

Telethonin, a novel sarcomeric protein of heart and skeletal muscle

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Abstract In this paper we describe a novel 19 kDa sarcomeric protein named telethonin. The cDNA sequence discloses an open reading frame of 167 amino acids that does not resemble any known protein. Antibodies against a recombinant telethonin fragment were used for Western blot analysis, confirming the presence of this 19 kDa protein in heart and skeletal muscle and revealing an immunofluorescence pattern typical of sarcomeric proteins, overlapping myosin. The frequency of specific cDNA clones in different libraries indicates that the telethonin transcript is amongst the most abundant in skeletal muscle. In human, telethonin maps at 17q12, adjacent to the phenylethanolamine *N*-methyltransferase gene.

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Key words: Skeletal muscle; Sarcomeric protein; Expressed sequence tag

1. Introduction

The systematic sequencing of expressed sequence tags (ESTs) is accelerating the discovery of new genes at an unprecedented rate [1,22]. Comparison of the frequency of ESTs obtained from different tissues allows the inference of the pattern of gene expression [5,14] and in some cases may also be indicative of the relative abundance of the mRNAs [9,15].

We are currently engaged in a project for systematic sequencing of ESTs from human skeletal muscle [9]. The cDNA library used in this project was not subtracted or normalised, thus allowing a reliable correlation between the frequency of an EST and the relative abundance of the corresponding mRNA in the muscle tissue. From this study many novel genes have been discovered, including some that appear to be expressed at a high level.

In this paper we present a comprehensive study of a new gene expressed in human skeletal muscle, which we named telethonin since this work is entirely supported by Telethon, Italy. This gene corresponds to the most frequent EST that does not show any similarity to known genes, ranking amongst the 12 most abundant nuclear mRNAs transcribed in skeletal muscle.

The sequence of telethonin has been submitted to the EMBL data library (accession number AJ000491) and is also accessible at our Web site <http://eos.bio.unipd.it>.

2. Materials and methods

2.1. Cell culture and human tissue samples

The following human lines were obtained from the American Type Culture Collection: BeWo (choriocarcinoma), Detroit551 (skin, fibroblasts), HepG2 (hepatoma), IMR32 (neuroblastoma) and Tera-2 (teratocarcinoma). The lines HL60 (human lymphoblasts) and K562 (erythroleukaemia) were obtained from the Karolinska Institutet, Stockholm, Sweden. Samples of human skeletal muscle and heart were obtained from the Hospital of Padua. The RNA was extracted by the acid guanidium thiocyanate method [6].

2.2. Northern blot analysis

The Northern blot analysis was performed on two filters supplied by Clontech. One filter was the 'Human muscle multiple tissue Northern blot' (cat. 7765-1) containing approximately 2 µg of poly A⁺ RNA per lane, from the following eight different human tissues: skeletal muscle, uterus (no endometrium), colon (no mucosa), small intestine, bladder, heart, stomach and prostate. The second filter was the 'Human multiple tissue Northern blot' (cat. 7760-1) containing approximately 2 µg of poly A⁺ RNA per lane, from the following eight different human tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas.

The hybridisation protocol and solutions were provided by Clontech (ExpressHyb Hybridisation Solution, cat. S0910). The probe was obtained by random priming, using a PCR fragment obtained from the 3'-end untranslated region of the mRNA, using [³²P]dATP.

2.3. Construction and screening of a human skeletal muscle cDNA library by PCR

A cDNA library suitable for the identification of full-length transcripts was produced with a kit obtained from Invitrogen. The double-stranded cDNA was ligated to *Bst*XI-*Eco*RI adaptors (Invitrogen) and size-fractionated on an agarose gel in order to select fragments longer than 350 bp. The resulting preparation was inserted into the pCDNAII plasmid (Invitrogen) and used to transform the electrocompetent *Escherichia coli* strain TOP10F⁺.

A part of the library was aliquoted in 480 tubes, at a density of 50 transformed bacteria per tube. Bacteria were grown to saturation and the plasmid DNA was extracted from each tube using the QIAwell system (Qiagen) and placed in five dishes of 96 wells, corresponding to 480 pools of DNA, each consisting of the plasmids from about 50 different bacterial clones.

