

Cloning and Characterization of a cDNA Encoding Transformation-Sensitive Tropomyosin Isoform 3 from Tumorigenic Human Fibroblasts

CHING-SHWUN LIN* AND JOHN LEAVITT

Armand Hammer Cancer Research Center, Linus Pauling Institute of Science and Medicine, 440 Page Mill Road, Palo Alto, California 94306

Received 9 July 1987/Accepted 13 October 1987

We isolated a cDNA clone from the tumorigenic human fibroblast cell line HuT-14 that contains the entire protein coding region of tropomyosin isoform 3 (Tm3) and 781 base pairs of 5'- and 3'-untranslated sequences. Tm3, despite its apparent smaller molecular weight than Tm1 in two-dimensional gels, has the same peptide length as Tm1 (284 amino acids) and shares 83% homology with Tm1. Tm3 cDNA hybridized to an abundant mRNA of 1.3 kilobases in fetal muscle and cardiac muscle, suggesting that Tm3 is related to an α_{fast} -tropomyosin. The first 188 amino acids of Tm3 are identical to those of rat or rabbit skeletal muscle α -tropomyosin, and the last 71 amino acids differ from those of rat smooth muscle α -tropomyosin by only 1 residue. Tm3 therefore appears to be encoded by the same gene that encodes the fast skeletal muscle α -tropomyosin and the smooth muscle α -tropomyosin via an alternative RNA-splicing mechanism. In contrast to Tm4 and Tm5, Tm3 has a small gene family, with, at best, only one pseudogene.

Tropomyosins are a family of proteins that were first isolated from muscle (2) but which, like actin, are also abundant cytoskeletal proteins of nonmuscle cells (8). In striated muscle, tropomyosin mediates the effect of calcium on the actin-myosin interaction through the binding of troponin to specific sites on the tropomyosin peptide (16, 35, 44, 49). In nonmuscle cells tropomyosin is a major structural component of cytoskeletal microfilaments (32, 33), but the function of tropomyosin with regard to contraction in these cells is not known. We and others have shown that human and rodent fibroblasts contain at least six isoforms of tropomyosin (10, 20) and that expression of these tropomyosins is modulated in neoplastic transformation (5, 10, 14, 15, 20, 26, 32). In general, the rates of synthesis of the three higher-molecular-weight tropomyosin isoforms 1, 2, and 6 (Tm1, Tm2, and Tm6, respectively) are greatly reduced. The rates of synthesis of the two lower-molecular-weight tropomyosin isoforms 4 and 5 (Tm4 and Tm5, respectively) are less affected. Expression of Tm3 is of particular interest in that it is stimulated on the transformation of rat fibroblasts by human adenovirus (10, 32) and on the transformation of human fibroblasts by chemical mutagenesis to an immortalized nontumorigenic state (20). Further transformation of these human fibroblasts to a fully tumorigenic state, however, leads to a reduction of the rate of synthesis of Tm3 (20, 22, 23). To study the relationship of tropomyosins to neoplastic transformation, we attempted to isolate cDNA clones from the fully tumorigenic fibroblast cell line HuT-14. We present here evidence of the isolation of a nearly full-length Tm3 clone. We examined the expression of Tm3 in various cell types, compared its sequence with related Tm sequences, and determined its genomic pattern with regard to its family size and relationship with muscle-related tropomyosin genes.

MATERIALS AND METHODS

Preparation of cellular RNA and DNA. Total cellular RNA was prepared by the guanidine hydrochloride method, as described previously (12). Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography (1). Genomic DNA was prepared as described previously (18). Muscle RNAs were kindly provided by L. Kedes and R. Wade.

Preparation of hybridization probes. Human skeletal muscle α - and β -tropomyosin cDNAs and human skeletal muscle β -actin cDNA were kindly provided by P. Gunning. These cDNAs and cDNAs isolated in this study were labeled with ³²P by the procedure described by Rigby et al. (39) and used as probes in the Northern and genomic analyses.

Construction and screening of the cDNA library. By using the method described by Huynh et al. (17), a λ gt10 cDNA library was constructed from poly(A)⁺ RNA isolated from human fibroblast cell line HuT-14 (19). A portion (20,000 PFU) of the library was plated onto LB plates, and phage DNA was transferred to nitrocellulose paper by the method described by Benton and Davis (3). The nitrocellulose papers were incubated in a solution of 4 \times SSC (1 \times SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7])–5 \times Denhardt solution (7)–50 mM phosphate buffer (pH 7)–10% (wt/vol) dextran sulfate–2 \times 10⁵ to 2 \times 10⁶ cpm of probe 504-HA (see below) per ml at 65°C for 20 h. The nitrocellulose papers were then washed twice with 1 \times SSC–0.1% sodium dodecyl sulfate at room temperature for 5 min each and twice with 0.5 \times SSC–sodium dodecyl sulfate at 65°C for 30 min each. The papers were dried in air and exposed to XAR-5 films (Eastman Kodak Co., Rochester, N.Y.).

Northern analysis. Total cellular RNA was size fractionated in a 1% agarose gel containing 50 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7), 1 mM EDTA, and 2.2 M formaldehyde; transferred to nitrocellulose paper; and hybridized to ³²P-labeled probes under the same conditions described above.

Genomic analysis. Genomic DNA was digested with restriction enzymes to completion, electrophoresed on a 0.7% agarose gel, and transferred to nitrocellulose paper by the

* Corresponding author.

method described by Southern (46). Hybridization and washing of the nitrocellulose paper were the same as described above.

DNA sequencing. cDNA was subcloned into M13mp8 and M13mp9 (36). Progressive deletion clones were prepared by the method described by Dale et al. (6) and sequenced by the method described by Sanger et al. (42).

In vitro message selection. The purified cDNA fragment was bound to nitrocellulose paper essentially as described by Parnes et al. (37), except that the DNA solution was not subjected to boiling and was added to nitrocellulose paper with the aid of a Minifold (Schleicher & Schuell, Inc., Keene, N.H.). Hybridization to mRNA and elution of hybridized mRNA were done as described by Maniatis et al. (31), except that tRNA was not included in the hybridization solution or during the elution of mRNA. The eluted mRNA was precipitated in the presence of calf liver tRNA (15 $\mu\text{g/ml}$) in 70% ethanol. The mRNA was suspended in water and subjected to in vitro translation.

In vitro transcription. The entire cDNA insert was cloned into the *EcoRI* site of an in vitro transcription vector pSPT18 (purchased from Pharmacia Fine Chemicals, Piscataway, N.J.). In vitro transcription was carried out by the method described by Schenborn and Mierendorf (43) with T7 RNA polymerase. A small portion (1%) of the transcription product was subjected to in vitro translation.

