TEF-1 DEPENDENT EXPRESSION OF THE ALPHA-TROPOMYOSIN GENE IN THE THREE MUSCLE CELL TYPES

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In vertebrates, the actin binding proteins tropomyosins are encoded by four distinct genes that are expressed in a complex pattern during development and muscle differentiation. In this study, we have characterized the transcriptional machinery of the α-tropomyosin gene in muscle cells. Promoter analysis reveals that a 284-base pair proximal promoter region of the *Xenopus laevis* α-tropomyosin gene is sufficient for a maximal activity in the three muscle cell types. The transcriptional activity of this promoter in the three muscle cell types depends on both distinct and common cis-regulatory sequences. We have identified a 30-base pair conserved sequence unique to all vertebrate α-tropomyosin genes and that contains an MCAT site that is critical for the expression of the gene in all muscle cell types. This site can bind TEF-1 present in muscle cells both *in vitro* and *in vivo*. In serum-deprived differentiated smooth muscle cells, TEF-1 is redistributed to the nucleus and this correlates with an increased activity of the α-tropomyosin promoter. Overexpression of TEF-1 mRNA in *Xenopus* embryonic cells leads to the activation of both the endogenous α-tropomyosin gene and the exogenous 284-base pair promoter. Finally we show that, in transgenic embryos and juveniles, an intact MCAT sequence is required for the correct temporal and spatial expression of the 284-base pair gene promoter. These studies represent the first analysis of the transcriptional regulation of the α-tropomyosin gene *in vivo* and highlight a common TEF-1-dependent regulatory mechanism necessary for expression of the gene in the three muscle lineages.

Tropomyosin (Tms) constitute a family of actin filament binding proteins found in all eukaryotic cells. They are present in both muscle and non-muscle cells. In striated muscle cells, Tms play a central role in contraction by regulating calcium-sensitive interaction of actin and myosin. In non-muscle cells, Tms regulate actin filament organization and dynamics (1). The Tm genes exhibit extensive cell type specific isoform diversity. In vertebrates, the diversity is generated in part by the existence of several genes, containing alternative promoters. In addition, these genes exhibit alternative splicing variants of the primary RNA transcripts (2). Null mutations in mouse have shown that Tms are essential for both development and for normal structure and function of muscle cells. Furthermore, several point mutations associated with abnormal protein function have been found in myopathies (3-5).

We have previously identified three orthologues Tms genes, namely α-Tm, β-Tm and Tm4 (also call δ-Tm), in the amphibian *Xenopus laevis* (6-8). We have shown that each of the Tm genes has a distinct temporal and spatial pattern of expression during early development and
differentiation. The amphibian β-Tm gene is structurally related to its mammalian orthologues. This gene contains one promoter and two sets of alternatively spliced exons, and can produce both skeletal and smooth muscle isoforms (8,9). The amphibian Tm4 gene is identical to the avian orthologue, with two promoters and two sets of alternatively spliced exons. The distal promoter is used in cardiac cells while the internal one is used to produce the ubiquitously expressed non-muscle Tm isoform (7). The *Xenopus laevis* α-Tm gene is by far the most complex of all. Using RNA expression and genomic DNA analysis, we have shown that in common with its avian and mammalian orthologues, the frog α-Tm gene contains two promoters (internal and distal) and three sets of alternatively spliced exons. However, we have not found evidence of a brain specific exon (10,11). The low molecular, weight non-muscle Tm transcripts are found throughout oogenesis, embryogenesis and in adult tissues. In contrast, the muscle α-Tm transcripts exhibit a restricted expression pattern. Transcripts accumulate from late neurula stage in both the somites and heart and later during development in smooth muscle tissues. The spatio-temporal expression of the gene is regulated through the two promoters. The internal promoter is ubiquitously active whereas the distal promoter is only active in muscle cells. In the adult frog, the α-Tm gene is expressed in the three muscle cell types like its avian and mammalian orthologues. Transcriptional regulation of the α-Tm gene therefore provides a suitable model for the characterization of the cis-regulatory sequences involved in the transcriptional program that operates in all three muscle lineages.

Although the transcriptional regulation of several sarcomeric protein-coding genes has been studied in detail, very little is known about sequences that may regulate tropomyosin expression in the different muscle types. In *Drosophila*, MEF2 has been shown to be a positive regulator of the expression of the Tm gene (12), but very few studies have addressed the transcriptional control of Tm genes in vertebrates. It has been shown that the chicken β-Tm gene transcription relies on several cis-regulatory elements. Among them, we find an MCAT sequence that is embedded in a highly conserved 30-bp module unique to all vertebrate α-Tm genes. This MCAT sequence can bind TEF-1 protein in vitro and in vivo in tissue-specific expression pattern of the two (14). It is now established that multiple, independent cis regulatory regions, or modules, are required to direct the complete developmental pattern of expression of individual muscle-specific genes (15). For instance, the MyoD family members that govern skeletal muscle differentiation and constitute the paradigm for cell type-specific transcription factors, function by interacting with co-factors (16). Moreover, muscle differentiation, like other developmental processes, relies on a transcriptional circuit that is dependent on combinatorial associations of cell type-specific and widely expressed transcription factors. These associations interpret cell identity, extracellular signals, and positional information within the embryo (17). Indeed, some factors involved in muscle gene activation are not restricted to muscle cell types. Among them, ubiquitously expressed TEF-1 (Transcriptional Enhancer Factor 1) has emerged as a transcription factor implicated in the specific activation of several muscle genes (18). Originally described as a factor binding to non-muscle specific cis-elements of the SV-40 enhancer, TEF-1 has also been found to bind to the MCAT (5’-CATTCCT-3’) regulatory sequence (19,20). There are four TEF-1 genes in vertebrates which are expressed in a complex pattern and can produce several isoforms (21-23). Importantly, the MCAT sequence that bind TEF-1 is present in the regulatory regions of several mammalian and avian cardiac and skeletal muscle specific genes, including those encoding the cardiac troponin T, the skeletal α-actin, the α and β-myosin heavy chain and β-acetylcholine receptor (24-36). The MCAT sequence has also been implicated in the regulation of the mammalian smooth muscle α-actin gene, in a complex mechanism that involves several single strand DNA-binding proteins (37,38).

