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A muscle-type tropomyosin in human fibroblasts: Evidence for expression by an alternative RNA splicing mechanism

(cytoskeleton/skeletal muscle/alternative splicing)

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ABSTRACT **We have isolated a cDNA clone from a human fibroblast cDNA library that contains the entire protein-coding region of a 1.1-kilobase mRNA. This mRNA encodes a 284-amino acid tropomyosin, the primary structure of which most closely resembles smooth muscle tropomyosin. Thus, the expression of both 284-amino acid muscle-type and 247-amino acid non-muscle-type tropomyosins appears to be a normal feature of human non-muscle cells. We also present evidence to suggest that this cytoskeletal tropomyosin and a human skeletal muscle β-tropomyosin are derived from a common structural gene by an alternative RNA splicing mechanism.**

Tropomyosins are proteins that were first isolated from skeletal muscle (1) but which, like actin, are also found in other types of muscle and most non-muscle tissues (2). In skeletal muscle, tropomyosin serves to mediate the effect of Ca^{2+} on the actin–myosin interaction. It does so, not by binding Ca^{2+} directly but, through interaction with the troponins, one of which (troponin T) binds to tropomyosin at a specific site in the carboxyl-terminal region of the skeletal muscle tropomyosin molecule (3, 4). In smooth muscle and non-muscle tissues, which lack troponins, tropomyosin has a characteristic and different carboxyl-terminal primary structure (5, 6).

Tropomyosins from skeletal and smooth muscle have a monomeric size of 284 amino acids, whereas characterized tropomyosins from non-muscle sources have a monomeric size of only 247 amino acids (7). This variation in size is commonly thought to represent fundamental differences in the functions of tropomyosin in muscle and non-muscle tissues. Recent studies have revealed a surprising heterogeneity of non-muscle tropomyosins (8–10). We have found that cultured human fibroblasts contain at least four distinct polypeptides having the acidic isoelectric point and resistance to denaturation characteristic of tropomyosin (11). We have called them heat-stable cytoskeletal proteins (hscp) and numbered them according to their apparent molecular weight $\times 10^{-3}$: 30,000 (hscp30), 32,000 (hscp32), 34,000 (hscp34), and 36,000 (hscp36) (11). High resolution two-dimensional gel electrophoresis has revealed that the hscp30 component is itself heterogeneous, being composed of at least two distinct polypeptides (12). A similar striking heterogeneity of tropomyosin-like proteins has also been observed in rat fibroblasts and other cells. To assess the significance of this heterogeneity, we have undertaken a characterization of the tropomyosin-like proteins of human non-muscle cells at the molecular level. In this paper, we show that, in addition to 247-amino acid tropomyosins of the classic non-muscle type, a variety of human cells also express a 284-amino acid muscle-type tropomyosin. Furthermore, we present evidence to suggest that this tropomyosin is derived from a gene that also encodes a skeletal muscle β-tropomyosin.

METHODS

RNA Blot Hybridization Analysis. RNA samples were denatured with formaldehyde, electrophoresed in a 1.2% agarose gel, and transferred to nitrocellulose as described (13). Hybridization was carried out as described (14) with a final wash in 0.5 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 67°C. Three different probes were used, all derived from human RNA copy pseudogenes containing non-muscle tropomyosin sequences as determined by sequence analysis after insertion into phage M13 mp8. Probe M29 hybridizes to a 2.5-kilobase (kb) mRNA and has been described in detail (14). Probe M1401 hybridizes to a 3.0-kb mRNA and will be described in detail elsewhere. Probe M1558 was isolated during a screen of the human genome with pβTM7, a chicken cDNA clone that hybridizes to a 1.1-kb mRNA from human fibroblasts (11). Like pβTM7, M1558 also hybridizes to this 1.1-kb mRNA.