In vitro translation and two-dimensional gel electrophoresis. All in vitro translation experiments were carried out in a rabbit reticulocyte system purchased from New England Nuclear Corp. (Boston, Mass.). The translated products were then electrophoresed in a two-dimensional polyacrylamide gel and visualized by autoradiography as described previously (20).

Computer analysis. DNA and protein sequences were analyzed with Bionet programs (IntelliGenetics, Inc., Palo Alto, Calif.).

RESULTS

Identification of tropomyosin cDNA clones. By using a human skeletal muscle α -tropomyosin-coding probe, 504-HA (a *HindIII-AvaI* fragment from codon 65 to nucleotide 45 of 3'-untranslated region [29]) we initially obtained 10 positive clones from our HuT-14 cDNA library. After further purification and Northern analysis, six clones were found to have tropomyosin cDNA inserts. Four of them were found to be Tm4 (TM30_{pl} [30]) clones by sequencing. The remaining two clones (T7 and T8) hybridized predominantly to a previously unidentified 2-kilobase (kb) mRNA in Northern analysis with RNA isolated from diploid human fibroblast KD cells and tumorigenic human fibroblast HuT-14 cells (Fig. 1A and B). Probe 504-HA also detected this mRNA (weakly), as well as mRNAs of 1.1, 2.5, and 3 kb (Fig. 1C) that are known to code for Tm1, Tm5, and Tm4, respectively (28, 29, 30). A human skeletal muscle β -tropomyosin-coding probe, 417-2 (an *NcoI-ClaI* fragment from codons 1 to 252 [28]), detected the same mRNAs, except for the 2.5-kb mRNA (Fig. 1D). T7 and T8 also detected Tm1 and Tm4 mRNAs and a 4-kb mRNA which possibly encodes Tm2. The consistently weaker hybridization signal for Tm1 mRNA in HuT-14 cells than in KD cells agrees with previous observations by us and others (20, 28) that expression of Tm1 is reduced following neoplastic transformation. At first we did not understand what the small mRNA (0.6 kb) was that was specifically detected by T7. Only after we sequenced T7 and compared its sequence with the data base of

the National Institutes of Health (Bethesda, Md.) did we realize that T7 is a fusion clone between tropomyosin and ubiquitin (27). We have used the ubiquitin portion of T7 as a probe in other Northern analyses and found it indeed detected the 0.6-kb mRNA and not any of the tropomyosin mRNA (data not shown).

Sequence of a novel fibroblast tropomyosin cDNA. The 2-kb mRNA that was detected by our cDNA clones T7 and T8 has not been reported before. We determined the nucleotide sequence of both clones. Clone T7 is 1,579 base pairs (bp) long, 389 bp of which are the ubiquitin sequence and the remaining 1,190 bp of which are the tropomyosin sequence. Clone T8 is 1,633 bp long and contains 286 bp of the 5'-untranslated sequence, 852 bp that encode a tropomyosin sequence, and 495 bp of 3'-untranslated sequence (Fig. 2). The tropomyosin portion of T7 is identical to T8, including the 3' end. The fact that two independently isolated clones have identical 3' ends suggests the existence of an *EcoRI* site in the original cDNA prior to *EcoRI* digestion during the construction of the cDNA library. This possible *EcoRI* site also explains the lack of a polyadenylation signal sequence in the cloned cDNA. The coding sequence of T8 shares 77% homology with that of Tm1, and their deduced amino acid sequences are 83% homologous (Fig. 2). The dissimilar amino acids, however, occur randomly. Therefore, it is unlikely that the cDNAs of T8 and Tm1 are derived from mRNAs that are encoded by the same gene via alternative RNA splicing.

Tm3 is the translational product of the 2-kb mRNA. To reveal which tropomyosin isoform the 2-kb mRNA encodes, in vitro message selection-translation experiments were carried out. In these experiments, the entire cDNA insert isolated from clone T8 was used to select for specific mRNAs among mRNAs isolated from either KD or HuT-14 cells. These specific mRNAs were then translated in vitro, and their products were visualized in two-dimensional gels. When KD mRNAs were used in the selection, all four large Tm isoforms (Tm1, Tm2, Tm3, and Tm6) were selected, with Tm2 and Tm3 being better selected than Tm1 and Tm6 (Fig.