In this report, we examine the molecular mechanisms that regulate the *Xenopus laevis* α-Tm gene in cardiac, skeletal and smooth muscle cells. For this analysis, we have used a genomic clone that encompassed the 5’ region of the gene (10). We identify a 284-bp promoter fragment whose activity in the three muscle types is dependent on the presence of multiple cis-regulatory elements. Among them, we find an MCAT sequence that is embedded in a highly conserved 30-bp module unique to all vertebrate α-Tm genes. This MCAT sequence can bind TEF-1 protein in vitro and in vivo in
differentiated muscle cells. In smooth muscle, cells TEF-1 can be redistributed to the nucleus when cells are cultured under serum deprived conditions that maintains them in a differentiated state. We have found that overexpression of TEF-1 mRNA in embryonic cells activates both endogenous and exogenous α-Tm genes. In the later case, we demonstrate that this activation occurs through the MCAT sequence. Finally, mutation of the MCAT sequence leads to a deregulation of the 284-bp transgene in the embryo and juvenile tissues. Taken together, our data suggest that correct regulation of the vertebrate α-Tm gene in all the three muscle cell types depends on TEF-1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and transfections** – Rabbit U8A4 smooth muscle cells, rat PAC1 and A7r5 smooth muscle cells were cultured as previously described (39,40). The C2/7 and C2C12 skeletal muscle cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum (FCS) at low density and, induced to differentiate when approaching confluence with DMEM-2% FCS (41). Neonatal rat myocardial cells were prepared according to standard protocol (42). Briefly, myocytes were dispersed from ventricles of 1-3-day-old Sprague-Dawley rats by digestion with collagenase type II (SIGMA) and pancreatin (GIBCO) at 37 °C during four periods of 20 min. Cell suspensions were then separated on a discontinuous Percoll gradient to obtain primary cell cultures containing over 99% of myocytes. The myocytes were plated in 10-cm culture dishes (Falcon) at a density of 1.2-2.0 x 10^6 cells/cm^2 when cultured in defined medium and at a density of 25,000 cells/cm^2 when cultured in medium supplemented with 10% FCS. 400 ng of luciferase reporter gene constructs along with pHook2-LacZ (Invitrogen) were transfected per well using 2.4 µl of Transfast (Promega) in OptiMEM (GIBCO-BRL). After 1 h, 1 ml of culture medium was added and cells were cultured for 48 h before analysis. C2/7 cells were cultured for 48 h before preparing cell extracts. For the transfected cells, cell extracts were used to determine luciferase activity.

**Construction of reporter plasmids** – Promoter fragments of the α-Tm gene were generated from a previously isolated genomic clone (10). A BamHI/BamHI fragment (-1763/+40) was excised from the genomic clone and inserted into the BglII site of pGL3-Basic vector (Promega) to generate the pGL1763LUC construct. A SacI/BamHI fragment (-284/+40) was excised from the genomic clone and subcloned into the SacI and BglII sites of the pGL3-Basic vector to generate the pGL284LUC construct. pGL235LUC, pGL120LUC and pGL78LUC constructs were generated by PCR, from the genomic clone, using oligonucleotide primers containing SacI and BglII linkers such that PCR products could be directionally cloned into the pGL3-Basic vector. Site directed mutagenesis was performed using an in vitro Site-Directed Mutagenesis System (Promega) following manufacturer’s instructions. The primer mutant sequences were as follows (the mutated sequences are underlined): Ebox1 5'-GAAGCGTGGAAAAAGTTAGGTTGCTCACTTC-3', Ebox2 5'-AAATGCAAGTTCTAGAGTCGCCAGAAGC-3', Ebox3 5'-CCCTATATATAGTCTAGAGTCACAATGTC-3', box4, 5'-GCTCCCTTCAGATCTAGACCTGGAGTTT-3', A/T rich 5'-AGCCATCTCTTAAGGGAACCCAGCAATTG-3', CarG-like element 5'-AAGGCAGGCTCCCCAGTACCTATTGGTGCT-3', MCAT 5'-TTGGGTTGCAGGTTACCTGTGTGTCGCC-3', GC-box 5'-AATGTGTTGCTGATCTTCCGCTGCCCCTTC-3'. All plasmids were confirmed by sequencing.

**DNA transfections** – U8A4, NIH-3T3, A7r5 and PAC-1 cells were seeded the day before transient transfection assays into 12-well plates at a density of 25,000 cells/cm^2 when cultured in defined medium and at a density of 15,000 cells/cm^2 when cultured in medium supplemented with 10% FCS. 400 ng of luciferase reporter gene constructs along with 400 ng of β-galactosidase control vector pHook2-LacZ (Invitrogen) were transfected per well using 2.4 µl of Transfast (Promega) in OptiMEM (GIBCO-BRL). After 1 h, 1 ml of culture medium was added and cells were cultured for 48 h before analysis. C2/7 cells were grown in 12-well plates at a density of 5,000 cells/cm^2 until reaching 50% confluence and then transfected as described above. Following transfection, cells were induced to differentiate during 48 h before preparing cell extracts.

