The mRNA and RNA-copy pseudogenes encoding TM30_{nm}, a human cytoskeletal tropomyosin

A.R.MacLeod, C.Houlker, F.C.Reinach and K.Talbot

Ludwig Institute for Cancer Research, MRC Centre, Hills Road, Cambridge CB2 2QH, UK

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ABSTRACT

We have determined the sequence of a 2.5 kb mRNA in human fibroblasts encoding a 248 amino acid cytoskeletal tropomyosin. The protein product of this mRNA is TM30_{nm}, one of five tropomyosin-like proteins in human fibroblasts. The structural gene encoding this mRNA can also produce a 1.3 kb mRNA encoding a 285 amino acid skeletal muscle α -tropomyosin by tissue-specific alternative mRNA splicing. However, the multiple RNA-copy pseudogenes of this gene family are derived largely if not exclusively from transcripts processed according to the pattern observed in non-muscle cells.

INTRODUCTION

Human fibroblasts contain five distinct polypeptides which possess the physical and chemical characteristics of tropomyosin (1,2). We have described three different mRNAs encoding human cytoskeletal tropomyosins, each one the product of a separate non-overlapping gene (3). Sequence analysis of these mRNAs has revealed that human fibroblasts express not only tropomyosins of the classic non-muscle type (4) but also protein isoforms whose structural organization is analogous to tropomyosins of muscle origin (5).

We have described a family of human genomic DNA sequences encoding a cytoskeletal tropomyosin (6). This gene family was defined initially by hybridization to an RNA-copy pseudogene hTM_{nm}-1 isolated from human genomic DNA using a heterologous chicken muscle tropomyosin probe. The product of the structural gene of this gene family in human fibroblasts is a 2.5 kb mRNA encoding a 30,000 molecular weight tropomyosin. However, this structural gene can also produce a 1.3 kb mRNA encoding a 285 amino acid α -tropomyosin in skeletal muscle by tissue-specific alternative mRNA splicing (7). Thus alternative splicing of tropomyosin genes yields not only different isoforms of the muscle-type tropomyosins (3,8,9,10) but also different isoforms of muscle and non-muscle tropomyosin. We have also determined that this structural gene is involved in the rearrangement giving

rise to the *trk* oncogene, a fusion of truncated tropomyosin sequences with tyrosine kinase sequences (11). In this paper we describe the structure of the 2.5 kb fibroblast mRNA deduced from sequence analysis of cDNA clones and compare its structure with that of the alternative 1.3 kb muscle transcript. We also show that the multiple RNA copy pseudogenes of this gene family are derived largely if not exclusively from the non-muscle transcript.

MATERIALS AND METHODS

Cell free translation and electrophoretic analysis of protein

mRNA selection and translation in a micrococcal nuclease-treated reticulocyte lysate cell free system (12) was carried out as described (1). Two-dimensional gel analysis was carried out using isoelectric focussing in the first dimension with LKB ampholytes (90% pH 3.5 - 10 with 10% pH 2.5 - 4) (13) followed by electrophoresis in the second dimension using sodium dodecyl sulphate (SDS)/8-18% polyacrylamide gradient gels (14). After electrophoresis, labelled proteins were detected by fluorography (15,16) using Kodak XAR-2 film.

Molecular cloning and DNA sequence analysis

A cDNA library of human fibroblast RNA in λ gt10 (3) was screened with probes derived from the coding and non-coding region of hTM_{nm}-1 (6). From an initial screen of 200,000 events only three positive clones were obtained, the largest of which contained 1.1 kilobases of 3' untranslated sequence including the poly A tail. A 20 base oligonucleotide CTG CTT CAG GAC TGT GGA AT complementary to a sequence near the 5' end of this fragment was prepared using an Applied Biosystems 340 B oligonucleotide synthesizer. This was annealed to human fibroblast mRNA in a 10 μ l reaction containing 100 μ g/ml poly A-containing RNA, 10 pmoles of primer (the oligonucleotide was not kinased), 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS. The mixture was sealed in a capillary and allowed to anneal at 55°C for 15 minutes. The contents were removed, ethanol-precipitated and resuspended in 20 μ l of water. This was used to prepare a cDNA library using the protocols described previously (3,17). Use of the specific primer increased the abundance of cDNA clones of the 2.5 kb fibroblast mRNA at least 20-fold. This library was screened as described above and yielded overlapping clones from which the majority of the sequence of the 2.5 kb mRNA could be determined. The λ gt10 recombinants were digested with EcoRI and the cDNA fragments inserted into M13mp18. Processively shortened molecules were generated by unidirectional digestion with exonuclease III (18) and

sequenced using the dideoxy chain-termination procedure (19,20). Sequences were aligned and decoded using the computer programs described (21,22). Nucleic acid sequence comparisons were carried out using the program DIAGON (23).