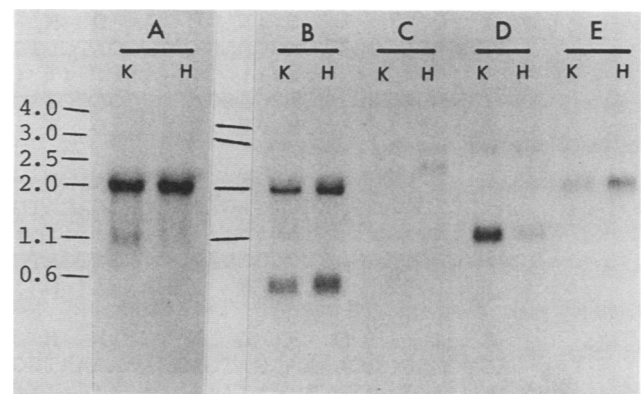


FIG. 1. Identification of tropomyosin cDNA clones. Total cellular RNA of KD (lanes K) and HuT-14 (lanes H) cells were size fractionated in formaldehyde gel, transferred to nitrocellulose paper, and hybridized to ^{32}P -labeled probes T8 (A), T7 (B), 504-HA (C), 417-2 (D), and β -actin (E). (B, C, D, and E) A total of 3 μg of KD RNA and 5 μg HuT-14 RNA were used. (A) A total of 5 μg of each RNA was used. Sizes of RNA (in kilobases) indicated to the left of the blots were determined by comparison with the size of the β -actin mRNA (2.1 kb) and by referring to the sizes given in other studies (27, 28, 29).

```

                                                    CAGAATCTCCGGCAGTTTT Tm3
TG TACCTCAAGAAGTAAGTGG AACACCTTTCCCTGTCATAGTTATTTTCATCCAGACATC Tm3
TGGTGG AAGCATCAGATTCCTTACAGATATAAGAGAGGCATCATT TAAAGGTAGAACAG Tm3

GATCGACAAACAAGGATTTATGTCAGGATCTCTCAGCCTCTGTGTTACCGAGGGCATTTC Tm3
          : : : : : : : : : : : : : : :
          TGCTGCTCTCCTCCCCTCCGTCCTCCTCGC Tm1

TAACAGTCTTCTTACTACGGCCTCCGCCGACCGCGCTCGCCCCGCCGCTCCTGCTGCA Tm3
          : : : : : : : : : : : : : : :
CTGCCACCGGTGCACCCAGTCCGCTCACCCAGCCAGTCCGTCCGGTCCTCACCCGCTGC Tm1

          M D A I K K K M Q M L 11
GCC CAGGGCCCCTCGCCGCCGCCACCATGGACGCCATCAAGAAGAAGATGCAGATGCTG Tm3
          : : : : : : : : : : : : : : :
CGGCCGGCCCAACCCCAACCGCAGGCCATGGACGCCATCAAGAAGAAGATGCAGATGCTG Tm1
          M D A I K K K M Q M L

K L D K E N A L D R A E Q A E A D K K A 31
AAGCTCGACAAGGAGAACGCCTTGGATCGAGCTGAGCAGGCGGAGGCCGACAAGAAGGCC Tm3
: : : : : : : : : : : : : : :
AAGCTGGACAAGGAGAACGCCATCGACCGCGCCGAGCAGGCCGAAAGCCGACAAGAAGCAA Tm1
K L D K E N A I D R A E Q A E A D K K Q

A E D R S K Q L E D E L V S L Q K K L K 51
GCCGAAGACAGGAGCAAGCAGCTGGAAGATGAGCTGGTGTCACTGCAAAAAGAAACTCAAG Tm3
: : : : : : : : : : : : : : :
GCTGAGGACCGCTGCAAGCAGCTGGAGGAGGAGCAGCAGGCCCTCCAGAAGAAGCTGAAG Tm1
A E D R C K Q L E E E Q Q A L Q K K L K

G T E D E L D K Y S E A L K D A Q E K L 71
GGCACCGAAGATGAACTGGACAAATACTCTGAGGCTCTCAAAGATGCCAGGAGAAGCTG Tm3
: : : : : : : : : : : : : : :
GGGACAGAGGATGAGGTGAAAAGTATTCTGAATCCGTGAAGGAGGCCAGGAGAAACTG Tm1
G T E D E V E K Y S E S V K E A Q E K L

E L A E K K A T D A E A D V A S L N R R 91
GAGCTGGCAGAGAAAAAGGCCACCGATGCTGAAGCCGACGTAGCTTCTCTGAACAGACGC Tm3
: : : : : : : : : : : : : : :
GAGCAGGCCGAGAAGAAGGCCACTGATGCTGAGGCAGATGTGGCCTCCCTGAACCGCCGC Tm1
E Q A E K K A T D A 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 111
ATCCAGCTGGTTGAGGAAGAGTTGGATCGTGCCAGGAGCGTCTGGCAACAGCTTTGCAG Tm3
: : : : : : : : : : : : : : :
ATTAGCTGGTTGAGGAGGAGCTGGACCGGGCCAGGAGCGCCTGGCTACAGCCCTGCAG Tm1
I Q L V E E E L D R A Q E R L A T A L Q

K L E E A E K A A D E S E R G M K V I E 131
AAGCTGGAGGAAGCTGAGAAGGCAGCAGATGAGAGTGAGAGAGGCATGAAAGTCATTGAG Tm3
: : : : : : : : : : : : : : :
AAGCTGGAGGAGGCCGAGAAGGCCGCTGATGAGAGCGAGAGGAATGAAGGTCATCGAA Tm1
K L E E A E K A A D E S E R G M K V I E

S R A Q K D E E K M E I Q E I Q L K E A 151
AGTCGAGCCCAAAAAGATGAAGAAAAATGAAATTCAGGAGATCCAAGTGAAGAGGCA Tm3
: : : : : : : : : : : : : : :
AACCGGGCCATGAAGGATGAGGAGAAGATGAACTGCAGGAGATGCAGCTGAAGGAGGCC Tm1
N R A M K D E E K M E L Q E M Q L K E A

K H I A E D A D R K Y E E V A R K L V I 171
AAGCACATTGCTGAAGATGCCGACCGCAAATATGAAGAGGTGGCCCGTAAGCTGGTCATC Tm3
: : : : : : : : : : : : : : :
AAGCACATCGCTGAGGATTCAGACCGCAAATATGAAGAGGTGGCCAGGAAGCTGGTGATC Tm1
K H I A E D S D R K Y E E V A R K L V I