Molecular Cloning and Sequence Analysis. A λgt10 library of human MRC-5 fibroblast cDNA was constructed using standard procedures (15). This was screened with probe M1558. The recombinant containing the largest insert was isolated, then the cDNA fragment was inserted into phage M13 mp18. Replicative form was prepared, and processively shortened molecules were generated by unidirectional digestion with exonuclease III according to the procedure of Henikoff (16). Sequence analysis was carried out using the dideoxy chain-termination procedure (17), and the sequences were aligned and decoded using the computer programs of Staden (18, 19). A library of human skeletal muscle cDNA (20) constructed according to the method of Okayama and Berg (21) was generously provided by L. Kedes. This was screened with a rabbit β-tropomyosin cDNA clone (22) provided by S. Putney.

cDNA clones corresponding to the 2.5- and 3.0-kb cytoskeletal tropomyosin mRNAs were also isolated from the human fibroblast λgt10 cDNA library using the M29 and M1401 probes, respectively. These cDNA clones were subject to sequence analysis and were also used to screen phage λ libraries of human fibroblast DNA at high stringency. Phage containing portions of the functional genes encoding the 2.5- and 3.0-kb mRNAs were isolated and characterized by DNA sequence analysis.

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Abbreviations: kb, kilobase(s); hscp, heat-stable cytoskeletal proteins.

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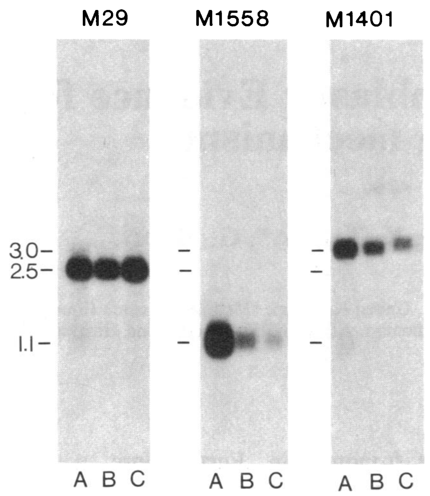

FIG. 1. Blot hybridization analysis of RNA from human cell lines. Equivalent amounts of poly(A)-containing RNA from human MRC-5 fibroblasts (lanes A), HeLa cells (lanes B) and HT-29 cells (lanes C) were electrophoresed in the presence of formaldehyde, transferred onto nitrocellulose sheets, and hybridized with the RNA-copy pseudogenes indicated. The numbers at the left refer to the apparent sizes in kb of the RNA hybridizing to the three different probes.

RESULTS AND DISCUSSION

Human Cytoskeletal Tropomyosin mRNAs. We can define three distinct mRNAs in human non-muscle cells that encode cytoskeletal tropomyosins by using probes derived from RNA-copy pseudogenes (Fig. 1). Analysis of an RNA copy pseudogene homologous to the 2.5-kb mRNA (M29) demonstrated that this mRNA encodes a 247-amino acid non-muscle tropomyosin, migrating with an apparent molecular weight of 30,000 on $NaDodSO_4$/polyacrylamide gels (14). A second RNA-copy pseudogene (M1401) hybridizes selectively to a 3.0-kb mRNA. Analysis of the corresponding full-length cDNA indicates that this mRNA encodes a 247-amino acid non-muscle tropomyosin which is distinct from the isoform encoded by the 2.5-kb mRNA (unpublished results). A third pseudogene, M1558, hybridizes to a 1.1-kb mRNA which we have shown previously encodes hscp36, the largest of the tropomyosin-like proteins in human fibroblasts (11). These three mRNAs are expressed in human fibroblasts, and in two cell lines derived from carcinomas, HeLa and HT-29 (23). The expression of the 2.5- and 3.0-kb mRNAs encoding 247-amino acid tropomyosin isoforms is relatively constant in the three cell lines examined (Fig. 1). By comparison, the expression of the 1.1-kb mRNA shows a dramatic variation. Furthermore, the protein encoded by this mRNA (hscp36) was the only protein in human fibroblasts to show a significant cross-reactivity with anti-skeletal muscle tropomyosin antiserum (11). It was, therefore, of considerable interest to determine the structure of the protein encoded by this mRNA.