The screening of the cDNA library was carried out on the pools of plasmids described above and based on PCR. Firstly, the 480 pools were processed by PCR using two telethonin-specific primers. The pools that produced a band of the expected size were considered to contain at least one telethonin cDNA and were further analysed by means of two parallel PCRs: one with the T15-REV and the Universal-FOR primer, the other with the T15-REV and the Universal-REV primer. The two 'Universal' primers corresponded to two sequences of the plasmid, located on opposite sides of the cloning site. Depending on whether an amplified band was produced with the Universal-FOR or the Universal-REV primer, it was possible to deduce the orientation of the insert. The agarose gel analysis also allowed the identification of the longest cDNA inserts that could be selected for further processing and sequencing.

DNA sequencing was carried out directly on 5 µl of the PCR reactions using either the Dye-deoxy-terminator chemistry or the Dye-primer chemistry (Applied Biosystems) and run on an ABI377 DNA sequencer (Applied Biosystems).

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2.4. Immunocytochemistry and immunofluorescence

A cDNA fragment of telethonin with appropriate restriction enzyme sites was obtained by RT-PCR and inserted into the prokaryote expression vector pQE9 (Qiagen). Telethonin in pQE9 was sequenced to confirm that there were no major changes from the original transcript. The recombinant protein was expressed in *E. coli* after induction by IPTG and was purified by affinity chromatography on Ni-NTA (nickel-nitrilotriacetic acid) resin. The eluted protein was dialysed against Tris-buffered saline (TBS) pH 8 to remove the 8 M urea. The recombinant telethonin was used to immunise three mice for the production of polyclonal antibodies. Mice were injected six times, at intervals of approximately 10 days.

Polyadenylated mRNA from human skeletal muscle was translated in vitro using the Promega wheat germ translation system and [³⁵S]methionine. Equal amounts of labelled skeletal muscle proteins were mixed with 2 µl of appropriate antibody (anti-telethonin or pre-immune mouse serum) and then immunoprecipitated with protein A Sepharose CL-4B beads (Pharmacia) in a buffer containing 50 mM HEPES pH 8.0, 250 mM NaCl, 0.1% NP40. The resulting immunoprecipitated samples were run on 15% SDS-polyacrylamide gels and autoradiographed.

For Western blotting, fragments of frozen heart muscle were homogenised under liquid nitrogen using a mortar and pestle. The resulting powder was resuspended in SDS-PAGE sample buffer, boiled and run on a preparative 15% SDS-acrylamide gel (approximately 20 µg of total protein per lane). The polyclonal antibody to telethonin was used at a 1:200 dilution. A goat anti-mouse alkaline phosphatase was used as a second antibody (Sigma A3562).

For immunofluorescence, frozen sections about 6 µm thick were prepared from human skeletal muscle (Vastus) using a Leica Jung/CM/1800 cryostat. As first antibodies we used the mouse anti-telethonin serum diluted 50 times and/or a rabbit anti-skeletal myosin antibody (Sigma M7523). As a second antibody we used a TRITC-labelled goat anti-mouse immunoglobulin (Sigma T7657) and/or a FITC-labelled goat anti-rabbit immunoglobulin (Sigma F0511). FITC-phalloidin (Sigma P5282) was used as a specific ligand of actin. All the commercial immunochemicals were diluted as recommended by the suppliers.

2.5. Genomic mapping

The genomic mapping was performed by PCR, with the radiation hybrids method, using the GeneBridge 4 whole-Genome Radiation Hybrid Panel (Research Genetics) consisting of 93 genomic DNA preparations from human-on-hamster somatic cell lines [26]. The screening results were processed with the RHMAPPER software program, available from the Whitehead Institute/MIT Center for Genomic Research (Cambridge, MA, USA).

3. Results

3.1. Molecular cloning and characterisation of the telethonin mRNA

From the systematic sequencing of human skeletal muscle ESTs carried out in our laboratory [9], many novel genes have been discovered, including some that appear to be expressed at a high level. Currently, more than 10 000 ESTs have been identified, representing well over 1500 different independent transcripts. The current list of the most frequent nuclear transcripts is given in Table 1 where the relative frequencies are also shown. It can be seen that telethonin is amongst the most abundant transcripts, ranking at the 12th position.

The cDNA libraries used for EST sequencing were especially designed to contain only the 3'-end of the transcripts [9]. Although this confers several advantages to the overall project, it makes the recovery of a full-length sequence more difficult. To overcome this problem we have produced a separate 'full-length' cDNA library and set up a fast screening method based on PCR, as described in Section 2. From the PCR screening of about 24 000 transformant plasmids we

found 105 positives. The sequence shown in Fig. 1 was produced from some selected cDNA inserts.