```

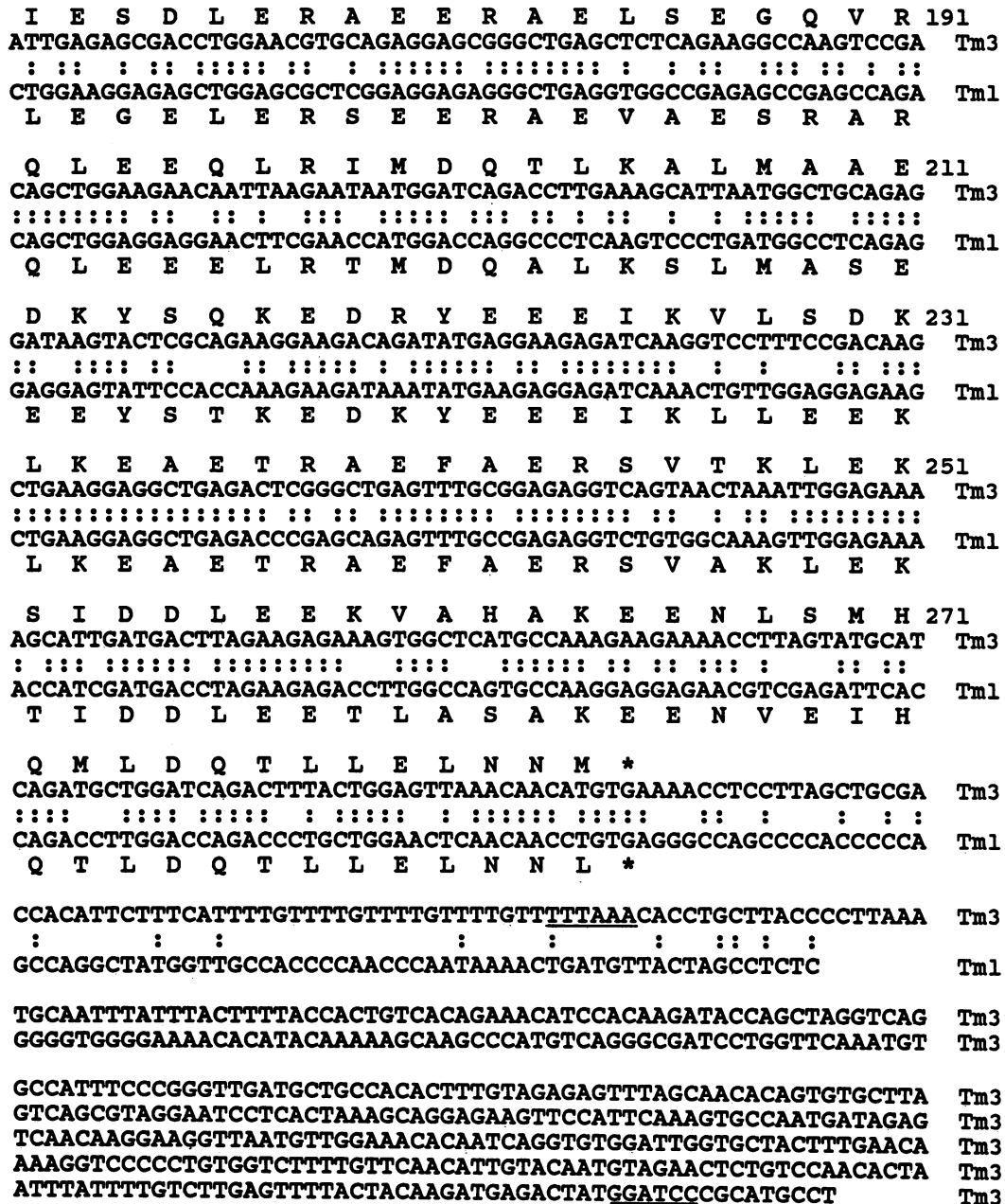


FIG. 2. Nucleotide and derived amino acid sequence comparison between Tm3 and Tm1 cDNA (28). The nucleotide sequence of Tm3 was determined from the cDNA insert of clone T8. Identical nucleotides are indicated by double dots. Amino acid numbers are indicated on the right, after the Tm3 sequences. The three underlined sequences are two *Dra*I sites and one *Bam*HI site that were used to generate Tm3 coding and 3'-untranslated sequence probes in later experiments.

3). When HuT-14 mRNAs were used, only Tm1 and Tm3 were selected, with Tm3 being better selected than Tm1. These results indicate that our T8 cDNA most likely encodes either Tm2 or Tm3 and that these two isoforms are highly homologous to each other.

Since in the *in vitro* message selection-translation experiments we were unable to distinguish between Tm2 and Tm3, we decided to use instead the *in vitro* transcription-translation technique, which has been used successfully in the identification of the cDNA that encodes Tm4 (30). By this technique, a single species of RNA was transcribed from the T8 cDNA; this RNA in turn was translated into a major

protein corresponding to Tm3 (Fig. 4). Two minor protein spots that formed a straight line with Tm3 in the two-dimensional gel did not correspond to any cellular protein and were possibly conformational isoforms of Tm3. From results of both the message selection-translation and the transcription-translation experiments, we conclude that the 2-kb mRNA encodes the Tm3 isoform.

Modulation of Tm3 accompanying neoplastic transformation. We have observed previously (20) that synthesis of Tm3 is elevated in the transformed human fibroblast HuT-12 and is reduced in the further transformed fibroblast HuT-14 when compared with the untransformed diploid parent fibro-

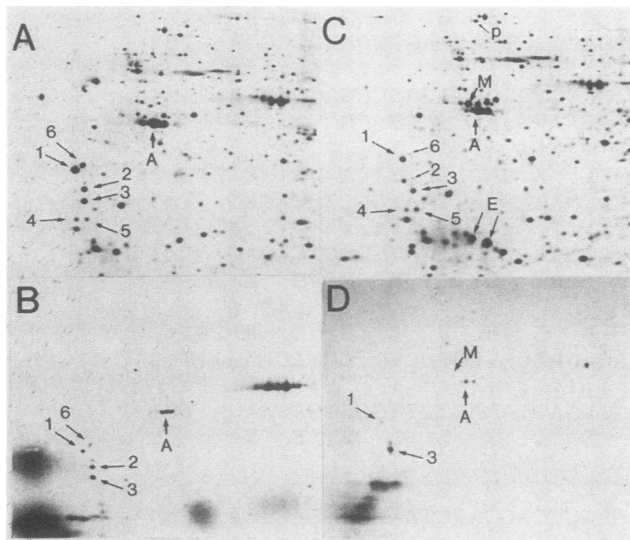


FIG. 3. Translation of mRNAs selected by Tm3 cDNA. In vitro translation products of total KD mRNA (A), total HuT-14 mRNA (C), T8-selected KD mRNA (B), or T8-selected HuT-14 mRNA (D) were separated in two-dimensional gels and visualized by autoradiography. Abbreviations: A, actins; M, mutant β -actin, E, epidermal growth factor-related polypeptides (4); P, plastin (11); 1 to 6, the six tropomyosin isoforms.

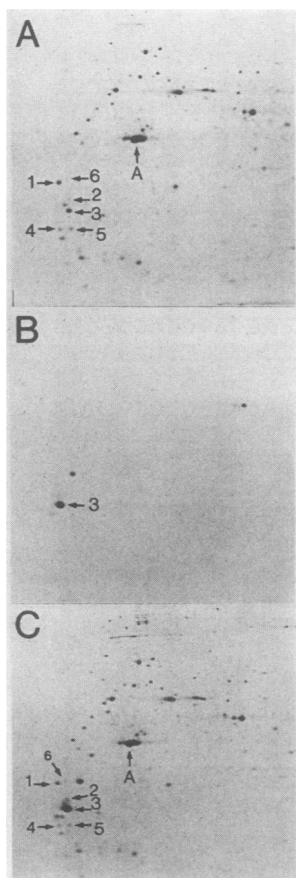


FIG. 4. Translation of in vitro-transcribed Tm3 RNA. (A) Cellular proteins of HuT-12 fibroblasts. (B) Translational products of in vitro-transcribed Tm3 RNA. (C) Mixture of proteins and products in panels A and B. Abbreviations: A, actins; 1 to 6, the six tropomyosin isoforms.

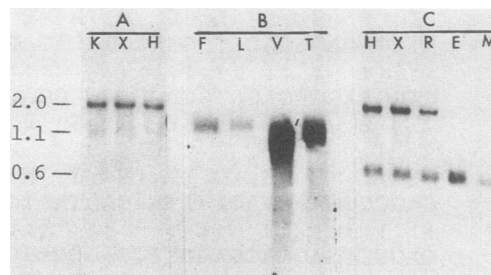


FIG. 5. Detection of Tm3 mRNA in different cells. Total cellular RNAs were size fractionated and transferred to nitrocellulose paper as described earlier (Fig. 1). (A) RNAs of KD (lane K), HuT-12 (lane X), and HuT-14 (lane H); the blot was hybridized to the T8-coding probe. (B) RNAs of 24-week-old human fetal leg muscle (lane F), human adult leg muscle (lane L), human heart ventriculum (lane V), and human heart atrium (lane T); the blot was hybridized to the T8-coding probe. (C) RNAs of HuT-14 (lane H), HuT-12 (lane X), R-17 (lane R) (a diploid human fibroblast; unpublished data), the CCRF-CEM human lymphocyte cell line (lane E), and Molt-4 (lane M); the blot was hybridized to the T7 probe. Sizes (in kilobases) are given to the left of the blots.

blast KD. The two-dimensional gels in Fig. 3 show that the reduction of Tm1, Tm2, Tm3, and Tm6 synthesis in HuT-14 cells is reproducible in the cell-free translation of purified mRNAs, strongly suggesting a transcriptional control in the modulation of Tm synthesis. We compared Tm3 mRNA synthesis among these three cell lines in a Northern analysis. Tm3 mRNA appeared to be synthesized at equal rates in these three cell lines (Fig. 5A). We do not know whether this is an indication that Northern analysis is less sensitive than two-dimensional gel analysis or whether there is indeed a translational control in the modulation of Tm3 synthesis.