Cardiomyocytes were seeded into 6-well plates at a density of 20,000 cells/cm^2 three days before transfection. Cells were incubated for 5 h using 4 µl of FuGENE6 transfection Reagent (Roche) along with 1µg of plasmid DNA. After rinsing with DMEM medium, cells were cultured for 48 h before preparing cell extracts. For the
luciferase assays, cell extracts were prepared using the Reporter Lysis Buffer following manufacturer’s instructions (Promega). Luciferase and β-galactosidase activities were measured according to the manufacturer’s instructions. All luciferase activity values were normalized to β-galactosidase activity. The figures were obtained from at least two independent experiments, with each construct tested in triplicate. pGL3-Control and pGL3-Basic vectors (Promega) were used as controls.

Comparative sequence analysis – The nucleotide sequences of vertebrate α-Tm genes promoters were analysed using sequence similarity computer programs (Infobiogen, Evry, France).

In Vitro Transcription and Translation – Chicken TEF-1 cDNA was kindly provided by Dr. C. Ordahl and subcloned into the pCS2+ vector (23). The resulting plasmid as well as the Xenopus laevis SRF cDNA cloned into the pSP64T vector (43) were used for in vitro transcription. Synthetic capped mRNA were made using the Ambion Message Machine Kit according to manufacturer’s instructions. The products were purified on Mini Quick RNA Columns (Roche). 200 to 400 ng of mRNA were in vitro translated in reticulocyte lysate (Promega) according to manufacturer’s instructions. The integrity and expected molecular weights of the proteins were assessed by resolving radiolabelled reaction products on a 10% polyacrylamide gel.

Western blot analysis – Total protein concentration in the samples was determined by Bradford assay (Bio-Rad Laboratories). 10μg of nuclear proteins or 20μg of total proteins were separated on a 10% SDS-PAGE followed by transfer onto Immobilon-P membrane (Millipore, Bedford, MA). The membranes were blocked for 1 h at room temperature in 5% nonfat dried milk made in 130 mM NaC, 25 mM Tris (pH 7.5) and containing 0.05% Tween-20 (TBST). After being washed in TBST, the membranes were incubated for 2 h in TBST supplemented with primary antibody. The immunoblots were then washed three times with TBST and incubated for 1 h with a 1:10,000 solution of a horseradish peroxidase-linked anti-mouse or anti-rabbit Ig antibody in TBST. The blots were then washed three times with TBST and developed using the ECL Western blot detection system (Amersham). Signals were visualized on X-ray film after exposures of membranes between 30 sec and 5 min. The primary antibodies used were the monoclonal tropomyosin antibody TM311 (44) (Sigma, 1:1,000), the monoclonal TEF-1 antibody (BD Transduction Laboratories, 1:1,000) and an anti-TEF-1A polyclonal antibody (generously provided by Dr I. Farrance).

Electrophoretic Mobility Shift Assay (EMSA) – Nuclear extracts were prepared from cells according to established methods (45) and protein concentration was determined by Bradford assay. All extracts were snap-frozen in liquid nitrogen and stored at -80 °C. 500 pmoles of each sense and antisense oligonucleotides, were annealed (95°C, 5 min), in 50 μl of 50mM NaCl, 10mM Tris-HCl, pH 7.5 and 1 mM EDTA and then cooled to room temperature. 25 pmoles of double-stranded oligonucleotides were end-labelled using [γ32P] ATP (6,000 Ci/mmol) in the presence of T4 polynucleotide kinase. Labelled double-stranded oligonucleotides were recovered after purification Quiagen column in 10mM Tris-HCl, pH 8, 1 mM EDTA.

For EMSAs, 5 to 10 μg of nuclear extracts or 1-2 μl of in vitro translated TEF-1 and SRF were incubated for 20 min in binding buffer (10 mM Tris-HCl, pH 7.5; 5 mM HEPES, 1 mM EDTA, 100 mM KCl, 1 mM DTT, 1 mg/ml BSA and 10% glycerol) containing 0.20 μg of poly (di-dC) and 4 x 10⁴ cpm of end-labelled probe. After a 20 min incubation at room temperature, the samples were subjected to electrophoresis on a 5 % nondenaturing polyacrylamide gels in 0.5X Tris borate/EDTA buffer (44.5 mM Tris-HCl, pH 8.3, 44.5 mM boric acid, and 1 mM EDTA) for 2 h at 150V. Gels were dried and exposed to films with an intensifying screen at -80 °C for 24-48 h. For supershift analysis, 750ng of monoclonal TEF-1 antibody (BD Transduction Laboratories) were pre-incubated with nuclear extracts for 10 min before addition of the probe.

The nucleotide sequence of the probes and competitor DNA were as follows (only upper strand is shown): MCAT, 5′-GTGTCGGAGGAATGTGTGGCCC-3′; MCATmut, 5′-GTGTCGGAGGATCCTGTGTGCCC-3′; CarG-like element, 5′-AAGGCAAGGCTCCCAAAAAAGTATTGGGTGT-3′; c-fos SRE (46), 5′-CAGGATGTCCATATTAGGACATCTGCGT-3′; CarG1 SM-MHC (47), 5′-GACTTCTTTATGGCCCTGA-3′; CarG4 desmin (48), 5′-AACACCCATATATGAAAAAT-3′; MCAT TnT (32) 5′-
CGTTGTCATTCCTCTCGGATC-3';
CArG/MCAT 5'-
GGGTGTGAGGCTCCCAAAAAAGT ATTGGGTGTCCGG A
GGAATG TGTGTGCCC-3'; Underlined nuc
leotides correspond to core binding sites. Bold nucleotides represent mutated nucleotides.