The total length of the nucleotide sequence shown in Fig. 1 is 959 bases. The translation of the sequence reveals an open reading frame of 501 bases, encoding a putative protein of 167 amino acids with a molecular weight of 19 051 Da.

Both nucleotide and amino acid sequences were used to perform extensive similarity searches in the EMBL nucleic acid database and in the SwissProt protein database using several programs such as FASTA [17], BlastN, BlastX [2], MPSearch [24] and BioSCAN [23]; however, these searches did not reveal significant similarity to any known gene.

Genomic mapping was performed with the radiation hybrids technique, as described in Section 2, revealing that the telethonin gene maps on the human genome on chromosome 17q12, with a very significant lod score (>15). Further evidence of the chromosomal position of the telethonin gene comes from the finding that the very last 50 bases found at the 3'-end of the transcript overlap the beginning of a genomic fragment that includes the human phenylethanolamine *N*-methyltransferase gene which is also known to map to chromosome 17 [8]. The distance between the end of the telethonin transcript and the beginning of the phenylethanolamine *N*-methyltransferase gene is approximately 1900 bases.

3.2. Northern blot analysis

The tissue specificity of the telethonin transcript was studied in several tissues by Northern blot analysis and the results are reported in Fig. 2. The expression of telethonin seems to be restricted to skeletal and heart muscle as under normal exposure conditions it cannot be detected in any other tissues including those containing smooth muscle such as stomach, bladder, intestine, colon, uterus and prostate. However, after

Table 1

List of the 24 most abundant nuclear transcripts from our human skeletal muscle EST work [9,16]

Transcript	Frequency/ 10 000 ESTs
1 Skeletal alpha-actin	830
2 Alpha globin	256
3 Skeletal muscle myosin light chain 2	208
4 Slow skeletal muscle troponin T	199
5 Creatine kinase	181
6 Glyceraldehyde-3-P dehydrogenase	92
7 Ribosomal protein L37a	76
8 Troponin I, slow-twitch isoform	64
9 Myoglobin	61
10 Fast skeletal muscle myosin H-chain	60
11 Skeletal muscle beta-tropomyosin	55
12 Telethonin	51
13 Ribosomal protein L41	51
14 Fast skeletal muscle troponin C	47
15 Fast skeletal muscle myosin L-chain 2	45
16 Skeletal muscle myosin alkali L-chain	42
17 Fructose 1,6-diphosphate aldolase A	41
18 Beta-globin	40
19 EST (skeletal muscle cDNA library)	39
20 Desmin	39
21 Cytochrome <i>c</i> oxidase subunit VIIa	38
22 Skeletal muscle troponin I fast-twitch	36
23 Fast 2a skeletal muscle myosin H-chain	36
24 Ribosomal protein S11	32

The frequency is calculated as the number of times that a transcript was found in 10 000 ESTs, counting also the mitochondrial transcripts (not shown in this list).