A cDNA Encoding a Fibroblast 284-Amino Acid Tropomyosin. A library of human fibroblast cDNA in λgt10 was screened with the M1558 probe, and positive clones were tested for insert size. The largest fragment obtained was inserted into phage M13 mp18, and DNA sequence analysis

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TGCTGCTCTCCTCCCGCTCCGTCCTCCTCGCCTGCCACCGGTGCACCCAGTCCGCTCACCCAGCCCAGTCCGTCCGGTCCTCACCGCCTG  1.1  TM mRNA
                                         5                                           20
                             M  D  A  I  K  K  K  M  Q  M  L  K  L  D  K  E  N  A  I  D  R
CCGGCCGGCCCACCCCCCACCGCAGGCCATGGACGCCATCAAGAAGAAGATGCAGATGCTGAAGCTGGACAAGGAGAACGCCATCGACCG  1.1  TM mRNA
                                        35                                           50
  A  E  Q  A  E  A  D  K  K  Q  A  E  D  R  C  K  Q  L  E  E  E  Q  Q  A  L  Q  K  K  L  K
CGCCGAGCAGGCCGAAGCCGACAAGAAGCAAGCTGAGGACCGCTGCAAGCAGCTGGAGGAGGAGCAGCAGGCCCTCCAGAAGAAGCTGAA  1.1  TM mRNA
                                        65                                           80
  G  T  E  D  E  V  E  K  Y  S  E  S  V  K  E  A  Q  E  K  L  E  Q  A  E  K  K  A  T  D  A
GGGGACAGAGGATGAGGTGGAAAAGTATTCTGAATCCGTGAAGGAGGCCCAGGAGAAACTGGAGCAGGCCGAGAAGAAGGCCACTGATGC  1.1  TM mRNA
                                        95                                           110
  E  A  D  V  A  S  L  N  R  R  I  Q  L  V  E  E  E  L  D  R  A  Q  E  R  L  A  T  A  L  Q
TGAGGCAGATGTGGCCTCCCTGAACCGCCGCATTCAGCTGGTTGAGGAGGAGCTGGACCGGGCCCAGGAGCGCCTGGCTACAGCCCTGCA  1.1  TM mRNA
                                        125                                          140
  K  L  E  E  A  E  K  A  A  D  E  S  E  R  G  M  K  V  I  E  N  R  A  M  K  D  E  E  K  M
GAAGCTGGAGGAGGCCGAGAAGGCGGCTGATGAGAGCGAGAGAGGAATGAAGGTCATCGAAAACCGGGCCATGAAGGATGAGGAGAAGAT  1.1  TM mRNA
                                        155                                          170
  E  L  Q  E  M  Q  L  K  E  A  K  H  I  A  E  D  S  D  R  K  Y  E  E  V  A  R  K  L  V  I
GGAACTGCAGGAGATGCAGCTGAAGGAGGCCAAGCACATCGCTGAGGATTCAGACCGCAAATATGAAGAGGTGGCCAGGAAGCTGGTGAT  1.1  TM mRNA
                                   *******************************************************
                                   ACATCGCTGAGGATTCAGACCGCAAATATGAAGAGGTGGCCAGGAAGCTGGTGAT  SKTM
                                        185                                          200
  L  E  G  E  L  E  R  S  E  E  R  A  E  V  A  E  S  R  A  R  Q  L  E  E  E  L  R  T  M  D
CCTGGAAGGAGAGCTGGAGCGCTCGGAGGAGAGGGCTGAGGTGGCCGAGAGCCGAGCCAGACAGCTGGAGGAGGAACTTCGAACCATGGA  1.1  TM mRNA
***************************************************   *    *  * ** ******** **   **   *
CCTGGAAGGAGAGCTGGAGCGCTCGGAGGAGAGGGCTGAGGTGGCCGAGAGTAAATGTGGGGACCTAGAGGAGGAGCTGAAAATTGTTAC  SKTM
                                                     K  C  G  D  L  E  E  E  L  K  I  V  T