Identification of proteins in gel shift complexes was performed as described elsewhere (24,49). The mobility shift reactions were scaled up fivefold in a final volume of 20 µl. After electrophoresis for 3 h, the gel was soaked twice for a total of 3 min in 2% SDS, 62.5 mM Tris, 25 mM dithiothreitol, and then dried. The DNA-protein complexes were then identified by a 2 h autoradiography, and the bands of interest were excised and loaded on a 10% SDS-PAGE gel. The immunoblot analysis was then carried out as described before with TEF-1 antibodies (BD Transduction Laboratories and Dr. I. Farrance’s TEF1 antibody).

Chromatin immunoprecipitation – Chromatin immunoprecipitation (ChIP) assays were performed on PAC1 smooth muscle cells cultured in 1% or 10% serum and from C2C12 myotblasts and myotubes according to published protocol (50). Briefly, 2.5x10^6 cells were used in the assays. The DNA and protein were cross-linked in situ by treatment in 1% (v/v) formaldehyde for 10 min at room temperature. Following chromatin sonication, the lysates were precipitated overnight using either 5µg of anti-TEF-1 mouse monoclonal antibody (BD Transduction Laboratories) or 5µg of anti-TEF-1 rabbit polyclonal antibody (AnaSpec, San Jose). Protein A-agarose beads were added to purify immune complexes. Crosslinking was reversed by heating the samples at 65 °C overnight. RNA was degraded with RNase A for 4 h at 65 °C and proteins were degraded by proteinase K treatment for 2 h at 45 °C. The DNA was further purified by phenol extraction and recovered in 10mM Tris-HCl, pH 8, 1mM EDTA.

After DNA purification, samples were subjected to PCR with primers designed for the α-Tm gene promoter (14) as follows: upper primer (U): 5’-GTCATATCCACCACCGGACTGG-3’ and lower primer (L): 5’-CPTGCTCCCTGATATGACTCTTTCC-3’. The amplified PCR product is 197-bp. For the smooth α-actin gene promoter (51), the primers were: U: 5’-CTCAAGCCCTTAGCTAATGGG-3’and L: 5’-AACTCCTTAACTGGTGGGC-3’. The amplified PCR product is 246-bp. PCR was performed for 40 cycles and 10µl of PCR products were analyzed by 2% agarose gel electrophoresis.

Immunofluorescence – Cells grown on coverslips were fixed for 10 minutes at 4 °C in methanol and then incubated for 2 h at 37 °C with the primary antibodies anti-TEF-1A or anti-SRF (SantaCruz, sc335X clone) at a 1:400 dilution. Cells were then incubated for one hour at room temperature with FITC conjugated secondary antibody. Nuclear staining was performed with iodide propidium. Negative controls were performed without primary antibody. Immunofluorescent staining was examined using a Nikon microscope.

DNA and RNA injections – Eggs were obtained from Xenopus laevis females, cultured, and microinjected as previously described (52). Embryonic stages were determined according to Nieuwkoop and Faber (53). Embryos were injected at the two-cell stage in each blastomere with pGL285LUC and pGLMCATLUC reporter constructs (45 pg/blastomere) along with synthetic TEF-1 mRNAs (100 pg/blastomere). Three pools of three embryos were collected at gastrula stage 12, homogenised in passive lysis buffer (Promega) and assayed for luciferase activity. Data were normalized per embryo. For RT-PCR analysis, 500 pg of TEF-1 mRNAs were injected in each blastomere of two-cell stage embryos. Animal caps were then excised at stage 8.5 and cultured until stage 11. RNA was extracted from 10 animal caps and analysed by RT-PCR as previously described (10) using the following primers: EF1α, U: 5’-CAGATTGGTGCTGGATATGC-3', L: 5’-A CTCGCCCTGAGTACTCTTAG-3’; α-Tm, U: 5’-AGATGTCAAACTGGACAAGG -3', L: 5’-CATCTGCAAGGCTCTCTACGCC -3’. PCR products were resolved on agarose gel.

Transgenesis – The PstI-BamHI fragments from pGL284LUC and pGLMCATLUC plasmids were subcloned into the GFP vector kindly provided by Dr. T. Mohun to create respectively the pGL284GFP and pGLMCATGFP constructs. α-Tm promoter plus GFP reporter sequences were excised from the backbone plasmid using NotI (New England Biolabs). The transgene was then used to generate transgenic Xenopus embryos using previously described protocols (54,55). Reporter gene expression was detected by fluorescence in live embryos using the Olympus SZX12 loupe couple to a ccd camera (Diagnostic instrument 3.2.0).
RT-PCR analysis of transgenic animals – RNA was extracted from juvenile tissues and analysed by RT-PCR as previously described (10) using the following primers: striated α-TM (U: 5’-CATTGAGGGTGATCTTGGAAC-3’, L: 5’-ATAGAGGTGACCATGGGAACG-3’); smooth α-TM (U: 5’-ATCTCCACCGAAACGAC-3’, L: 5’-TCTGGTCCACATGTGAC-3’); ODC (U: 5’-GTCAATGATGGAGTGTATGGATC-3’, L: 5’-TCCATTCCGCTCTGACCAC-3’) and GFP (U: 5’-GTGAAGGTGATGCAACATACG-3’, L: 5’-TCAAGAAGGACCATGTGGT-3’).

Whole-mount in situ hybridisation – Whole-mount in situ hybridisation was performed as described before using the XTMα2 probe (56).