<i>M A T S E L S C E V S E E N C E</i>	16
CGGCACGAGC ATG GCT ACC TCA GAG CTG AGC TGC GAG GTG TCG GAG GAG AAC TGT GAG	58
<i>R R E A F W A E W K D L T L S T R P E</i>	35
CGC CGG GAG GCC TTC TGG GCA GAA TGG AAG GAT CTG ACA CTG TCC ACA CGG CCC GAG	115
<i>E G C S L H E E D T Q R H E T Y H Q Q</i>	54
GAG GGC TGC TCC CTG CAT GAG GAG GAC ACC CAG AGA CAT GAG ACC TAC CAC CAG CAG	172
<i>G Q C Q V L V Q R S P W L M M R M G I</i>	73
GGG CAG TGC CAG GTG CTG GTG CAG CGC TCG CCC TGG CTG ATG ATG CGG ATG GGC ATC	229
<i>L G R G L Q E Y Q L P Y Q R V L P L P</i>	92
CTC GGC CGT GGG CTG CAG GAG TAC CAG CTG CCC TAC CAG CGG GTA CTG CCG CTG CCC	286
<i>I F T P A K M G A T K E E R E D T P I</i>	111
ATC TTC ACC CCT GCC AAG ATG GGC GCC ACC AAG GAG GAG CGT GAG GAC ACC CCC ATC	343
<i>Q L Q E L L A L E T A L G G Q C V D R</i>	130
CAG CTT CAG GAG CTG CTG GCG CTG GAG ACA GCC CTG GGT GGC CAG TGT GTG GAC CGC	400
<i>Q E V A E I T K Q L P P V V P V S K P</i>	149
CAG GAG GTG GCT GAG ATC ACA AAG CAG CAG CTG CCC CCT GTG GTG CCT GTC AGC AAG CCC	457
<i>G A L R R S L S R S M S Q E A Q R G -</i>	167
GGT GCA CTT CGT CGC TCC CTG TCC CGC TCC ATG TCC CAG GAA GCA CAG AGA GGC TGA	514
<i>GAGGGACTGTGACTTGGGCTCCGCTGTGCCCGCCCTGGGCTGGGCCCCTTCTGGCTAGGACTGTGG</i>	580
<i>AGGGGAGCTGCTGSCCATGGCTGCTTTGTAGTTTGCCCAAGGAGGGTGGCAGAGACAGTGGGCAC TAA</i>	646
<i>CAGAGGCCAGGATGCCTGAGCCCCCTGAGTTCCCAAGGGAGGGTGGCAGAGACAGTGGGCAC TAA</i>	712
<i>GGGTGAGAGTTGGGGCCAGCACAGCTGAGGACCTCAGCCCCAGGAGAAGGGACAAAAGGTACT</i>	778
<i>GCTGAGGGCAAGAGGTGCCTGGGAGGAGTGGCCCTGATCCAGGAAAATGTGAGGGGAATCTGGAAC</i>	844
<i>GCTCTAGGCAGAAGAAGCTGGGAGGGAGGGGAGGTGAAAAGGGCAGAGGCAAGGATGGTGGGGCC</i>	910
<i>CCCAGCACCTCTGTAGTGCCCAATAAATGCTCAATCATGTGCCAGA-poly(A)</i>	959

Fig. 1. Nucleic acid sequence of the telethonin transcript. The translated amino acid sequence is shown in italics above each codon. The TGA stop codon is represented by a dash.

a prolonged exposure of the filter, a very faint band was observed in the prostate lane (data not shown).

Further evidence that telethonin exhibits a very restricted pattern of expression came from RT-PCR [19] and RNase protection assay [27], performed on skeletal muscle, heart and a variety of cell lines (see Section 2). Also in these cases the telethonin transcript was only detected in skeletal muscle and heart (data not shown).

3.3. Antibodies, immunoprecipitation and Western blot analysis

A recombinant fragment of telethonin consisting of 128 C-terminus amino acids (SLHEE...EAQR) was produced in *E. coli* and used for the immunisation of three mice as described in Section 2.

The anti-telethonin antibodies were used for immunoprecipitation and Western blot analysis (Fig. 3) revealing a band of about 19–20 kDa. From the immunoprecipitation results (Fig. 3, left) it can be seen that the anti-telethonin antibodies bring down a single band. There are no other visible bands co-precipitating with telethonin under the experimental conditions used.

The Western blot analysis (Fig. 3, right) was repeated independently with sera from three different mice, producing a single band of the expected size, whereas the serum from a pre-immune mouse did not produce any detectable band. Immune sera produced in a similar way against other recombinant proteins obtained from pQE9 revealed different bands from those of Fig. 3 (data not shown). In conclusion, we can say that the antibodies raised against the putative telethonin protein displayed in Fig. 1 do actually recognise a band of the expected size; therefore, the sequence presented in Fig. 1 most likely covers the entire coding sequence of telethonin.

Further evidence that the sequence presented in Fig. 1 cov-

ers the full coding region of telethonin is given in Fig. 4, where the natural form of telethonin found in skeletal muscle and heart is compared with the recombinant form produced in bacteria, using Western blot analysis. It can be seen that the recombinant form of telethonin runs slightly more slowly as a result of the histidine tag used for affinity purification (see Section 2).

The intensity of the Western blot signal (Fig. 4) can be used as an indication of the relative amount of telethonin in skeletal muscle and heart, based on the comparison with known amounts of recombinant telethonin. The densitometric analysis of the bands (not shown) gives a perfect correlation with the amount of control telethonin loaded on the gel and indicates a relative amount of 5 ng of telethonin per 10 µg of total muscle protein, in both heart and skeletal muscle.