Determination of the start of transcription of the 2.5 kb mRNA

A BamHI-SmaI segment of exon I_{nm} (7) was inserted into M13 mp18. A standard protocol for DNA sequencing was followed except that primed synthesis was carried out in the presence of all four deoxynucleoside triphosphates. The reaction products were digested with EcoRI or NcoI and the labelled single-stranded fragments isolated by electrophoresis on a 40 cm polyacrylamide sequencing gel under denaturing conditions as described (24). Each probe was hybridized separately with 1 µg of human fibroblast poly A-containing mRNA in 60% formamide, 500 mM NaCl, 4 mM Pipes pH 6.4, 1 mM EDTA for 12 hours at 37°C. The hybridization reactions were ethanol-precipitated and resuspended in distilled water. The reaction containing the EcoRI prime-cut probe was digested with SI nuclease in a reaction containing 250 mM NaCl, 30 mM sodium acetate pH 4.5, 1 mM ZnSO₄, 5% glycerol and 300 units/ml of SI nuclease (BCL) at 37°C for 30 minutes. The hybridization mixture containing the NcoI prime-cut probe was incubated with reverse transcriptase (Anglian Biotechnology) in a reaction containing 50 mM NaCl, 50 mM Tris-HCl pH 8.3, 8 mM MgCl₂, 2 mM DTT and 0.5 mM of each of the deoxynucleoside triphosphates at 42°C for 30 minutes (25). Products were analyzed by electrophoresis on sequencing gels under denaturing conditions using size markers of pBR322 digested with MspI and end-labelled.

Characterization of genomic DNA clones

Cloning of human genomic DNA was carried out using a modification of procedures described previously (6). Fragments of DNA, 15 - 20 kb in size, were obtained by preparative electrophoresis of partial Sau3A or HindIII-digested human fibroblast (MRC-5) DNA. These were inserted into λ₁₀₅₉ (26) or λ₂₀₀₁ (27) and libraries screened using probes derived from the hTM_{nm}-1 RNA-copy pseudogene.

Restriction enzyme analysis was carried out using single and double digestions performed according to the manufacturers' recommendations. Restriction fragments were electrophoresed in 1.0% agarose gels and transferred to nitrocellulose (28). Radioactive probes were prepared by exonuclease III digestion and "filling-in" with DNA polymerase (Klenow fragment) as described (6). Hybridization to blots of genomic DNA was carried out in a buffer containing 50% formamide (BDH) 10% dextran sulphate

Figure 1. Identification of the protein product encoded by the 2.5 kb mRNA in fibroblasts. Total human fibroblast (MRC-5) poly-A containing mRNA was selected by hybridization to a probe derived from the coding region (M29) and from the non-coding region (M43) of the pseudogene $hTM_{nm}-1$ exactly as described (6). mRNA was translated in a reticulocyte lysate cell free system and protein products analysed by two dimensional gel electrophoresis using isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulphate (SDS)/8-18% polyacrylamide gel electrophoresis in the second dimension. a) Translation products of total MRC-5 mRNA. b) Translation products of mRNA selected by M29. c) Translation products of mRNA selected by M43. The arrows indicate the five proteins which possess the characteristics of tropomyosin. The subscripts pl and nm indicate the two isoforms of TM30.

(29), 5 x SCC (SCC is 150 mM NaCl, 15 mM sodium citrate pH 6.8), 0.1% SDS, 0.1% polyvinylpyrrolidone, 0.1% Ficoll and 50 μ g sonicated denatured calf thymus DNA/ml. Filters were incubated overnight at 42°C and washed successively with 2 x SCC, 0.1% SDS at 37°C and with 0.5 x SCC, 0.1% SDS at 67°C. Hybridization of blots of recombinant lambda DNA was carried out in a buffer containing 3 x SCC, 0.1% SDS, 0.1% polyvinylpyrrolidone and 0.1% Ficoll. Filters were incubated overnight at 67°C and washed successively at 67°C with 3 x SCC, 0.1% SDS and 0.5 x SCC, 0.1% SDS. Labelled bands were detected by exposure to prefogged X-ray film at -80°C using an intensifying screen (30).