RESULTS

Identification of a 284-bp promoter sequence of the α-tropomyosin gene that confers maximal activity in the three muscle cell types – We have previously shown that the 5’ region of the Xenopus laevis α-tropomyosin (α-Tm) gene is structurally related to its mammalian and avian orthologues. It contains two promoters flanking a pair of alternatively spliced exons, 2a/2b, exon 2a being a smooth muscle specific exon (10). mRNAs encoding muscle Tms originate from the distal promoter while mRNAs encoding low molecular weight non-muscle Tms originate from the internal one. As a first step to the identification of sequences required for maximum promoter activity in muscle cells, a series of deletions constructs were created from a genomic fragment, spanning 1763-bp upstream of the transcription initiation site. These different constructs were tested in skeletal, cardiac, smooth muscle cells as well as in fibroblasts. The C2/7 cell line was used as a model for skeletal muscle differentiation. Transcriptional activities were tested both at the myoblast stage and after differentiation when myotubes have formed. Transcriptional activities in cardiac and smooth muscle cell types were assessed in transfected rat primary neonatal cardiomyocytes and U8A4 cell line, respectively. We have previously demonstrated that U8A4 cells retain transcriptional and post-transcriptional potencies while maintaining a differentiated phenotype when expressing the smooth muscle α-Tm isoform (40). We have also shown that the U8A4 cells can be induced to de-differentiate when the NFAT signalling pathway is blocked (57).

Transient transfection assays with the different 5’ deletion constructs revealed that the maximum promoter activity in the three muscle cell types is obtained using the pGL284LUC construct (Fig. 1). In myoblasts, all the constructs tested retain a very low level of luciferase activity compared to the activity observed in myotubes (data not shown). The addition of 1500-bp of 5’-flanking DNA to the 284-bp promoter (pGL1763LUC construct) has no effect in skeletal or cardiac cells. However, this addition leads to a 50% reduction in promoter activity in smooth muscle cells. This indicates that this region contains one or several negative cis-acting elements specific to smooth muscle cells. The pGL235LUC DNA construct shows a 25 to 50% reduced activity in smooth and cardiac cells respectively when compared to the pGL284LUC construct. No difference is observed between these two constructs in skeletal muscle cells. Reducing the promoter sequences to 120 and 78-bp (pGL120LUC and pGL78LUC) leads to a dramatic loss of activity in all three muscle cell types.

Since smooth muscle cell lines can behave very differently with respect to promoter activity, we have tested the pGL284LUC construct in PAC1 and A7r5 smooth muscle cells. These cell lines have been considered to exhibit a differentiated phenotype (39). We found that the pGL284LUC construct was transcriptionally active in both cell lines, showing an efficiency equivalent to that observed in U8A4 cells. We obtained a similar result using the mouse SM22α promoter (data not shown). In contrast, in NIH-3T3 mouse fibroblasts, the 284-bp promoter yields no activity suggesting that this region is regulated in a muscle specific manner (data not shown). Together, these results indicate that the 284-bp proximal region of the α-Tm promoter can confer maximal activity in the three muscle cell types.

An MCAT sequence is essential for full α-Tm promoter activity in the three muscle cell types – Sequence analysis of the 284-bp promoter revealed the presence of several consensus DNA sequences known to be required for the binding of muscle specific transcription factors (Fig. 2A). Among them are four E-box motifs (hereafter referred to as E-box1, 2, 3 and 4 from proximal to distal positions), a GC-rich region, an A/T-rich region, a slightly imperfect CArG-box (CArG-like element) and one MCAT sequence.
sequences in mediating the transcriptional activity of the 284-bp promoter, each sequence was mutated and tested in a transient transfection assays in the three muscle cell types (Fig. 2B). In these experiments, the activity of the wild type 284-bp promoter was set as 100%. Mutation of E-box4 in the context of the promoter had no effect on its activity in cardiac muscle cells but rather stimulated its activity in smooth and skeletal muscle cells. Mutation of E-box3 had no effect on the activity of the promoter in skeletal or cardiac muscle cells, but resulted in a 50% reduction of activity in smooth muscle cells. Conversely, mutation of E-box2 had a more drastic effect on the promoter activity in cardiac and skeletal muscle but no significant effect in smooth muscle cells. Finally, E-box1 mutation led to a 80% reduced activity of the promoter in skeletal muscle and a 40 to 50% reduction in cardiac and smooth muscle respectively. Mutation of the GC-box led to about 70% reduction of promoter activity in the three muscle cell types. Mutation of the A/T rich region had a significant effect only in skeletal muscle cells, reducing the activity of the promoter by about 80%. Mutation of the CArG-like element had no effect on the activity of the promoter in smooth or cardiac cells but induced a 60% reduction of promoter activity in skeletal muscle cells. These results suggest that the α-Tm gene is controlled by both distinct and common cis-sequences in different muscle cell types.

The most striking results were obtained by mutation of the MCAT sequence. This had a dramatic effect on the transcriptional activity of the promoter in all three muscle cell types. The MCAT mutation led to an 80% reduction of activity in smooth (U8A4 or PAC1) and cardiac cells and an almost completely abolition of activity in skeletal muscle cells. Together, these results indicate that although multiple cis-regulatory elements are involved in the transcriptional activity of the 284-bp α-Tm gene promoter in muscle cells, the MCAT sequence appears as an important positive regulator in all muscle cell types.