Tm3-related mRNA is expressed abundantly in fetal and cardiac muscles. It is known that Tm1 and Tm5 have counterparts in muscle tissues (13, 28, 29, 38). We therefore examined whether the Tm3 counterpart exists in muscle. The Tm3 cDNA probe strongly detected a 1.3-kb mRNA in fetal, adult leg, and cardiac muscle tissues (Fig. 5B). A same-sized mRNA has been shown to encode skeletal and cardiac muscle α -tropomyosin (38, 41). Skeletal muscle α -tropomyosin exists in two forms, α_{fast} and α_{slow} , in fast muscle and slow muscle, respectively (R. Wade and L. Kedes, personal communication). The fast-form mRNA is induced early in development, whereas the slow-form mRNA is expressed more abundantly in adult muscle. Our results indicate that the Tm3-related mRNA is more abundantly expressed in fetal skeletal muscle than in adult skeletal muscle; therefore, Tm3 has a counterpart of α_{fast} -tropomyosin in muscle.

Tm3 is not expressed in lymphocytes. We have observed in two-dimensional gels that human lymphocytes express only Tm4 and Tm5 (unpublished data). The isolation of Tm3 cDNA enabled us to examine the expression of tropomyosin mRNAs in these cells. A probe derived from the cDNA of clone T7 hybridized strongly with the Tm3 and the ubiquitin mRNAs and weakly with the Tm1, Tm4, and the 4-kb mRNAs in three fibroblast cell lines (Fig. 5C), just as it did earlier (Fig. 1B). In contrast, the T7 probe detected essentially only the ubiquitin mRNA (a faint band corresponding to the 3-kb Tm4 mRNA is visible in the original autoradiograph) in two human lymphocyte cell lines, CCRF-CEM (ATCC CCL-119 [11]) and Molt-4 (11, 21). The Tm3 mRNA, which was clearly visible in the fibroblasts, was completely

Tm3	MDAIKKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLE	40
Sk α (rat)	-----	
Sk α (human)	M-E-----EQ-Q-E-----	
Sm α (rat)	-----	
Tm1	-----I-----Q---C----	
Tm3	DELVSLQKCLKGTEDELDKYSEALKDAQEKLELAEKKATD	80
Sk α (rat)	-----	
Sk α (human)	---AAM-----A-	
Sm α (rat)	EDISAKE-L-RAS---R-RVL-E-HK-EDS-LA-DETAAK	
Tm1	E-QQA-----VE---SV-E---Q-----	
Tm3	AEADVASLNRRIQLVEEELDRAQERLATALQKLEEAEKAA	120
Sk α (rat)	-----	
Sk α (human)	---E-----	
Sm α (rat)	-----	
Tm1	-----	
Tm3	DESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADR	160
Sk α (rat)	-----	
Sk α (human)	-----N--L-----L-----E---	
Sm α (rat)	-----	
Tm1	-----N--M-----L--M-----S--	
Tm3	KYEEVARKLVIIESDLERAEEAELSEGQVRQLEEQLRIM	200
Sk α (rat)	-----KCAE---E-KTV	
Sk α (human)	-----C---T-----A-SKCSE---E-KNV	
Sm α (rat)	-----KCAE---E-KTV	
Tm1	-----L-GE---S-----VA-SRA---E--T-	
Tm3	DQTLKALMAAEDKYSQKEDRYEEEEIKVLSDKLKEAETRAE	240
Sk α (rat)	TNN--S-E-QAE-----K-----	
Sk α (human)	TNN--S-E-QAE-----K-----I-T---E-----	
Sm α (rat)	TNN--S-E-QAE-----K-----	
Tm1	--A--S--S-EE--T--K-----L-EE-----	
Tm3	FAERSVTKLEKSIDDLEEKVAHAKEENLSMHQMLDQTLLE	280
Sk α (rat)	-----DELYAQ-LKYKAISEE--HA-ND	
Sk α (human)	-----A---T-----DELYAQ-LKYKAISEE--HA-ND	
Sm α (rat)	-----	
Tm1	-----A---T-----TL-S-----VEI--T-----	
Tm3	LNNM	284
Sk α (rat)	MTSI	
Sk α (human)	MTSI	
Sm α (rat)	----	
Tm1	---L	

FIG. 6. Comparison of amino acid sequences among Tm3 and other tropomyosins. Rat skeletal muscle α -tropomyosin (Sk α) and smooth muscle α -tropomyosin (Sm α) sequences are from previously published data (40). Tm1 sequence is also from previously published data (28). Human skeletal muscle α -tropomyosin (29) has 285 amino acids; its first amino acid is positioned as 0 instead of 1 in this comparison. An amino acid identical to that of Tm3 is indicated by a dash.

absent from the lymphocytes. This result not only further confirms the identity of our Tm3 clones but also demonstrates for the first time that lymphocytes do not express Tm3 mRNA (or Tm1 RNA) at a detectable level.

Tm3 is closely related to muscle α -tropomyosin. The existence of Tm3-related mRNAs in muscle tissues suggests that