RESULTS AND DISCUSSION

Identification of the protein product of the 2.5 kb fibroblast mRNA

We have described an RNA copy pseudogene $hTM_{nm}-1$ which is closely related in structure and nucleotide sequence to a 2.5 kb mRNA expressed in human fibroblasts. Selection of mRNA using probes derived from $hTM_{nm}-1$

revealed that the 2.5 kb mRNA encoded a 30,000 molecular weight polypeptide (6). Using high resolution two dimensional gel electrophoresis we have found that there are two distinct isoforms of cytoskeletal tropomyosin having a molecular weight of 30,000, designated TM30_{p1} and TM30_{nm} (2). To determine which isoform is encoded by the 2.5 kb fibroblast mRNA the products of mRNA selection and cell free translation were analyzed by two dimensional gel electrophoresis (Fig. 1). A probe from the coding region of hTM_{nm}-1 selects mRNAs encoding TM30_{nm} and TM36, while the probe from the non-coding region selects TM30_{nm} alone. We conclude that the protein product of the 2.5 kb fibroblast mRNA is TM30_{nm}. The co-selection of TM36 by the coding region probe is due to homology between tropomyosin coding sequences. It is not due to shared exon sequences as determined by a direct comparison (not shown) of the sequence of TM36 (3) with that of TM30_{nm} presented here.

Molecular cloning and DNA sequence analysis of the 2.5 kb fibroblast mRNA

We have used probes derived from hTM_{nm}-1 to screen cDNA libraries of human fibroblast RNA. In libraries generated by priming with oligo dT alone the representation of sequences encoding the 2.5 kb fibroblast mRNA was very poor and only fragments of sequence were obtained. The reasons for this are not clear since it has proved straightforward to isolate full-length cDNA clones for other tropomyosin mRNAs from the same libraries. From the available sequence a specific oligonucleotide was prepared and, used in addition to oligo dT, to prime the synthesis of cDNA. Screening of libraries of cDNA prepared in this way led to the isolation of overlapping clones containing 2,077 nucleotides of the 2.5 kb mRNA. The available sequence includes 53 nucleotides of 5' untranslated sequence, the complete protein coding sequence and the complete 3' untranslated sequence (Fig. 2).

The protein sequence of TM30_{nm} deduced from the sequence of the 2.5 kb mRNA is 248 amino acids long (numbering from the initiator methionine). This confirms our previous conclusions, based on structural analysis of hTM_{nm}-1 (6), that the 2.5 kb fibroblast mRNA encoded a protein related in structure to horse platelet tropomyosin (4), a 247 amino acid non-muscle tropomyosin. However the amino acid sequence of TM30_{nm} deduced from structural analysis of the 2.5 kb mRNA is significantly different from that of horse platelet tropomyosin, varying from it by more than 25%. A detailed comparison of these sequences will be presented elsewhere.

The gene from which the 2.5 kb fibroblast mRNA is transcribed can also produce a 1.3 kb mRNA encoding a 285 amino acid α -tropomyosin in skeletal

muscle by alternative mRNA splicing (7). Furthermore, this structural gene is involved in the generation of the *trk* oncogene, a fusion of cytoskeletal tropomyosin sequences to a truncated tyrosine kinase receptor (11). In the recombinant oncogene the tropomyosin portion of the oncogene is spliced according to the pattern observed in the non-muscle tissue. Thus, the 2.5 kb fibroblast mRNA shows the same pattern of identical and non-identical nucleotide sequences when compared to the 1.3 kb muscle mRNA as does the tropomyosin portion of *trk* (Fig. 2). The boundaries of the regions of