The MCAT sequence is embedded in a highly conserved 30-bp module unique to α-Tm genes – In studying genes such the α-Tm gene that show conservation in both function and expression domains across species, DNA sequence comparison can be useful in identifying conserved regulatory regions. We therefore compared the 284-bp amphibian promoter with known vertebrates α-Tm gene promoters. Although the overall sequence conservation between the different α-Tm gene promoters is poor, a highly conserved 30-bp region encompasses the MCAT sequence and the flanking CArG-like sequence in all promoters examined (Fig. 3). There are only two or three nucleotide substitutions over this region between the amphibian, avian and mammalian sequences. These substitutions are found in the 13 nucleotides linker sequence between the CArG-like element and the MCAT sequence. This 30-bp sequence is unique to the α-Tm genes and is found neither in the promoter regions of the others tropomyosin genes family members nor in other genes promoter regions. Moreover the location of this sequence is conserved with respect to the transcription initiation sites that have been mapped in the Xenopus, quail and rat genes. A similar spatial organization of the CArG-like element and the MCAT sequence is also found in all promoters. Together, these findings indicate that the 30-bp sequence has been highly conserved during evolution and suggests its potential importance in the regulation of the α-Tm gene expression.

The MCAT sequence binds TEF-1 in vitro – The MCAT sequence has been described as the recognition site for TEF-1 protein in non-muscle, cardiac and smooth muscle cells (18). To determine whether the MCAT sequence found in the promoter region of the α-Tm gene could function as a TEF-1 binding site, we tested the ability of double stranded oligonucleotides encompassing the MCAT sequence to bind TEF-1 in a gel shift assay. As shown in Fig. 4A, in vitro translated TEF-1 can bind very efficiently to the MCAT sequence (Fig. 4A, lane 1). The retarded complex can be competed by an excess of unlabelled probe (an oligonucleotide corresponding to the MCAT consensus sequence of the chicken TnT promoter), but not by a mutated version of the MCAT sequence (Fig. 4A, lanes 2-5). Using nuclear extracts from either cardiomyocytes, smooth muscle cells or myotubes, three major complexes named C1, C2 and C3 are observed using the MCAT probe (Fig. 4B-D, lanes 1). These complexes are competed by the unlabelled MCAT probe or the chicken cardiac TnT gene MCAT sequence (Fig. 4B-D, lanes 2 and lanes 3 respectively) but not by a mutated MCAT site (Fig. 4B-D, lanes 4). Our results are in agreement with previous observations that show the formation of three distinct complexes varying in intensity with...
nuclear extracts from striated and smooth muscle cells (24,38,49).

The addition of a TEF-1-specific antiserum produced a supershifted complex with much slower mobility and a depletion of complex C3 (Fig. 4B-D lanes 5). This indicates that TEF-1 proteins contribute to MCAT-binding activities in muscle cells. The addition of preimmune serum did not supershift any of the three mobility shift complexes (Fig. 4B-D, lanes 6).

We next performed EMSA with nuclear extracts from non-muscle cells myoblasts (Fig. 4E, lanes 1-3) and NIH-3T3 (Fig. 4E, lanes 4-6). As shown in Fig. 4E, the three complexes specific for the MCAT probe are formed and only the lower complex C3 is supershifted by TEF-1 antibody (Fig. 4E, lanes 3 and 6). Previous reports have mentioned the inability of some TEF-1 antibodies to work in supershift analysis, indicating that some MCAT binding activities may not be related to TEF-1 (38,49). To test this possibility more directly, we asked whether each of the three protein-MCAT DNA complexes contained polypeptides antigenically related to TEF-1. Briefly, protein-MCAT DNA complexes made with cardiomyocytes and U8A4 smooth muscle cells nuclear extracts were separated by EMSA and the position of each complex localized by autoradiography (Fig. 4 E, lanes 1-3) to C3 is supershifted by TEF-1 antibody (Fig. 4E, lanes 3 and 6). This suggests that complexes C1 and C2 contain protein antigenically related to TEF-1. Since the intensity of complexes C1 and C2 is higher than that of complex C3, we may hypothesize that the TEF-1 related polypeptide contained in C1 and C2 is either much prevalent than the TEF-1 in complex C3, or present at a lower level but with higher affinity for the MCAT sequence.

**The MCAT sequence binds TEF-1 in vivo** – To ascertain the presence of TEF-1 family members in the nuclear extracts we used in EMSA, we performed Western blot analysis with muscle and non-muscle extracts. Results of immunoblot analysis with the TEF-1 antibody showed that all nuclear extracts contain a 52 kDa polypeptide that is antigenically related to TEF-1 (Fig. 4H, lanes 1-5).

To directly address whether TEF-1 bound the Tm MCAT element in vivo, we employed ChIP assays. Since the promoter region of the smooth α-actin has been shown to contain MCAT elements active in smooth muscle myocytes, this promoter was used as a control in those experiments (38). PAC1 smooth muscle cells cultured either in 10% serum or 1% serum, C2C12 myoblasts and myotubes were fixed directly with formaldehyde. Cross-linked chromatin was immunoprecipitated with anti-TEF-1 antibody. The precipitated chromatin DNA was then purified and subjected to PCR analysis for enrichment of the target sequences. As shown in Fig. 5, anti-TEF-1 antibody specifically enriched the MCAT containing region of the α-Tm promoter in differentiated smooth muscle cells (1% serum) and in myotubes (Fig. 5, compare lanes 6 and 12 with lanes 5 and 11). In PAC1 smooth muscle cells cultured in 10% serum or in myoblasts there is no binding of TEF-1 to the α-Tm gene promoter region (Fig. 5, compare lanes 3 and 9 with lanes 2 and 8). Therefore the binding of TEF-1 to MCAT sequence seems to be specific to the differentiation status of the cells. The smooth α-actin gene region that contains MCAT sequences is enriched in samples immunoprecipitated with TEF-1 antibody (Fig. 5, lanes 3 and 6). These MCAT elements have been shown to contribute to cell type-specific regulation of the smooth α-actin gene (38). We show here for the first time that this is correlated to the in vivo binding of TEF-1 to that region. An apparently unexpected finding of our ChIP assays is that in myotubes, TEF-1 antibody is bound to MCAT sequences of the smooth α-actin gene promoter (Fig. 5, compare lanes 11 and 12 with lanes 8 and 9). However previous report has clearly shown the immunodetection of smooth α-actin in myoblast C2C12 cells within hours of exposure to differentiation medium (58).