                                        215                                          230
  Q  A  L  K  S  L  M  A  S  E  E  E  Y  S  T  K  E  D  K  Y  E  E  E  I  K  L  L  E  E  K
CCAGGCCCTCAAGTCCCTGATGGCCTCAGAGGAGGAGTATTCCACCAAAGAAGATAAATATGAAGAGGAGATCAAACTGTTGGAGGAGAA  1.1  TM mRNA
* *   * * **  *****  ****   * ***  *******************************************************
CAACAACTTGAAATCCCTGGAGGCCCAGGCGGACAAGTATTCCACCAAAGAAGATAAATATGAAGAGGAGATCAAACTGTTGGAGGAGAA  SKTM
  N  N  L  K  S  L  E  A  Q  A  D  K
                                        245                                          260
  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  E  T  L  A
GCTGAAGGAGGCTGAGACCCGAGCAGAGTTTGCCGAGAGGTCTGTGGCAAAGTTGGAGAAAACCATCGATGACCTAGAAGAGACCTTGGC  1.1  TM mRNA
*********************************************************************************     *
GCTGAAGGAGGCTGAGACCCGAGCAGAGTTTGCCGAGAGGTCTGTGGCAAAGTTGGAGAAAACCATCGATGACCTAGAAGATGAAGTCTA  SKTM
                                                                                D  E  V  Y
                                        275
  S  A  K  E  E  N  V  E  I  H  Q  T  L  D  Q  T  L  L  E  L  N  N  L  *
CAGTGCCAAGGAGGAGAACGTCGAGATTCACCAGACCTTGGACCAGACCCTGCTGGAACTCAACAACCTGTGAGGGCCAGCCCCACCCCC  1.1  TM mRNA
       ***  * ** **   *  ***  * **    ***** *  * **    **  *** *  *** ****  ***  **** *
TGCCCAGAAGATGAAGTACAAGGCCATTAGCGAGGAACTGGACAACGCACTCAATGACATCACCTCCCTCTGAGCCCCACGCCCAGCGTG  SKTM
  A  Q  K  M  K  Y  K  A  I  S  E  E  L  D  N  A  L  N  D  I  T  S  L  *

AGCCAGGCTATGGTTGCCACCCCAACCCAATAAAACTGATGTTACTAGCCTCTC-POLY A                                1.1  TM mRNA
   *      *   * *   **   **     *   **    *
CCACCTCAGCTCTCTTCTCTCCTCTCCTTTCCATTCTCTCTATGGGGAGGGGAGCAGGCAGGAGGAGCAGAAATTGCCAACATTGCACAG  SKTM

CCAGGCTGGGAGCAGCCTAGGGAGAGCCCCCATCATGCCCACCACCCACTCTGGCACTGGCTTCATCCTTTACCTATCCCCTTCCACCCT  SKTM mRNA

CCTTTGCTTGCTTAATAAATTCTGAACTTGG-POLY A                                                       SKTM mRNA
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FIG. 2. Sequence analysis of a cDNA complementary to the 1.1-kb human fibroblast tropomyosin mRNA (1.1 TM mRNA) and comparison with a human skeletal muscle β-tropomyosin cDNA (SKTM mRNA). Positions of nucleotide identity in the two sequences are indicated by *. The sequence AATAAA, the putative signal for polyadenylylation, is underlined.

Table 1. Percentage identical amino acid residues in tropomyosin sequences

	Human tropomyosin	
	Fibroblast	Skeletal muscle
Rabbit skeletal α-tropomyosin	76.8	77.2
Rabbit skeletal β-tropomyosin	86.3	100.0
Chicken gizzard β-tropomyosin	96.5	62.4

The amino acid sequences of the human fibroblast tropomyosin (284 amino acids) and the available sequence of the human skeletal muscle tropomyosin (101 amino acids) were compared to the known protein sequences of rabbit and chicken muscle tropomyosins. The number of identical amino acids was noted and is expressed as a percentage of the number of residues compared.

was carried out. This revealed that the insert was 1050 bases long, comprising a 5′ untranslated sequence of 120 bases, a protein-coding sequence of 852 bases, and a 3′ untranslated sequence of only 80 bases (Fig. 2). The structure of this mRNA is in striking contrast to that of the 2.5- and 3.0-kb mRNAs whose large size is due to exceptionally long 3′ untranslated sequences.