TCACAGGTTGAGCCTACCAACAGCCACTGCTCATGATGGAGGCCATCAAGAAAAAGATGCAGATGCTGAAGTTAGACAAGGAGAATGCTCT	1.3	ATM	mRNA
M M E A I K K K M Q M L K L D K E N A L			
	10		
CGCCGAGCGCAGGAGGCGAGAACCGGAGCGGAGCAGTAGCTGGGTGGGCACCATGGCTGGGATCACCAACCTGAGGCGGGTGAGGCGCAA	2.5	fib	mRNA
* * * * *			
GGATCGGGCTGAGCAAGCTGAAGCTGAGCAGAAAGCAGGCGAGAAGAAAGTAAACAGCTGGAGGATGAGCTGGCAGCCATGCAGAAAGAA	1.3	ATM	mRNA
D R A E Q A E A E Q K Q A E R S K Q L E D E L A A M Q K K			
	20	30	40
I Q V L Q Q Q A D D A E R R A E R L Q R E V E G E R E A R E			
GATCCAGGTTCTGCGACGAGCAGATGATGACAGGAGCGAGCTGAGCGCCTCCAGCGAGAAGTTGAGGGAGAAAGCGGGGCCGGGA	2.5	fib	mRNA
* * * * *			
GCTGAAAGGGACAGAGGATGAGCTGGACAAGTATTCTGAAGCTTTGAAGGATGCCAGGAGAAGCTGGAACTGGCAGAGAAGAAGGCTGC	1.3	ATM	mRNA
L K G T E D E L D K Y S E A L K D A Q E E L E L A E K K A A			
	50	60	70
Q A E A E E V A S L N E R I Q L V E E E R L D B A Q E R L A T A			
ACAGGCTGAGGCTGAGGTCCTCTTGAACCGTAGGATCCAGCTGGGTTGAAGAAGAGCTGGACCGTGCAGGAGCGCTGGCCACTGCG	2.5	fib	mRNA
* * * * *			
TGATGCTGAGGCTGAGGTGGCTCCTTGAACCGTAGGATCCAGCTGGTGAAGAAGAGCTGGACCGTGCAGGAGCGCTGGCCACTGCG	1.3	ATM	mRNA
D			
	80	90	100
L Q K L E E A K A A D E S E R G M K V I E N R A L K D E E			
CCTGCAAAAGCTGGAAGAGCTGAAAAGCTGCTGATGAGAGTGAGAGAGGTATGAAGGTTATTGAAAACCGGGCCTTAAAGATGAAGA	2.5	fib	mRNA
* * * * *			
CCTGCAAAAGCTGGAAGAGCTGAAAAGCTGCTGATGAGAGTGAGAGAGGTATGAAGGTTATTGAAAACCGGGCCTTAAAGATGAAGA	1.3	ATM	mRNA
	110	120	130
K M E L Q Q E I Q L K E A K H I A E E A D R K Y E E V A R K L			
AAAGAGTCCAGGAATCCAACTCAAAGAACTAAGCACATTGCGAGAAGAGGCAGATAGGAAGTATGAAGAAGTGCGCTCGTAAGTT	2.5	fib	mRNA
* * * * *			
AAAGATGGAATCCAGGAATCCAACTCAAAGAACTAAGCACATTGCGAGAAGAGGCAGATAGGAAGTATGAAGAAGTGCGCTCGTAAGTT	1.3	ATM	mRNA
	140	150	160
V I I E G D L E R T E E R A E L A E S R C R E M D E Q I R L			
GGTGATCATTGAAGGAGACTTGAACGCGACAGAGGAACGAGCTGAGCTGGCAGAGTCCCGTTGCCGAGAGATGGATGAGCAGAGTAGACT	2.5	fib	mRNA
* * * * *			
GGTGATCATTGAAGGAGACTTGAACGCGACAGAGGAACGAGCTGAGCTGGCAGAGTCTAAGTGTTCTGAGCTGGAGGAGGAGCTGAAGAA	1.3	ATM	mRNA
K C S E L E E E L K N			
	170	180	190
M D Q N L K C L S A A E E K Y S Q K E D K Y E E E I K I L T			
GATGACAGAGAACCTGAGAGTCTGAGTGTCTGAAGAAAGTACTCTCAAAAAGAGATAAATATGAGGAAGAAATCAAGATTCTTAC	2.5	fib	mRNA
* * * * *			
TGTACCAACAACCTCAAGTCTCTTGAGGCTCAGGCGGAGAGTACTCTCAAAAAGAGATAAATATGAGGAAGAAATCAAGATTCTTAC	1.3	ATM	mRNA
V T N N L K S L E A Q A E K			
	200	210	220
D K L K E A E T R A E F A E R S V A K L E K T I D D L E D K			
TGATAAAGCTCAAGGAGGACAGAGACCCGTGCTGAGTTTGTCTGAGAGATCGGTAGCCAAAGCTGGAAAAGACAATTGATGACCTGGAGAGATAA	2.5	fib	mRNA
* * * * *			
TGATAAAGCTCAAGGAGGACAGAGACCCGTGCTGAGTTTGTCTGAGAGATCGGTAGCCAAAGCTGGAAAAGACAATTGATGACCTGGAGAGATAA	1.3	ATM	mRNA
E D E			
	230	240	
L K C T K E E N L C T Q E M L D Q T L L D L N E M stop			
ACTGAAATGACCAAGAGGAGGACCTCTGTACAGAAAGATGCTGGACGACGACCTGCTGACCTGAATAGAGATGAGAGAGCCGAGT	2.5	fib	mRNA
* * * * *			
GCTCTATGCCAGAAAGTGAAGTACAAAGCCATTAGCGGAGGAGCTGGACCAAGCCCTCAATGACATGACCTCTATATAATTATCACGGTT	1.3	ATM	mRNA
L Y A Q K L K Y K A I S E E L D H A L N D M T S I stop			
CCACCCGTGCTGCTGCTCCTCCTCTGACCCAGACTCCGCTGAGGCCAGCTGCGGGAAGCTGACCTTTAACTGAGGCTGATCTTTAAC	2.5	fib	mRNA
* * * * *			
TCTGCTCTGTTGGATCTGCCCCCTTTACTCTCGGGGAAGCCAAAGGCCCACTCTCGCTCTGGATTCATTGGGTGAGCTGGCTGG	1.3	ATM	mRNA
TGGAAGGCTGCTTCTCCTTTACCACCCCTCTCTCCCTGTGTCTTTTTCGCCAAACTGTCTCTGCTCTTCCGGAGATCCAGCTGGG	2.5	fib	mRNA
* * * * *			
TCCCCAAGGCATTAGGATGGGGGAGCAAAAAGCAACTATGTATTTTCTCCACCCCAACCCCAAAATAAATGTTAAGCTGCTGG	1.3	ATM	mRNA