3.4. Immunofluorescence

To elucidate the intracellular localisation of telethonin we

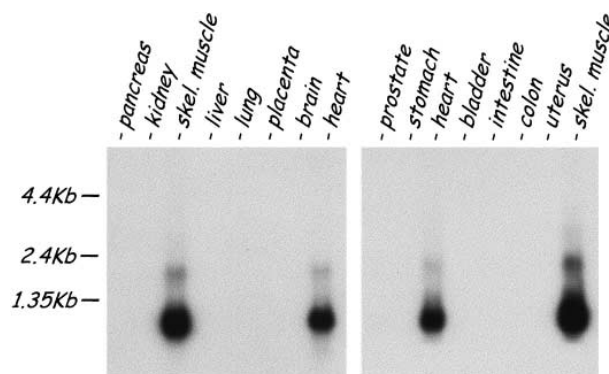


Fig. 2. Northern blot analysis of several human tissues using the 3' untranslated region of telethonin as a probe.

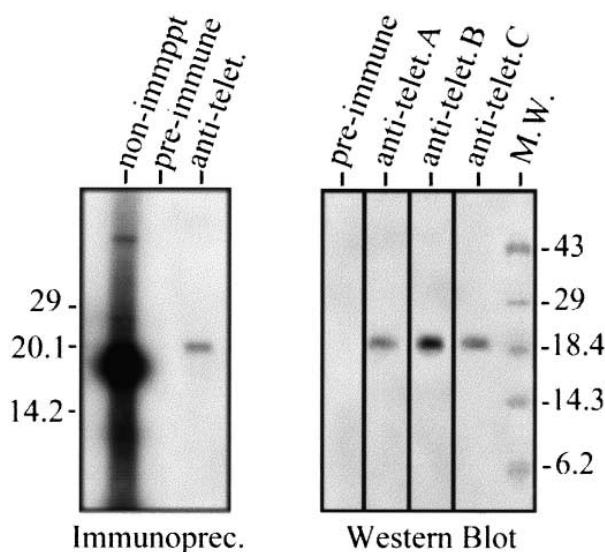


Fig. 3. Immunoprecipitation and Western blot analysis of telethonin. The immunoprecipitation (left) was carried out as described in Section 2, from a wheat germ *in vitro* transcription system, using polyadenylated RNA from skeletal muscle. The non-immunoprecipitated sample was obtained from 2 μ l of reaction, whereas the two immunoprecipitated samples were obtained from 15 μ l after incubation either with mouse pre-immune serum or with a serum from a mouse immunised with the recombinant telethonin. It can be seen that pre-immune serum does not precipitate any band, whereas the anti-telethonin brings down a single band of approximately 19–20 kDa. The Western blot analysis is shown on the right. A fragment of human heart muscle was homogenised and run on a 15% SDS-PAGE, as described in Section 2. After blotting, four identical nitrocellulose strips were cut and probed with four different mouse antisera diluted 200 times. Strip 1: pre-immune serum; strips 2, 3 and 4: polyclonal antibodies from three different mice immunised with the recombinant telethonin. The positions of the molecular weight markers (kDa) are indicated at the side of the figure.

performed some immunofluorescence analyses on skeletal muscle. An indirect immunofluorescence assay revealed a banding typical of sarcomeric proteins, visible as rhodamine red fluorescence in Fig. 5a,d. To establish the relationship between the banding of telethonin and that of other major sarcomeric proteins such as myosin and actin, we performed double immunofluorescence assays, where myosin and actin were revealed by fluorescein green staining, as described in Section 2. The results are presented in Fig. 5b,e, where the actin and myosin patterns are shown in the same sections as in Fig. 5a,d. From the overlapping of the two images it can be seen that the banded pattern displayed by telethonin overlaps that of myosin (Fig. 5f) and alternates with actin (Fig. 5c). Frames g, h and i of Fig. 5 show several negative controls that support the double immunofluorescence data and confirm that the pattern observed is not due to artefacts that sometimes occur in double immunofluorescence staining.

4. Discussion

The main point from the work presented in this paper is that a new major sarcomeric protein has been discovered. Therefore, the understanding of how this new protein relates to other known sequences is of primary interest. As was anticipated in Section 3, the sequence similarity searches did not reveal any significant similarity to any known gene or protein.

The amino acid sequence was also analysed for the presence of known amino acid domains and motifs, against respectively the Sbase [18] and the Prosite [4] databases. The only finding from the Prosite search is the presence of several putative phosphorylation sites, whereas a similarity search against Sbase uncovered a weak correlation with 25 amino acids from the catalytic domain of the KIR serine/threonine protein kinase receptors, with 48% identity. Although the possible involvement of kinase activities is very fascinating, it is far from being proven and these data should only be taken as the best matches found by the above computer searches.