Tm3 may share a common gene with its muscle counterpart, as is the case for Tm1 and Tm5 (13, 28, 29). It is possible to recognize such arrangements by comparing the sequences between muscle and nonmuscle forms (28, 29, 41). Tm3 was entirely identical to rabbit or rat skeletal muscle α -tropomyosin (40, 41, 45, 47) from amino acids 1 to 188 (Fig. 6). In

```

TGAAAACCTCCTTAGCTGCGACCACATTCTTTCATTTTGT---TTGTTTTGTTTTGTTTTTA
::::: ::::::::::::::: ::::::::::: : : :::      ::::::::::: ::::::::::: ::::
TGAAAACCTCCTTAGCTGCGGCCACATTCTCTCGTTTCCCCCTGTTGTTTTATTTTGTCTTTA

AACACCTGCTTACC-----CCTTAAATGCAATTTATTACT-----TTTTAC
::::: :::::::::::      ::::: ::::: : : : :      :::::
AACACATGCTTACCATGAAAACCCCTTCAATGCTTTTTTTTTTTTATATATATATACCTTTAC

CACTGTACAGAAAACATCCACAAGATACCAGCTAGGTCAGGGGGTGGGAAAACACATACAAA
:::::::::: : ::::: : : : : : : : : : : : : : : : : : : : : : : : : :
CACTGTCACTGCAACATCTGC-AG---CC---AGGGCAGGGGGTGGGG-AAAGACACACAGA

AAGCAAGCCCATGTCAGGGCGATCCTGGTTCAAATGTGCCATTTCCCGGGTTGATGCTGCCAC
::: : :      : : : : : : : : : : : : : : : : : : : : : : : : : : :
AAGTCAG-----GGTACTTGTATTATTTAAATGTGCCATTTCCAAGTTGGCATTGCCGC

ACTTTGTAGAGAGTTTAGCAACACAGTGTGCTTAGTCAGCGTAGGAATCCTCACTAAAGCAGG
: : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
GCTTCGCGGAG--TTTGGGCATGCAGTTTGCTTAGCCAGTGTAGGAAGCCTCACGAAAACAAA

AGAAGTTCCATTCAAAGTGCCAATGATAGAGTCAACAAGGAAGGTTAATGTTGGAAACACAAT
: : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
AGGATTGACAGCCTAAGTGCCAATGGTGGGGTCATCA-GGAAGACTGATGTTGGAGACA--AT

CAGGTGTGGATTGGTGCTACTTTGAACAAAAGGTCCCCCTGTGGTCTTTTGTTCAA
::::: ::::::::::: : : : : : : : : : : : : : : : : : : : : : : :
CAGGTGCAGATTGGTGCTAATTTAAACAAAAGAA-CCCCTAGTGTCTTTTGTTCAG

```

FIG. 7. Comparison of 3'-untranslated sequences between Tm3 and rat smooth muscle α -tropomyosin. The Tm3 (upper) and the rat (lower) sequences (40) are aligned for maximal homology with appropriate insertion of dashes. Identical nucleotides are indicated by double dots. The first trinucleotide (TGA) of each sequence is the translation termination codon.

this same stretch of sequence, the homology between Tm3 and human skeletal muscle α -tropomyosin (29, 38) is less (a difference of 18 amino acids), adding another piece of evidence that this human skeletal muscle tropomyosin is most likely the slow form, whereas the rabbit and rat sequences belong to the fast form. From amino acids 189 to 213, Tm3 differs from other tropomyosins considerably, although the sequences for muscle are identical or nearly identical to each other. From amino acid 214 to the end of the sequence, Tm3 still differs from the skeletal muscle sequences considerably but is entirely identical to the smooth muscle sequence, except for one residue at amino acid 220. At amino acid 220 all tropomyosins that we compared had a lysine; Tm3 was the only one that had an arginine. By assuming that this difference was due to mutation (see below), it becomes apparent that Tm3 shares with the fast skeletal muscle and smooth muscle α -tropomyosins a common gene which has an exon (encoding amino acids 189 to 213) that is specifically expressed in fibroblasts.

Tm3 and smooth muscle α -tropomyosin have homologous 3'-untranslated sequences. The 3'-untranslated sequence of skeletal muscle β -tropomyosin is conserved between rats and humans, suggesting that there is a functional role for the 3'-untranslated sequence (13). We found that the 3'-untranslated sequences of human Tm3, rat smooth muscle α -tropomyosin, and quail smooth muscle α -tropomyosin are homologous to each other (Fig. 7; quail sequence not shown). It has been shown that the last 27 codons and the 3'-untranslated sequence of rat smooth muscle α -tropomyosin are contained in the last exon of the rat gene (40). Since Tm3 is homologous to rat smooth muscle α -tropomyosin in the last 27 codons and in the 3'-untranslated sequences, it appears that Tm3 and smooth muscle α -tropomyosin share this last exon.

Tm3 genomic sequences are limited and are different from the Tm4 and Tm5 gene families. Rat skeletal muscle and smooth muscle α -tropomyosins are encoded by the same gene (39). Our Northern analysis (Fig. 5B) and sequence comparisons presented above suggest that this same gene also encodes Tm3 and that this gene is different from the gene that encodes the reported human skeletal muscle α -tropomyosin (29, 38). To further confirm this, we compared the genomic hybridization patterns by using Tm3 cDNA and 504-HA (cDNA of human skeletal muscle α -tropomyosin) as probes. These two probes detected very different sets of sequences (Fig. 8A and B), confirming that Tm3 and slow skeletal muscle α -tropomyosin are encoded by different genes.

Results of this genomic sequence analysis also show that Tm3 has a limited number of gene sequences, in contrast to Tm4 and Tm5, which have many pseudogenes in their gene families (29, 30). Some of the sequences detected by the T8-coding probe were probably not true Tm3 gene sequences, since cross-hybridization among different tropomyosin nucleotide sequences is to be expected. To determine the actual number of Tm3 gene sequences, we used a 3'-untranslated sequence probe (Fig. 2), which is specific for Tm3 mRNA in Northern analyses (data not shown), to reexamine the genomic pattern. This probe detected two sequences in each of the genomic DNAs that were digested with three different enzymes (Fig. 8C). This result suggests that there are only two Tm3 loci in the genome; one of them could be a pseudogene, as is the case for Tm1 in the rat genome (13).

DISCUSSION

Our interest in isolating Tm3 cDNA originated from our observation in two-dimensional gels that Tm3 is modulated

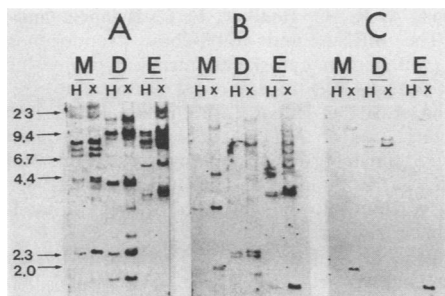


FIG. 8. Genomic representation of Tm3 and skeletal muscle α -tropomyosin genes. Genomic DNAs of HuT-14 (lanes H; 7 μ g) and HuT-12 (lanes X; 10 μ g) were digested to completion with *Eco*RI (lanes E), *Hind*III (lanes D), or *Bam*HI (lanes M); size fractionated in a 0.7% agarose gel; transferred to nitrocellulose paper; and hybridized to 504-HA (A), the Tm3-coding probe (B); or the Tm3 3'-untranslated sequence probe (C). Size markers (in kilobases) on the left are *Hind*III fragments of λ DNA.