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CTAGAGGCTGAGCACCTTTGGAAACAACATTTAAGGGAATGTGAGCACAAATGCATAATGTCTTTAAAAAGCATGTTGTGATGTACACATT 2.5 fib mRNA
TTGTAATTACCTTTTTTGTGTTTGTAGCAACCAATTTGTAAGCAATTCCTCAATATCCACAGTCTGTAAGCAGCAATCGAATCCCTTT 2.5 fib mRNA
CTCACCTTTTGGAAAGGTGACTTTTCACCTTAATGCATATTCCTCTCCATAGAGGAGAGGAAAAAGGTGTAGGGCTGCCCTTACCGAGAGGCC 2.5 fib mRNA
AAACAGAGCCCAGGGAGACTCCGCTGTGGGAAACCTCATTGTTCTGTACAAAGTACTAGCTAAACCAGAAAGGTGATTCCAGGAGGAGTT 2.5 fib mRNA
AGCCAAACAACAACAAAAAATAATGTGCTGTTCAAGTTTTCAGCTTTAAGATATCTTTGGATAATGTTATTCTATTATTTTATTTTTT 2.5 fib mRNA
TTCATTAGAAGTTACCAATTAAGATGGTAAGACCTCTGAGACCAAAATTTGTCCCATCTCTACCCCCCTCACAACCTGCTTACAGAATGGA 2.5 fib mRNA
TCATGTCCCCCTTATGTTGAGGTGACCACCTTAATTGCTTTCTGCTCCTTGAAAAGAAAGAAAGAAAGAAAGACTGTGTTTTTGCCACTGA 2.5 fib mRNA
TTTAGCCATGTGAAACTCATCTCATCACCCCTTTCTGGGTTTGAAGCTGCTGCTCTAGAAGTGCCATCTCAATTGTGCTTTGTATCAGT 2.5 fib mRNA
CAGTGTGGAGAAAATCTTGAATAAGCTTATGTACAAAACCTTTTAAATTTTATATTATTTTGAACCTTTGCTTTGGGTTTGTGGCACCCTG 2.5 fib mRNA
GCCACCCCATCTGGCTGTGACAGCCTCTGCAGTCCGTGGGCTGGCAGTTTGTGTGACTCTTTTAAGTTTCTCTCCCTACCCAGTCCCCATT 2.5 fib mRNA
TTCTGGTAAGGTTTCTAGGAGGTCTGTTAGGTGTACATCTGCAGCTTATTGGCTTAAATGTACTCTCCTTTTATGTGTCTCTTTGGG 2.5 fib mRNA
GCCGATTGGGAGAAAGAGAAATCAATAGTCAACTGTTTTGATACTGAATATTGACAAGTGTCTTTTGAATAAAGAACCAAGTCCCTCC 2.5 fib mRNA
AACCCCTCAAAAAAAAAAAAA 2.5 fib mRNA