The 852-base reading frame of the fibroblast 1.1-kb mRNA encodes a tropomyosin that is 284-amino acids long. Thus, the higher apparent molecular weight of hscp36 results from a real difference in size: it is identical in length to tropomyosins from muscle sources. Comparison of the deduced protein sequence with that determined for tropomyosins of skeletal muscle and smooth muscle origin indicates that it most closely resembles chicken gizzard smooth muscle β-tropomyosin (5, 6) (Table 1). This similarity presumably accounts for its cross-reactivity with anti-muscle tropomyosin antiserum. Note that the sequence referred to as chicken gizzard smooth muscle β-tropomyosin in ref. 6 is identical to a sequence referred to as chicken gizzard α-tropomyosin in ref. 5.

Thus, not only do non-muscle cells express two distinct tropomyosins of the classic 247-amino acid non-muscle type, they also express a 284-amino acid muscle-type tropomyosin. This muscle-type tropomyosin is expressed in human cells of very different embryological origins, albeit at different levels (Fig. 1). Muscle-type and non-muscle-type tropomyosins differ not only in actin binding capacity (24) but also in their head-to-tail polymerization properties (25). It is, therefore, reasonable to expect that these differences will be reflected in different functional roles for the two types of tropomyosin in the cytoskeleton.

Giometti and Anderson (26) have recently described a protein in human fibroblasts designated TM:3 that has characteristics of smooth muscle tropomyosin. The similarity of hscp36, the product of the 1.1-kb fibroblast mRNA, to smooth muscle tropomyosin suggests that hscp36 and TM:3 are one and the same protein. However, it remains to be established whether the protein expressed in human non-muscle tissues is in fact identical to the protein expressed in human smooth muscle.

A cDNA Encoding Human Skeletal Muscle β-Tropomyosin. Tropomyosin isoforms can be generated not only by expression of related functional genes but also by alternative splicing of the transcripts of these functional genes. Generation of isoform diversity by alternative RNA splicing is now well documented, particularly for expression of proteins in muscle tissues such as tropomyosin (27–29), troponin T (30), and myosin light chain (31). In *Drosophila melanogaster*, alternative splicing of an exon encoding amino acids 258–284 generates tropomyosin isoforms that are identical from amino acids 1–257 but have different carboxyl-terminal sequences (27, 28). In rat, alternative RNA splicing apparently generates a skeletal muscle tropomyosin in skeletal muscle and a smooth muscle tropomyosin in smooth muscle from a single structural gene (29). While tropomyosin protein sequences are normally very conserved, the important and characteristic feature of tropomyosins derived by alternative RNA splicing mechanisms is that they are identical over long regions, not only at the protein level but also at the nucleotide