*The CarG like element of the 30-bp module does not bind SRF efficiently –* CarG boxes are 10-bp elements having the sequence CC(A/T)GG that are present in promoter regions of immediate early growth-response genes. CarG boxes are also found in many muscle specific gene promoters where they regulate transcriptional activity by binding the serum response factor (SRF) (59). Several studies have described divergent CarG boxes, named CarG-like elements, that have a single base mismatch from the original sequence but
still bind SRF although with a lower affinity. Some CArG-like elements present a C or G substitution in the (A/T)n core such as the CArG-like element (5'-CCTTTTATGG-3') found in the mouse SM-MHC gene promoter (47). Other elements exhibit a slight change at the 5' or 3' end of the sequence such as the CArG4 element (5'-CCATATATGT-3') of the mouse desmin gene promoter (48). The CArG like element 5'-CCAAAAAAGT-3' located 13-bp upstream of the MCAT site within the 30-bp module of the α-Tm gene promoter belongs to this type of element. We performed gel shift assay in order to know whether this CArG-like element could bind SRF. A double stranded oligonucleotide containing the CArG-like sequence, as well as 8-bp and 12-bp of 5'- and 3'-flanking sequence respectively, was labelled and incubated with muscle nuclear extracts. Our first attempts failed to detect any binding between nuclear extracts and the CArG-like element (data not shown). We then turned to in vitro translated Xenopus laevis SRF protein and failed to detect any binding even when increasing amount of protein or after extensive exposure of the autoradiogram (data not shown). In order to control whether the in vitro translated SRF was able to bind to bona fide CArG box and to CArG-like sequence, we used the CArG1 box sequence (5'-CCTTTTATGG-3') of the mouse SM-MHC and the CArG4 element (5'-CCATATATGT-3') of the mouse desmin gene as probes (Fig. 6A and Fig. 6B). The in vitro translated SRF can bind CArG1 box sequence efficiently, giving a complex which can be competed by an excess of either the unlabelled probe, the CArG box of the c-fos gene or the CArG-like element of the desmin gene (Fig. 6A, lanes 2, 4 and 5). The CArG-like element of the α-Tm promoter can compete the complex, albeit not completely, even when using a 200 fold excess of competitor (Fig. 6A, lane 3). As shown in Fig. 6B, SRF can form a complex with the CArG4 desmin probe that is competed by the unlabelled probe, the CArG boxes of SM-MHC and the c-fos gene (Fig. 6B, lanes 2-5; 8-13). The CArG-like element of the α-Tm gene cannot compete the complex totally even when in large excess (Fig. 6B, lanes 5-7). Together, these results demonstrate that the CArG like element of the 30-bp module of the α-Tm gene promoter cannot bind SRF or does so with a very low affinity. This is similar to previous findings, showing, for example, that the CArG like element of the cardiac TnT gene does not compete efficiently when analysed by competition footprint (33).

Previous reports have suggested the importance of CArG box and MCAT sites within the avian troponin T and mammalian skeletal actin gene promoters (30,60). It has also been shown that SRF and TEF-1 can interact through a direct and stable interaction (60). In order to know whether SRF could help to the binding of TEF-1 to the MCAT sequence, we performed EMSA using a double stranded oligonucleotide of 44-bp covering the 30-bp module containing the CArG-like and the MCAT sequence with 9-bp and 5-bp of 5'- and 3'-flanking sequence. We used a fixed amount of in vitro translated TEF-1 and increased the amount of in vitro translated SRF. As shown in Fig. 6C, in vitro translated SRF cannot bind to the probe while TEF-1 does (Fig. 6C, lanes 1, 2 and 6). When increasing amount of SRF are added to the reaction, an increase in the TEF-1 MCAT complex is observed (Fig. 6C, lanes 3-5). Together these results suggested that at least in vitro, SRF can help TEF-1 to bind to its sequence or prevent its dissociation from its binding site.

**MCAT sequence is required for maximum transcriptional activity of the α-Tm gene promoter in differentiated smooth muscle cells** – Unlike skeletal or cardiac muscle cells that are terminally differentiated, smooth muscle cells retain a remarkable plasticity and can undergo reversible changes in phenotype in response to changes in local environment cues, such as exposure to growth factors (61). These changes in smooth muscle cell phenotype are referred to as phenotypic modulation. For example, after injury, mature cells exhibiting a contractile phenotype switch to a synthetic state characterized by an increased in the rate of proliferation migration and synthesis of extracellular matrix. We have previously shown that the U8A4 cells, when cultured in absence of serum have retained a differentiated phenotype and express several smooth muscle markers amongst them the α-Tm smooth muscle specific isoform (40). In order to know whether the expression of the α-Tm gene promoter is sensitive to the phenotypic modulation of smooth muscle cells, we transfected the pGL284LUC DNA construct, either wild type or mutated at the MCAT sequence (pGLMCATLUC), in U8A4 cells that were cultured in the presence or absence of 10% serum. In these experiments, the activity of the pGL3 control vector was set as 100%. As expected, the pGL284LUC construct
showed a high level of expression in U8A4 cells when the cells are cultured in absence of serum (Fig. 7A). However, when the cells are cultured in 10% serum, the pGL284LUC construct showed a 70% decrease in activity. The pGLMCATLUC showed a very low level of activity whether the cells were cultured in the presence or absence of serum. Thus, the TEF-1 transcription promoting activity of the α-Tm gene is selectively increased in serum-deprived myocytes. We have also observed a similar increase in the activation of the pGL284LUC DNA construct in the PAC1 smooth muscle cell line when cultured in low serum conditions (1% serum) compared with high serum conditions (10% serum) (data not shown). Together with the ChIP data, these results demonstrate the absolute requirement for binding of TEF-1 to the MCAT sequence for transcriptional activity of the α-Tm gene in smooth muscle cells.