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Figure 2. Sequence analysis of the 2.5 kb mRNA in human fibroblasts. The sequence of the 2.5 kb fibroblast mRNA (2.5 fib mRNA) is shown compared to the sequence of the 1.3 kb muscle mRNA (1.3 ATM mRNA) encoding a 285 amino acid skeletal muscle α -tropomyosin. The protein sequence of TM30_{nm} is numbered from the initiator methionine residue. The protein sequence encoded by the 1.3 kb muscle mRNA is shown only where it varies from that encoded by the 2.5 kb fibroblast mRNA. The asterisks indicate identical nucleotides. Note the identity of nucleotide sequence encoding amino acids 45-152 and 177-222 of the 248 amino acid molecule with those encoding the homologous sequences of the 285 amino acid muscle molecule.

Figure 3. Determination of the 5' end of the 2.5 kb mRNA. Experimental strategy: a BamHI-SmaI fragment of genomic DNA containing exon I_{nm} (7) was subcloned into M13mp18 (A). Single-stranded probes were generated by the prime cut method (24). The labelled probes are defined at the 5' end by the sequencing primer and at the 3' end by restriction enzyme cleavage. Digestion with EcoRI was used to produce a probe suitable for SI nuclease protection (B). The hatched box indicates the region of the probe protected by hybridization to the 2.5 kb mRNA. Digestion with NcoI was used to produce a probe suitable for primer extension by reverse transcriptase after hybridization to the 2.5 kb mRNA. C) The hatched box indicates the extension of the original probe. A scale of the region in base pairs is given (D).

Figure 4. Determination of the 5' end of the 2.5 kb mRNA. Experimental results: products of SI protection of the EcoRI probe after hybridization in the presence (lane 2) and absence (lane 3) of human fibroblast mRNA were compared to the undigested EcoRI probe (lanes 4,5) on a 6% acrylamide/8M urea sequencing gel. Note the 195 nucleotide fragment protected by hybridization to mRNA. Extension of the 173 nucleotide NcoI probe with reverse transcriptase to 241 nucleotides (arrowed) is shown in lane 8. Lanes 1,6,7,9 are size markers of pBR322 digested with MspI and end-labelled.


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TGAGTTCGCGTATTTTCAGGCGGTAGCAGGCGGAAGTAAGGGTGAGAGGAGGCTGCAACGCCGAGCGGAGGAGGCAGGAACCGGAGCGCGA exon Inm
*** * **** * * *** * *****
ATTCTTCTTGGAATTTTCCCATGCGCAAAAmctttgccGGTGAGAGGAGGCTGCAACACCGGAGCGGAGGAGGCAGGAACCGGAGCGCGA hTMnm-1
                               30                               60                               90

      M A G I T T I E A V K R K I Q V L Q Q Q A D D
GCAGTAGCTGGGTGGGCACCATGGCTGGGATCACCACCATCGAGGCGGTGAAGCGCAAGATCCAGGTTCTGCAGCAGCAGGCAGATGATG exon Inm
*****
GCAGTAGCTGGGTGGGCACCATGGCTGGGATCACCACCATCGAGGCGGTGAAGCGCAAGATCCAGGTTCTGCAGCAGCAGGCAGATGATG hTMnm-1
                               120                               150                               180