in neoplastic transformation (20). We have presented evidence here that we obtained a Tm3 cDNA clone that contains the entire coding sequence and 781 bp of untranslated sequences. From results of Northern analyses, we learned that Tm3 cDNA cross-hybridizes to Tm1 and Tm4 mRNAs and a 4-kb mRNA that presumably encodes Tm2 or Tm6. We observed a great reduction of the Tm1 mRNA in transformed cells, an observation that is consistent with the Northern analysis of MacLeod et al. (28) and our two-dimensional gel analysis (20). We observed no difference in the amount of Tm3 and Tm2 (or Tm6) mRNAs between transformed and untransformed cells (Fig. 1 and 5A), however, although these three isoforms are, like Tm1, less abundant in HuT-14 than in KD cells (20). This observation initially led us to believe that the modulation of Tm3 and Tm2 (or Tm6) expression is at the translational rather than the transcriptional level. Results of our later experiments showed, however, that modulation of tropomyosin isoforms is entirely reproducible in the cell-free translation of mRNAs isolated from HuT-14 and KD cells (Fig. 3A and C). This later finding strongly argues against our earlier interpretation of the results of Northern analyses. To accommodate both findings, we considered the following alternatives. (i) In the case of Tm3, of which modulation is minimal, Northern analysis may not be sensitive enough to detect its small difference in modulation. (ii) In the case of Tm2 or Tm6, of which modulation is strong, the lack of difference in Northern analysis might be due to poor cross-hybridization, or alternatively, the 4-kb mRNA might not be Tm2 or Tm6 mRNA. (iii) Modulation is indeed translational and occurs at the initiation step, with HuT-14 and KD mRNAs being different in the translational initiation sequences. We have no clue as to the answer to these questions and may have to isolate more tropomyosin cDNAs from both KD and HuT-14 cells to solve this puzzle.

It has been shown previously (15, 25, 32, 48) that each tropomyosin isoform is encoded by a separate mRNA. Each mRNA could be encoded by a separate gene, or some of the mRNAs may be encoded by a single gene via alternative RNA processing. Tm1, Tm4, and Tm5 are now known to be encoded by three different genes (28, 29, 30). Our results show that Tm3 is encoded by yet another gene. The Tm1 and Tm5 genes also encode the muscle β - and α -tropomyosins, respectively (13, 28, 29). We found that skeletal and cardiac muscles express Tm3-related mRNAs and that, except for one region between amino acids 189 and 220, the Tm3

sequence is identical to skeletal muscle α -tropomyosin at the amino terminus and to the smooth muscle α -tropomyosin at the carboxy terminus. It is known that these two muscle isoforms are encoded by the same gene and that their amino acids 189 to 213 are encoded from one exon (40). In fact, the Tm3-specific sequence could be reduced to a region between amino acids 189 and 212 if the difference at amino acid 220 had not existed. At amino acid 220, all tropomyosins that we compared had a lysine; Tm3 was the only tropomyosin that had an arginine. Since our Tm3 clone was isolated from a mutagenized cell which has already been shown to have a mutated β -actin gene (18, 24), we consider mutation (from AAA to AGA) to be a possible explanation for this unusual amino acid at amino acid 220 of Tm3. If this is the case, then it becomes apparent that Tm3 mRNA is likely generated via alternative RNA splicing of the same transcript that produces skeletal and smooth muscle α -tropomyosins. This view is further supported by our finding that Tm3 and smooth muscle α -tropomyosin cDNAs are homologous in the 3'-untranslated sequence. When we searched the two introns that flank the exon encoding amino acids 189 to 213 in the rat gene, however, we failed to identify a sequence that might encode the Tm3-specific sequence. Whether such a sequence exists in the corresponding human gene or whether Tm3 has its own gene requires further investigation.

ACKNOWLEDGMENTS

We are grateful to L. Kedes, P. Gunning, and R. Wade of Stanford University (Stanford, Calif.) for providing muscle RNA and cDNA and for communicating valuable information to us.

This study was supported by Public Health Service grant CA-34763 from the National Cancer Institute (to J.L.) and by funds donated by the Japan Shipbuilding Foundation.

LITERATURE CITED

1. Aviv, H., and P. Leder. 1972. Purification of biologically active messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* **69**:1408-1412.
2. Bailey, K. 1948. Tropomyosin: a new asymmetric protein component of the muscle fibril. *Biochem. J.* **43**:271-278.
3. Benton, W. D., and R. W. Davis. 1977. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* **196**:180-182.
4. Burbuck, S., G. Latter, E. Metz, and J. Leavitt. 1984. Neoplastic human fibroblast proteins are related to epidermal growth factor precursor. *Proc. Natl. Acad. Sci. USA* **81**:5360-5363.
5. Cooper, H. L., N. Feuerstein, M. Noda, and R. H. Bassin. 1985. Suppression of tropomyosin synthesis, a common biochemical feature of oncogenesis by structurally diverse retroviral oncogenes. *Mol. Cell. Biol.* **5**:972-983.
6. Dale, R. M., B. A. McClure, and J. P. Houchins. 1985. A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to sequencing the corn mitochondrial 18S rDNA. *Plasmid* **13**:31-40.
7. Denhardt, D. T. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**:641-646.
8. Fine, R. E., and A. L. Blitz. 1975. A chemical composition of tropomyosin from muscle and non-muscle tissues. *J. Mol. Biol.* **95**:447-454.
9. Flach, J., G. Lindquister, S. Berish, K. Hickman, and R. Devlin. 1986. Analysis of tropomyosin cDNAs isolated from skeletal and smooth muscle mRNA. *Nucleic Acids Res.* **14**:9193-9211.
10. Franza, B. R., and J. I. Garrels. 1984. Transformation-sensitive proteins of REF52 cells detected by computer-analyzed two-dimensional gel electrophoresis. *Cancer Cells* **1**:137-146.
11. Goldstein, D., J. Djeu, G. Latter, S. Burbuck, and J. Leavitt. 1985. Abundant synthesis of the transformation-induced protein