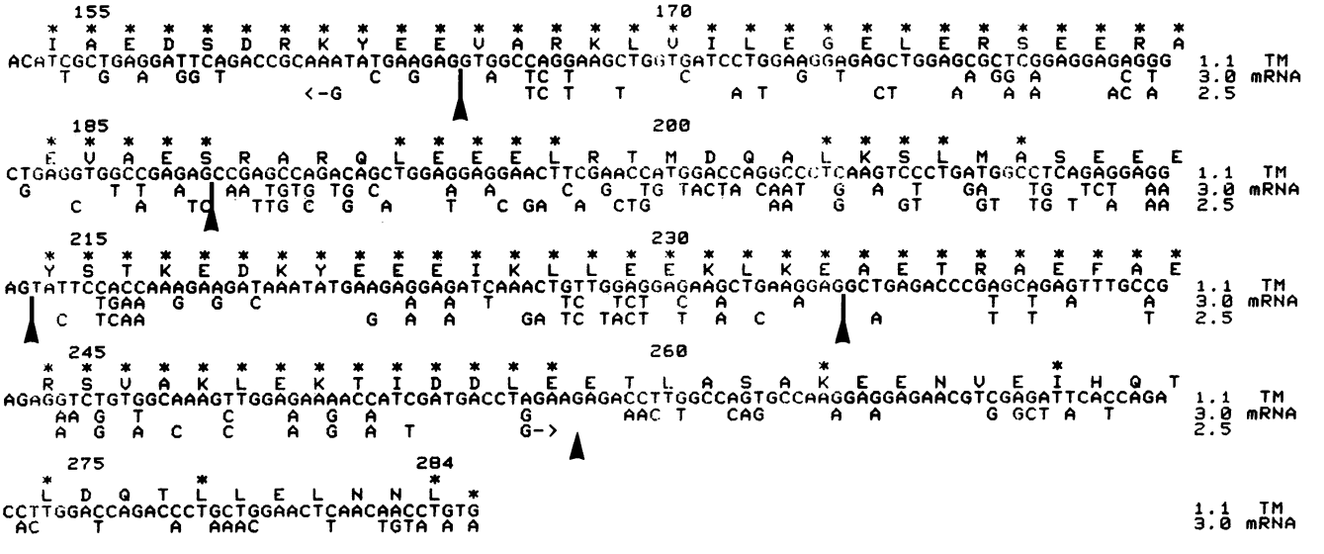

FIG. 3. Comparison of the nucleotide sequences of the 1.1-, 2.5-, and 3.0-kb cytoskeletal tropomyosin (TM) mRNAs. The sequences of the 2.5- and 3.0-kb mRNAs are derived from an analysis of both cDNA and genomic DNA. The functional genes encoding the 2.5- and 3.0-kb mRNAs are very large and have not yet been isolated in a complete form. The positions of splice junctions are indicated (↓). The splice junction at amino acid-257/8 has been confirmed only for the gene encoding the 2.5-kb mRNA. This region of the gene encoding the 3.0-kb mRNA has not yet been isolated, the sequence indicated being derived entirely from cDNA. < > indicates the limits of the available sequence of the 2.5-kb mRNA in this region. The comparison of the three sequences is over the length of the molecule for which a comparison with the skeletal muscle β-tropomyosin can be made. Only where the nucleotide sequence of the 2.5- and 3.0-kb mRNAs varies from the 1.1-kb mRNA is the difference noted. The protein sequence shown is that of the 1.1-kb mRNA. The * indicates amino acids that are identical in the skeletal muscle β-tropomyosin sequence and the fibroblast 284-amino acid cytoskeletal tropomyosin sequence.

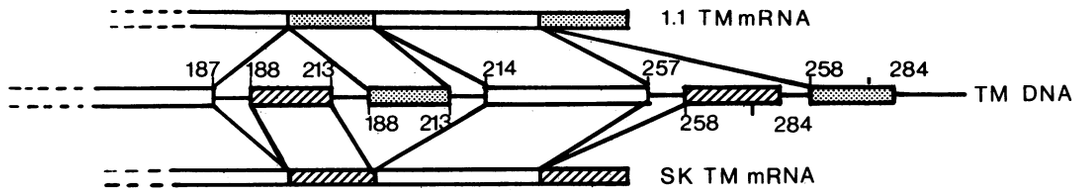

FIG. 4. Model for alternative splicing of the transcript of a tropomyosin (TM) structural gene. The model assumes the existence within the functional gene of at least two exons encoding the amino acid-188 to -213 region, one containing the fibroblast (1.1) sequence, one containing the skeletal (SK) muscle sequence. There must also be a similar duplication of an exon encoding the amino acid-258 to -284 region and the 3′ untranslated region as well since this is specific to each mature mRNA. The model depicts the minimum number of exons required to produce the regions of identity and nonidentity seen in Fig. 2. It would seem likely that the structure of the common regions up to amino acid-188 and from amino acid-214 to -257 will be more complex given the presence of splice junctions in the corresponding regions of the functional genes encoding the 2.5- and 3.0-kb mRNAs.

level. Furthermore, points of divergence in the protein sequence are identical with the positions of splice junctions in the functional gene.

As part of a study of human muscle tropomyosin sequences we have isolated and sequenced a cDNA encoding a human skeletal muscle tropomyosin. This cDNA clone contains the 3′ coding region of the mRNA corresponding to amino acids 153–284, in addition to the entire 3′ noncoding region (Fig. 2). Comparison of the deduced protein sequence with known muscle tropomyosins indicates that it is identical to rabbit skeletal muscle β-tropomyosin (32) over the 101 amino acids available for comparison (Table 1).