A E E R A E R L Q R E V E G E R R A R E Q
CAGAGGAGCGAGCTGAGCGCTCCAGCGAGAAAGTTGAGGGAGAAAGCGGGCCCGGGAACAGGTACGGAGATGGTGAGGCTCATGAGGGA exon Inm
*****
CAGAGGAGCGAGCTGAGCGCTCCAGCGAGAAAGTTGAGGGAGAAAGCGGGCCCGGGAACAGGTACGGCTGAGGTGGCCTCTCTGAACC hTMnm-1
                               210                               240                               270

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Figure 5. Comparison of the DNA sequences of the structural gene and the RNA-copy pseudogene hTM_{nm}-1. The sequence of the structural gene containing exon I_{nm} (7) is shown compared to the sequence of hTM_{nm}-1 (6). Asterisks indicate identical nucleotide residues. The coding sequence of exon I_{nm} extends from nucleotide 111 to 242. The 5' limit of homology of the two sequences is nucleotide 40. This corresponds to 5' boundary of the pseudogene defined by the 9 nucleotide direct repeat ACTTTTGCC (lower case letters). Note that this is within the sequence AGGGTG to which the 5' end of the 2.5 kb mRNA had been mapped by SI protection and primer extension. The cDNA sequence begins at nucleotide 58.

sequence identity and non-identity correspond to the boundaries of exons which are alternatively spliced in the structural gene encoding the 2.5 kb fibroblast and 1.3 kb muscle mRNA'S (7).

Determination of the start of transcription of the 2.5 kb fibroblast mRNA

The apparent start of transcription of the 2.5 kb fibroblast mRNA was determined by SI nuclease protection and primer extension experiments (Fig. 3,4). A BamHI-SmaI fragment of genomic DNA containing the 5' coding sequences of the 2.5 kb fibroblast mRNA was inserted into M13mp18. This was used as template to prepare two single-stranded probes by the prime-cut method (24). For the SI nuclease protection experiment a 398 nucleotide EcoRI probe was used (Fig. 3). A 195 nucleotide fragment of this probe is protected from SI nuclease digestion by hybridization to human fibroblast mRNA (Fig. 4). For the primer extension experiment a 173 nucleotide NcoI probe was used. This can be extended a further 68 nucleotides by elongation with reverse transcriptase after hybridization to mRNA. Both procedures map the 5' end of the 2.5 kb fibroblast mRNA to the same point in the BamHI-SmaI segment of genomic DNA, about 70 nucleotides upstream of the initiator methionine within the sequence AGGGTG. This is 18 nucleotides upstream of the 5' limit of the cDNA sequence. It corresponds to the 5' limit of homology between the genomic sequence containing exon I_{nm} and the hTM_{nm}-1 RNA copy pseudogene sequence (Fig. 5). It further corresponds to the 5' boundary of the hTM_{nm}-1 RNA-copy pseudogene sequence as defined by the 9

Figure 6. Restriction enzyme analysis of lambda clones containing sequences hybridizing to hTM_{nm}-1. Positions of restriction enzyme cleavage sites were determined by analysis of single and double digests using EcoRI (R), BamHI (B) and HindIII (H). The approximate region of sequence hybridizing to hTM_{nm}-1 is indicated by the heavy bar. The vertical arrows mark the boundaries of the human DNA insert in each clone. The phage λ241 and λ242 were isolated from a λ1059 library and therefore contain a HindIII and EcoRI site in the right arm of the vector. The other phage was isolated from a λ2558 library and contain natural HindIII cleavage sites at the point of insertion of the human DNA into the vector.

nucleotide sequence ACTTTTGCC which is repeated at the 3' boundary (6). Therefore, we conclude that the start of transcription of the 2.5 kb fibroblast mRNA occurs within the AGGGTG sequence approximately 70 nucleotides upstream of the initiator methionine codon in the exon I_{nm} sequence. This suggests that the promoter from which transcription of the 2.5 kb fibroblast mRNA is initiated occurs directly upstream of this point. This possibility is now being tested.

