residue 250 (5). Cross-linking experiments suggested that the actin-binding domain interacts with actin at two sites, between residues 1–12 and 86–119. These regions correspond to the first three exons of the human gene.

Sequence comparisons show that the two human exon-exon junction positions in the cDNA, ex1/ex2 and ex7/ex8, are both conserved in *Caenorhabditis elegans* (ex1/ex2 and ex3/ex4 of W04D2 gene) (18) and *Drosophila melanogaster* (ex1/ex2 and ex3/ex4 of 1-2Cb gene) (19). The forth human junction corresponds to the second junction in *Caenorhabditis,* whereas the fifth and eighth human splice sites are conserved in *Drosophila* (ex2/ex3 and ex4/ex5 junctions, respectively). Because actin gene is highly conserved across all species investigated, the actin-binding domain of α-actinin is also expected to be highly conserved. Indeed, protein and cDNA sequence comparisons among species confirmed this expectation (data not shown). Moreover, this last observation suggests that a high level of homology might exist also at the genomic level. However, only the proximal part of the gene shows an high conservation through evolution, whereas in the remaining part of the gene a divergence from the genomic organization of *Caenorhabditis* and *Drosophila* is noticed.

TABLE 2

Intronic Primer Pairs for Mutation Screening of the ACTN2 Gene

Exon No.	Primer pair 5′ → 3′	Product size (bp)
1	Ex1-F: CGTTTGCCAGTCAGCCCGTG	193
	Ex1-R: CTTCCTCTGCTGCTTCTCCC	
2	Ex2-F: TCAAGTGTCGTCTGTGAGGA	225
	Ex2-R: CACATGAAAGCATAGGATGG	
3	Ex3-F: TGTCTCTATGTCTGCATGATTC	196
	Ex3-R: TCAGTGTGGCAGACAGGAC	
4	Ex4-F: TCCAATAGCTCTGAAGTCAACA	309
	Ex4-R: GAACCCACTTACATGTCTCTCA	
5	Ex5-F: AGTGAACTAAGCGGCATTTC	191
	Ex5-R: ACCCACAGATTCCACTGAGA	
6	Ex6-F: ACACGTGTTCCTGTTCTTCT	164
	Ex6-R: ATGTTGAGACGCGTGGCTG	
7	Ex7-F: GTCCGGCCTAAAGTGAGGTA	175
	Ex7-R: CTTCAGCATCCAACATTTTA	
8	Ex8-F: TATTTTCTCCCCCTTCAG	145
	Ex8-R: AGAGAGTAACACAACAGCCC	
9	Ex9-F: CATTCCCGTCGACAGAGC	211
	Ex9-R: GTTTTTCACCCTCTGTGCCT	
10	Ex10-F: GCTGGTGTCTTCAGCAGTAT	293
	Ex10-R: AGGGCTTGGCGCTTGCTAC	
11	Ex11-F: TACACATTTGCTTCCCTTGG	234
	Ex11-R: TGGCTCAACTCTGGTTTTTC	
12	Ex12-F: CATGCTTTCTTGCTACCACC	203
	Ex12-R: GAGGACGGAGGGCATCTG	
13	Ex13-F: TCTCTCATCATCTGGGAAAGTT	189
	Ex13-R: TCATGTCAACAAGTGGCTTC	
14	Ex14-F: TGATAATGCTTGCTTCTCTTT	196
	Ex14-R: GCATACAGAGTTACGGTTCCA	
15	Ex15-F: TTTCTCCACTTGTGTCTCGG	279
	Ex15-R: CTTCAAGAATGCCCCCTG	
16	Ex16-F: GCCTCTAACCCTTGTTGTCC	220
	Ex16-R: AGGGACTTATCGCAAGGCT	
17	Ex17-F: TCACTCTGCTTCTCTCCCTG	261
	Ex17-R: GTGAGAAGTGAGCGGCAC	
18	Ex18-F: GCAGAGTTGACATGTGGAGA	240
	Ex18-R: TTAATGTCCCCAGTATTGCC	
19	Ex19-F: GCTCACCTGCTCTGTCCTT	187
	Ex19-R: CCTTGGTTTGAGCTTGTCAT	
20	Ex20-F: TGAGAGTTGTGTACCGTTCG	243
	Ex20-R: CCGCTAAAGCAGAAGGAAAT	
21	Ex21-F: CTGCAACTGACTGCAAACAC	235
	Ex21-R: TGCATTCTGATGGGATGAGT	

Note. A series of oligonucleotides is shown, specifically designed to PCR amplify all the exons of ACTN2, starting from human genomic DNA. An optimal annealing temperature around 58°C is recommended for most primer pairs.

The central rod of ACTN2, composed by four internal 122 amino acid repeats, spans exons 9 to 17. Repeat 1, the most highly conserved repeat across species, coincides with exons 9 and 10 and is precisely separated from the other repeats by ex10/ex11 junction. This repeat is interrupted by ex9/ex10 junction, a splice site conserved in *Drosophila* (ex5/ex6 in the fly gene). Repeats 2–4 span the exons from 11 to 17; the last repeat

exactly ends at the ex17/ex18 junction. All the repeats are variously interrupted by introns. Interestingly, all these splice sites correspond to amino acid sites poorly or non-conserved through evolution and/or within the α-actinin isoforms of a given species.