The nucleotide sequence of the skeletal muscle cDNA clone shows an extraordinary homology to the cDNA sequence encoding the fibroblast 284-amino acid cytoskeletal tropomyosin. The two sequences are identical from the start of the comparison to amino acid-188, diverge through to amino acid-213, are identical again to amino acid-257 and diverge again through the remainder of the protein coding sequence and 3′ noncoding region (Fig. 2). This is in striking contrast to the sequences that encode the other two cytoskeletal tropomyosin mRNAs. When compared over the same region, the 1.1-, 2.5-, and 3.0-kb mRNAs, although closely related, possess no significant regions of identity (Fig. 3). This nonidentity, which extends through the entire protein coding region (not shown), indicates that the three cytoskeletal tropomyosin mRNAs are the products of distinct and different functional genes. These different functional genes have incorporated random nucleotide substitutions principally at the third position of the codon such that the mRNAs, while possessing rather different nucleotide sequences, nevertheless encode protein sequences which are closely related (not shown). The absence of random third-base variation in large regions of the mRNA sequences encoding the fibroblast 284-amino acid cytoskeletal tropomyosin and the skeletal muscle β-tropomyosin implies that these mRNA sequences are transcribed, at least in part, from the same DNA sequence. Although we have not yet isolated the functional gene encoding the 1.1-kb cytoskeletal tropomyosin mRNA, we have isolated and characterized the homologous regions of the functional genes encoding the 2.5- and 3.0-kb mRNAs. In the region available for comparison, the positions of the splice junctions found in these two genes are conserved exactly (Fig. 3). The boundaries of the region of nonidentity between the fibroblast 284-amino acid cytoskeletal tropomyosin and the skeletal muscle β-tropomyosin correspond exactly to splice junctions in the two functional genes. Furthermore, the boundary of the region of nonidentity from amino acid-258 to -284, which is a splice junction in the functional gene encoding the 2.5-kb cytoskeletal tropomyosin mRNA (Fig. 3), corresponds exactly to the splice junction used for alternative splicing of *Drosophila* tropomyosin mRNAs (27, 28).

These data support a model in which the skeletal muscle β-tropomyosin and the fibroblast 284-amino acid cytoskeletal tropomyosin are produced from a common transcript by alternative splicing of exons encoding the region 258–284 as in *Drosophila* tropomyosins and of exons encoding the region 188–213 (Fig. 4). It is interesting to note that this mechanism results in expression of an exon containing cysteine-190 as well as an exon containing the carboxyl-terminal 27 amino acids in the skeletal muscle sequence. These are features of the skeletal muscle tropomyosin sequence that are required for the binding of troponin T (3, 4, 33–37), a protein necessary to the function of tropomyosin in skeletal muscle but that is absent from non-muscle tissues.

CONCLUSIONS

Human fibroblasts express at least three distinct mRNAs encoding cytoskeletal tropomyosins. Two of these, a 2.5- and 3.0-kb mRNA, encode non-muscle tropomyosins of the classic 247-amino acid type. The third, a 1.1-kb mRNA, encodes a 284-amino acid muscle-type tropomyosin previously characterized as hscp36 (14). The presence of both muscle and non-muscle type tropomyosins in the cytoskeleton is striking and implies a complex functional role for tropomyosin in non-muscle cells.

Sequence analysis reveals that the three mRNAs encoding cytoskeletal tropomyosins in human fibroblasts are transcribed from distinct functional genes. However, a comparison of the 1.1-kb mRNA sequence with a human skeletal muscle β-tropomyosin cDNA sequence reveals long regions of complete identity interrupted by regions of nonidentity in the nucleotide sequence. The boundaries of the regions of sequence divergence are identical with the positions of splice junctions found in other non-muscle tropomyosin genes. These observations support a model in which the fibroblast 284-amino acid cytoskeletal tropomyosin and the skeletal muscle β-tropomyosin are expressed from a common structural gene by alternative splicing. This model is consistent with the expression of tropomyosin in rat muscle in which a skeletal muscle and a smooth muscle tropomyosin are produced by alternative RNA splicing. The important and distinguishing feature of the observations presented here is that alternative splicing can apparently produce different proteins from the same gene in cells as different as fibroblasts and skeletal muscle and, therefore, is probably not a mechanism restricted to terminally differentiated cells of related lineages.

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