The Psort program [13] indicated that telethonin is a probable cytoplasmic protein and the Predict program [21] showed that it does not contain any transmembrane domain, thus confirming the hypothesis of a sarcomeric protein, as indicated by the immunofluorescence data.

4.1. Relative abundance of the transcript and pattern of expression

The second major question that arises from this work is whether the telethonin transcript is really as abundant as it appears from our EST work, ranging around 0.51% of the polyadenylated mRNA molecules. The method that has been developed in our laboratory for the construction of the cDNA libraries used for EST sequencing gives a good correspondence between the relative amount of the mRNAs and the number of cDNA inserts found in the library [9]. Furthermore, very similar results were found from two independent libraries produced in our laboratory and used for EST sequencing. The first library was obtained from a pectoral muscle of a woman after mastectomy, the second from a gastrocnemium of a man after leg amputation.

Further evidence of the high level of telethonin mRNA comes from the frequency of the positive clones in the full-length cDNA library. As described in Section 2, the library was screened by PCR. A total of 24 000 cDNA clones were probed in 480 groups of 50, giving 105 positives. Although 105/24 000 makes 0.44%, it must be calculated that the screening was done in groups of 50 and that some groups may

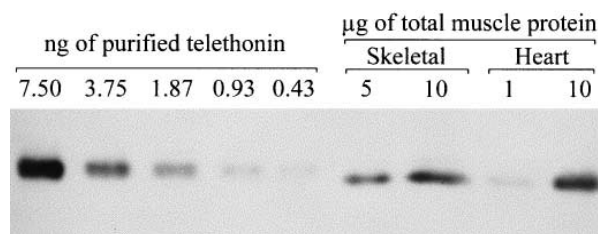


Fig. 4. Western blot analysis of known amounts of full-length recombinant telethonin produced and purified from bacteria (on the left) and known amounts of total protein from human heart and skeletal muscle (right). The slightly larger size of the recombinant protein is due to the N-terminal histidine tag used for affinity purification (see Section 2). The recombinant telethonin was purified by electro-elution from a preparative polyacrylamide gel; then it was quantified by a Bio-Rad protein assay and finally loaded on the gel for Western blot analysis. The anti-telethonin antibodies were obtained from a portion of telethonin that was lacking about 20 amino acids at the N-terminus. From a densitometric analysis of the bands it can be calculated that the telethonin signal from 10 μ g of total muscle proteins (from heart or skeletal muscle) is approximately equivalent to 5 ng of purified telethonin.