It is interesting to note that the structural organization of the 5' untranslated sequence of the tropomyosin coding sequence in the *trk* oncogene is different from that of the 2.5 kb fibroblast mRNA (comparison not shown). The *trk* transcript contains all the 5' untranslated sequences of the 2.5 kb fibroblast mRNA directly upstream of the initiator methionine codon but contains additional nucleotide sequence which extends the *trk* transcript in the 5' direction (11). This implies that the *trk* transcript and the 2.5 kb fibroblast mRNA's are transcribed from different promoters possibly as a result of sequence rearrangements occurring during the generation of the *trk*

Figure 7. Genomic representation of the sequences present in lambda clones hybridizing to hTM_{nm}-1. Human fibroblast DNA (A) was digested with BamHI and Hind III and compared to similar digests of λ 242 (B), λ H211 (C), λ 241 (D), λ H81 (E), λ H132 (F), λ H232 (G) and λ H51 (H) by electrophoresis in a 1.0% agarose gel. Restriction fragments were transferred to nitrocellulose and hybridized to M2, an M13 subclone of hTM_{nm}-1. The arrows indicate the positions of fragments present in genomic DNA but not represented in the cloned lambda DNA presented here. The arrow marked (f) indicates the fragment now determined to contain functional gene sequences.

oncogene. However, it may also be due to differences in post-transcriptional processing of the two transcripts.

Structure of the RNA-copy pseudogenes

We have characterized seven RNA-copy pseudogenes of this gene family by molecular cloning, restriction enzyme analysis and DNA sequence analysis. Each of the cloned sequences possesses a unique pattern of restriction enzyme sites (Fig. 6) indicating that each one represents a unique chromosomal locus rather than an allelic variant of another sequence. Digestion of each cloned sequence with BamHI and HindIII produces a unique fragment which comigrates with a fragment found in a similar digest of human genomic DNA (Fig. 7). Together with the previously characterized functional gene sequences these pseudogenes account for most if not all of the genomic complexity of this gene family. All the pseudogenes contain sequences hybridizing to probes from both the coding and non-coding regions of the hTM_{nm}-1 pseudogene (not shown). We have determined the complete DNA sequences of three of these pseudogenes and carried out a partial sequence analysis of the 5' coding sequences of the remaining four. All the cloned

Figure 8. Comparison of the sequence of the RNA-copy pseudogenes with that of the 2.5 kb fibroblast mRNA. The comparison is carried out using the program DIAGON (22), scoring for 8 identical nucleotides in a span of 11. The sequence of the 2.5 kb fibroblast mRNA is the vertical axis. The sequences of genomic DNA containing the RNA-copy pseudogenes $hTM_{nm}-1$ and $hTM_{nm}-AL$ are on the horizontal axis. Note that the homology to the genomic DNA sequences extends across the entire length of the sequence of the 2.5 kb mRNA. The discontinuities in $hTM_{nm}-AL$ arise as a consequence of Alu insertions into the RNA-copy pseudogene sequence.

sequences were found to lack intron sequences and to contain errors in the reading frame of the protein coding sequence, features characteristic of RNA-copy pseudogenes. The sequences of the pseudogenes for which a complete DNA sequence analysis had been carried out were compared to the cDNA sequence of the 2.5 kb fibroblast mRNA using the program DIAGON (23). The sequences of two of these pseudogenes ($hTM_{nm}-1$, $hTM_{nm}-2$) are co-linear with that of the 2.5 kb mRNA and very closely related but not identical to it at the nucleotide level (Fig. 8). The sequence of the third pseudogene $hTM_{nm}-AL$ is also very closely related at the nucleotide level to the 2.5 kb mRNA in both coding and non-coding sequences. However, it is not co-linear in sequence as is apparent from the interruptions in the diagonal relationship (Fig. 8). This is due to the presence of three consensus Alu repeat sequences (31) which have become inserted into the pseudogene sequence. However, in all other respects these three pseudogene sequences reflect the pattern of alternative mRNA splicing of this tropomyosin structural gene in non-muscle tissue. DNA sequence analysis (not shown) of the 5' coding sequence of the remaining four pseudogenes revealed that all contained nucleotide sequences encoding the N-terminal amino acids characteristic of the 248 amino acid cytoskeletal tropomyosin. Thus, the

structure of the cloned RNA-copy pseudogenes reflects the pattern of splicing of the non-muscle transcript of this gene to the exclusion of the muscle transcript. This supports the notion that most such RNA-copy pseudogenes arise by information transfer (retroposition) from processed mRNAs normally expressed in germ-line tissue (32).

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