- of neoplastic human fibroblasts, plastin, in normal lymphocytes. *Cancer Res.* **45**:5643-5647.
12. Gunning, P., T. Mohun, S.-Y. Ng, P. Ponte, and L. Kedes. 1984. Evolution of the human sarcomeric-actin genes: evidence for units of selection within the 3' untranslated regions of the mRNAs. *J. Mol. Evol.* **20**:202-214.
 13. Helfman, D. M., S. Cheley, E. Kuismanen, L. A. Finn, and Y. Yamawaki-Kataoka. 1986. Nonmuscle and muscle tropomyosin isoforms are expressed from a single gene by alternative RNA splicing and polyadenylation. *Mol. Cell. Biol.* **6**:3582-3595.
 14. Hendricks, M., and H. Weintraub. 1981. Tropomyosin is decreased in transformed cells. *Proc. Natl. Acad. Sci. USA* **78**:5633-5637.
 15. Hendricks, M., and H. Weintraub. 1984. Multiple tropomyosin polypeptides in chicken embryo fibroblasts: differential repression of transcription by Rous sarcoma virus transformation. *Mol. Cell. Biol.* **4**:1823-1833.
 16. Hitchcock, S. E., H. E. Huxley, and A. G. Szent-Gyorgyi. 1973. Calcium sensitive binding of troponin to actin-tropomyosins: a two-site model for troponin action. *J. Mol. Biol.* **80**:825-836.
 17. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in λ gt10 and λ gt11. *DNA Cloning* **1**:49-78.
 18. Leavitt, J., P. Gunning, P. Porreca, S.-Y. Ng, C.-S. Lin, and L. Kedes. 1984. Molecular cloning and characterization of mutant and wild-type human β -actin genes. *Mol. Cell. Biol.* **4**:1961-1969.
 19. Leavitt, J., and T. Kakunaga. 1980. Expression of a variant form of actin and additional polypeptide changes following chemical induced in vitro neoplastic transformation and human fibroblasts. *J. Biol. Chem.* **255**:1650-1661.
 20. Leavitt, J., G. Latter, L. Lutowski, D. Goldstein, and S. Burbeck. 1986. Tropomyosin isoform switching in tumorigenic human fibroblasts. *Mol. Cell. Biol.* **6**:2721-2726.
 21. Leavitt, J., A. Leavitt, and A. Attallah. 1980. Dissimilar modes of expression of β -actin and γ -actin in normal and leukemic human T-lymphocytes. *J. Biol. Chem.* **255**:4984-4987.
 22. Leavitt, J., S.-Y. Ng, U. Abei, M. Varma, G. Latter, S. Burbeck, L. Kedes, and P. Gunning. 1987. Expression of transfected mutant β -actin genes: alterations of cell morphology and evidence for autoregulation in actin pools. *Mol. Cell. Biol.* **7**:2457-2466.
 23. Leavitt, J., S.-Y. Ng, M. Varma, G. Latter, S. Burbeck, P. Gunning, and L. Kedes. 1987. Expression of transfected mutant β -actin genes: transitions toward the stable tumorigenic state. *Mol. Cell. Biol.* **7**:2467-2476.
 24. Lin, C.-S., S.-Y. Ng, P. Gunning, L. Kedes, and J. Leavitt. 1985. Identification and order of sequential mutations in β -actin genes isolated from increasingly tumorigenic human fibroblast strains. *Proc. Natl. Acad. Sci. USA* **82**:6995-6999.
 25. Lin, J. J.-C., D. M. Helfman, S. H. Hughes, and C. S. Chou. 1985. Tropomyosin isoforms in chicken embryo fibroblasts: purification, characterization and changes in Rous sarcoma virus-transformed cells. *J. Cell Biol.* **98**:116-127.
 26. Lin, J. J.-C., S. Yamashiro-Matsumura, and F. Matsumura. 1984. Microfilaments in normal and transformed cells: changes in the multiple forms of tropomyosin. *Cancer Cells* **1**:57-65.
 27. Lund, P. K., B. M. Moats-Staats, J. G. Simmons, E. Hoyt, A. J. D'Ercole, F. Martin, and J. J. Van Wyk. 1985. Nucleotide sequence analysis of a cDNA encoding human ubiquitin reveals that ubiquitin is synthesized as a precursor. *J. Biol. Chem.* **260**:7609-7613.
 28. MacLeod, A. R., C. Houlker, F. C. Reinach, L. B. Smillie, K. Talbot, G. Modi, and F. S. Walsh. 1985. A muscle-type tropomyosin in human fibroblasts: evidence for expression by an alternative RNA splicing mechanism. *Proc. Natl. Acad. Sci. USA* **82**:7835-7839.
 29. MacLeod, A. R., C. Houlker, F. C. Reinach, and K. Talbot. 1986. The mRNA and RNA-copy pseudogenes encoding TM30_{nm}, a human cytoskeletal tropomyosin. *Nucleic Acids Res.* **14**:8413-8426.
 30. MacLeod, A. R., K. Talbot, L. B. Smillie, and C. Houlker. 1987. Characterization of a cDNA defining a gene family encoding TM30_{pl}, a human fibroblast tropomyosin. *J. Mol. Biol.* **194**:1-10.
 31. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning, a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 32. Matsumura, F., J. J.-C. Lin, S. Yamashiro-Matsumura, G. P. Thomas, and W. C. Topp. 1983. Differential expression of tropomyosin forms in the microfilaments isolated from normal and transformed rat cultured cells. *J. Biol. Chem.* **258**:13954-13964.
 33. Matsumura, F., and S. Yamashiro-Matsumura. 1985. Purification and characterization of multiple isoforms of tropomyosin from rat cultured cells. *J. Biol. Chem.* **260**:13851-13859.
 34. Matsumura, F., S. Yamashiro-Matsumura, and J. J.-C. Lin. 1983. Isolation and characterization of tropomyosin containing microfilaments from cultured cells. *J. Biol. Chem.* **258**:6636-6644.
 35. McLachlan, A. D., and M. Stewart. 1976. The troponin binding region of tropomyosin. Evidence for a site near residues 197-217. *J. Mol. Biol.* **106**:1017-1022.
 36. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20-78.
 37. Parnes, J. R., B. Velan, A. Felsenfeld, L. Ramamathan, U. Ferrini, E. Appella, and J. G. Sidman. 1981. Mouse β_2 microglobulin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. *Proc. Natl. Acad. Sci. USA* **78**:2253-2257.
 38. Reinach, F. C., and A. R. MacLeod. 1986. Tissue-specific expression of the human tropomyosin gene involved in the generation of the trk oncogene. *Nature (London)* **322**:648-650.
 39. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-251.
 40. Ruiz-Opazo, N., and B. Nadal-Ginard. 1987. α -Tropomyosin gene organization: alternative splicing of duplicated isotype-specific exons accounts for the production of smooth and striated muscle isoforms. *J. Biol. Chem.* **262**:4755-4765.
 41. Ruiz-Opazo, N., J. Weinberger, and B. Nadal-Ginard. 1985. Comparison of α -tropomyosin sequences from smooth and striated muscle. *Nature (London)* **315**:67-70.
 42. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
 43. Schenborn, E. T., and R. C. Mierendorf. 1985. A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. *Nucleic Acids Res.* **13**:6223-6236.
 44. Smillie, L. B. 1979. Structure and functions of tropomyosins from muscle and non-muscle sources. *Trends Biochem. Sci.* **4**:151-155.
 45. Sodek, J., R. S. Hodges, and L. B. Smillie. 1978. Amino acid sequence of rabbit skeletal muscle alpha-tropomyosin. *J. Biol. Chem.* **253**:1129-1136.
 46. Southern, E. 1975. Detection of specific DNA sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
 47. Stone, D., and L. B. Smillie. 1978. The amino acid sequence of rabbit skeletal muscle alpha-tropomyosin. *J. Biol. Chem.* **253**:1137-1148.
 48. Talbot, K., and A. R. MacLeod. 1983. Novel form of non-muscle tropomyosin in human fibroblasts. *J. Mol. Biol.* **164**:159-174.
 49. Taylor, E. W. 1979. Mechanism of actomyosin ATPase and the problem of muscle contraction. *Crit. Rev. Biochem.* **6**:103-163.