The two EF-hand-like calcium binding motifs, at the C terminal region of ACTN2, correspond to exons 18-20. Ex19/ex20 junction, a splice site also conserved in *G. gallus* (20), exactly separates the two domains. The first EF-hand is interrupted by an intron between the liganding oxygen residues, denoted as X and Y (5). Residue Y is a non conserved amino acid among α-actinin isoforms in different species, which is believed to explain the differences in calcium binding properties between the muscle and non-muscle isoforms (1).

Part of exon 20 and/or the exon 21 are likely to contain the essential sequences mediating the Z-disc titin binding, which have been located from about the second EF-hand up to the extreme C terminus of ACTN2 (6). The last exon also includes the 3′ UTR of the gene.

It is interesting to notice that no information is available so far on the genomic organization of actinin genes in Vertebrates, if we exclude the short segment of DNA sequence obtained in *G. gallus* (20).

ACTN2 has been proposed as a candidate gene for some inherited neuromuscular and cardiac diseases, mapped to 1q42-q43, where it was localized by FISH (1). Among those, Arrhythmogenic Right Ventricular Cardiomyopathy type 2, significantly linked to an intronic CA repeat within the ACTN2 gene (21). The mutation screening in patients affected by this disease was hindered so far by the difficulty in obtaining myocardial biopsies and by the lack of expression of the gene in human lymphocytes (data not shown). The present characterization of the exon/exon boundaries opens the way to the mutation screening of the gene, based on genomic DNA, without the need of invasive biopsies. Table 2 reports the list of intronic primers specifically designed to amplify all the exons of ACTN2, directly from genomic DNA.

The knowledge of the sequence and genomic organization of the ACTN2 gene is expected to facilitate the study of the genomic organization of other human, mammalian or vertebrate α-actinin genes, thus clarifying their phylogenetic relationships.

ACKNOWLEDGMENTS

This work was financially supported by Grant E.743 from the Telethon Italian Foundation to A. Nava, by Telethon Grant B41 to G. Lanfranchi and G. Valle (CRIBI Biotechnology Centre), and by a generous donation from the Lions Club Association of Verona "Europa" to G. A. Danieli. Natascia Tiso is a recipient of a postdoctoral fellowship from University of Padova.

REFERENCES

1. Beggs, A. H., Byers, T. J., Knoll, J. H., Boyce, F. M., Bruns, G. A., and Kunkel, L. M. (1992) *J. Biol. Chem.* **267**(13), 9281–9288.
2. Youssoufian, H., McAfee, M., and Kwiatkowski, D. J. (1990) *Am. J. Hum. Genet.* **47**(1), 62–71.
3. Honda, K., Yamada, T., Endo, R., Ino, Y., Gotoh, M., Tsuda, H., Yamada, Y., Chiba, H., and Hirohashi, S. (1998) *J. Cell Biol.* **140**(6), 1383–1393.
4. Chan, Y., Tong, H., Beggs, A. H., and Kunkel, L. M. (1998) *Biochem. Biophys. Res. Commun.* **248,** 134–139.
5. Blanchard, A., Ohanian, V., and Critchley, D. (1989) *J. Muscle Res. Cell Motil.* **10,** 280–289.
6. Sorimachi, H., Freiburg, A., Kolmerer B., Ishiura, S., Stier, G., Gregorio, C. C., Labeit, D., Linke, W. A., Suzuki, K., and Labeit, S. (1997) *J. Mol. Biol.* **270,** 688–695.
7. Hance, J. E., Fu, S. Y., Watkins, S. C., Beggs, A. H., and Michalak, M. (1999) *Arch. Biochem. Biophys.* **365,** 216–222.
8. Beggs, A. H., Phillips, H. A., Kozman, H., Mulley, J. C., Wilton, S. D., Kunkel, L. M., and Laing, N. G. (1992) *Genomics* **13,** 1314–1315.
9. Walter, M. A., Spillet, D. J., Thomas, P., Weissenbach, J., and Goodfellow, P. N. (1994) *Nat. Genet.* **7,** 22–28.
10. Slonim, D., Stein, L., Kruglyak, L., and Lander, E. RHMAPPER. http://www-genome.wi.mit.edu/ftp/pub.
11. Collins, A., Frezal, J., Teague, J., and Morton, N. E. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14771–14775.
12. Scherer, S. W., and Tsui, L.-C. (1991) *in* Advanced Techniques in Chromosome Research (Adolph, K. W., Ed.), pp. 33–72, Dekker, New York.
13. Solovyev, V. V., Salamov, A. A., and Lawrence, C. B. (1994) *Nucleic Acids Res.* **22**(24), 5156–5163.
14. Rychlik, W., and Rhoads, R. E. (1989) *Nucleic Acids Res.* **17,** 8543–8551.
15. Rozen, S., and Skaletsky, H. J. (1996, 1997) Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.
16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
17. Green, M. R. (1991) *Annu. Rev. Cell Biol.* **7,** 559–599.
18. Wilson, R., Ainscough, R., Anderson, K., and Wohldman, P. (1994) *Nature* **368**(6466), 32–38.
19. Fyrberg, E., Kelly, M., Ball, E., Fyrberg, C., and Reedy, M. C. (1990) *J. Cell Biol.* **110**(6), 1999–2011.
20. Parr, T., Waites, G. T., Patel, B., Millake, D. B., and Critchley, D. R. (1992) *Eur. J. Biochem.* **210**(3), 801–809.
21. Rampazzo, A., Nava, A., Eberhard, P., Vian, E., Slomp, P., Tiso, N., Thiene, G., and Danieli, G. A. (1995) *Hum. Mol. Genet.* **4,** 2151–2154.