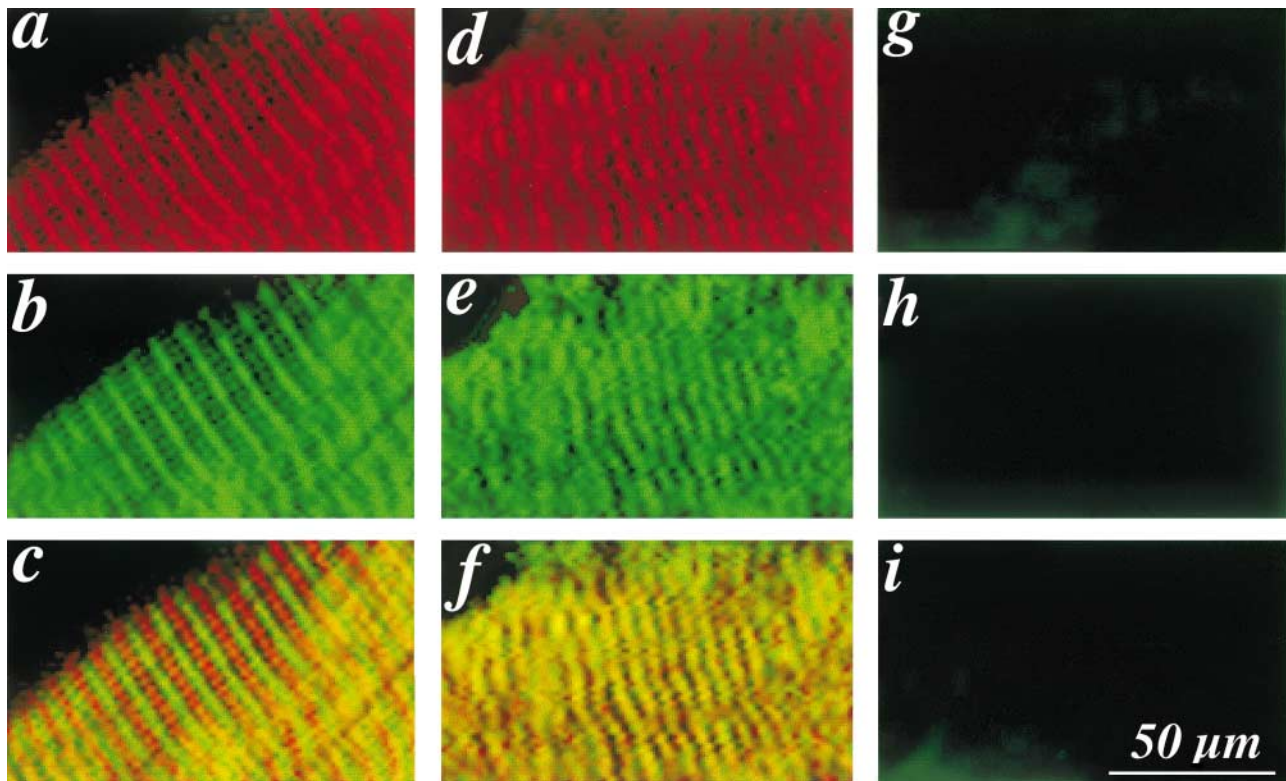


Fig. 5. Immunofluorescence analysis of skeletal muscle to detect telethonin (red), actin (green, left) and myosin (green, centre). The three frames on the left (a, b, c) show the same field of a section of human skeletal muscle where rhodamine (red) corresponds to telethonin while fluorescein (green) corresponds to actin. Frame a shows the rhodamine channel, frame b shows the fluorescein channel and frame c shows the two superimposed channels. Similarly, frames d, e and f show another section of skeletal muscle where rhodamine corresponds to telethonin while fluorescein corresponds to myosin. It can be seen that the red fluorescence (telethonin) coincides with the green myosin banded pattern (frame f), whereas it displays an alternate green/red banded pattern when compared with actin (frame c). The three frames on the right display the negative controls. g: Pre-immune mouse serum. h: Section incubated with mouse anti-telethonin as a first antibody, followed by goat anti-rabbit IgG as a second antibody, to show that the anti-rabbit IgG does not cross-react with the mouse IgG. i: Section incubated with rabbit anti-myosin as a first antibody, followed by goat anti-mouse IgG as a second antibody, to show that the anti-mouse IgG does not cross-react with the rabbit IgG. Further details of the double immunofluorescence protocol are described in Section 2.

contain multiple positives, bringing the corrected value to about 0.52%: a very similar figure to that found by sequencing the EST libraries.

Although the telethonin sequence did not show any similarity to known genes, it matches several ESTs identified in other laboratories. Fourteen such matches were found in Release 49 of the EMBL-EST library. In these cases the frequencies of the ESTs cannot be taken as an indication of the relative abundance of the transcript because these cDNA libraries were subtracted or normalised. However, the fact that 13 out of 14 ESTs are from skeletal muscle [3] or heart [11,25] is further evidence of the pattern of expression restricted to muscle.

At the protein level telethonin appears to be also present at a high amount, although not quite as high as at the RNA level. The 5 ng of telethonin per 10 μ g of total muscle protein (Fig. 4) gives a figure of 0.05% which may seem low when compared with the 0.5% found at the RNA level. However, the RNA was measured as relative number of molecules whereas the protein was measured as relative mass. This is particularly relevant since the molecular weight of telethonin is only 19 kDa which is much smaller than the average protein. It is also plausible that telethonin is not as stable as most muscle proteins. This possibility is substantiated by the analysis of the Instability Index [7], which gives a value of 69.35,

when values above 40 are indicative of a possible instability of the protein.

4.2. Genomic mapping and possible link to genetic diseases

The radiation hybrids assay allowed us to position the telethonin gene on the human chromosome 17q12. Several genetic disorders map to this region, including some responsible for myopathies such as malignant hyperthermia susceptibility-2 [10] and adhalinopathy [12,20]. The finding that telethonin maps to 17q12 opens interesting new possibilities of investigation on the molecular characterisation of these diseases.

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