The subcellular localisation of TEF-1 in smooth muscle cells is dependent on serum – It has been described that SRF-dependent gene expression in smooth muscle cells can be regulated through nuclear translocation of SRF (62,63). To test whether cytoplasmic redistribution of TEF-1 could account for the diminished TEF-1 transcription-promoting activities observed when cells are cultured in high serum, we analyzed TEF-1 localisation by immunofluorescence staining in U8A4 cells cultured in either presence or absence of serum. As a control experiment, we also analysed the cellular distribution of SRF in the same conditions. TEF-1 immunoreactivity is restricted to the nucleus of U8A4 cells cultured in absence of serum (Fig. 7C, a) whereas in serum fed cells, TEF-1 is partially redistributed to the cytoplasm (Fig. 7C, c). In contrast, and as previously reported, SRF protein appeared strictly localized to the cytoplasm of cells in the absence of serum but found in the nucleus of cells cultured in presence of serum (Fig. 7C, e,g) (62). We have observed similar results using PAC1 smooth muscle cells (data not shown). In order to know whether there is modification of TEF-1 content in cells in response to culture conditions we performed Western blot analysis. As shown in Fig. 7B, TEF-1 protein content is not modified in U8A4 smooth muscle cells cultured either in presence of in absence of serum. As a control there is no variation of the ubiquitously expressed Tm proteins. Together, these data indicate that the full activity of the α-Tm gene in smooth muscle cells is not correlated with new synthesis of TEF-1 in the cytoplasm but with TEF1 nuclear localization.

TEF-1 can activate the α-Tm gene in embryonic cells – We next asked whether TEF-1 could activate the α-Tm gene in Xenopus embryonic cells. For this purpose, we have taken advantage of the animal cap assay that has been widely used for testing gene activation by transcription factors. For instance, we have shown that in this model the skeletal myosin light chain 1/3 gene and the α-Tm gene are induced by the MyoD family of transcription factors (10,52). As shown in Fig. 8A, animal cap explants from TEF-1 mRNA microinjected embryos showed a higher level of expression of muscle α-Tm mRNA when compared to uninjected control embryos. This suggests that TEF-1 can activate the endogenous α-Tm gene in embryonic cells. To know whether TEF-1 could activate the pGL284LUC DNA, we injected two-cell stage embryos with the DNA construct together with TEF-1 mRNA and then measured the luciferase activity in gastrula stage embryos. Embryos injected with TEF-1 mRNA showed a 3.5 fold increase in luciferase activity compared to embryos injected with the pGL284LUC DNA alone (Fig. 8B). The luciferase activity observed in embryos injected with pGLMCATLUC DNA is equivalent to the activity resulting from the injection of the wild type pGL284LUC DNA. Together these data suggest that TEF-1 can activate the α-Tm gene in embryonic cells through the MCAT site found in the minimal promoter.

The MCAT sequence is required for the correct expression of the α-Tm transgene in vivo – We have previously shown, by in situ hybridization and RT-PCR analyses, that the α-Tm gene is expressed in the somites, the embryonic heart and later in the smooth muscle tissues of the embryo (10). As shown in Fig. 9, whole-mount in situ hybridization with a striated α-Tm probe confirm expression of the gene in the somites of late neurula and tadpole embryo (Fig. 9A, a-c) and in the tadpole embryonic heart (Fig. 9A, c).

Because a relatively small upstream region of the Xenopus laevis α-Tm gene promoter is sufficient to direct high levels of cardiac, skeletal and smooth muscle muscle-specific gene expression in vitro, we asked whether this same region functions in an equivalent manner in the in vivo environment of the Xenopus laevis embryo and juvenile. Preliminary transient
expression experiments using microinjection of DNA luciferase constructs indicated that the maximum reporter gene expression was observed in embryos injected with the pGL284LUC construct. In these experiments, transcription of the transgene was correctly initiated (data not shown).

We then used a permanent transgenesis assay (54,55) with a GFP reporter gene placed under the control of the 284-bp promoter. As shown in Fig. 9B the pGL284GFP gene was expressed in the embryo in a correct temporal and spatial pattern (Fig. 9B, a,b,e,f). The expression of the transgene was not detected in early neurula (Fig. 9A, b) but was first detected in late neurula stages within the somites (data not shown). Later during development, GFP expression became obvious within the somites and embryonic heart from the tadpole stage (Fig. 9B, f). Therefore, the 284-bp promoter can recapitulate the expression of the α-Tm endogenous gene in somites and embryonic heart (Fig. 9A, a-c). In order to determine whether the MCAT binding site located in the 284-bp is required for correct expression of the reporter gene in transgenic embryos, we made a pGLMCATGFP construct in which the MCAT sequence has been mutated and showed no binding of in vitro translated TEF-1 (see Fig. 4A). The resulting pGLMCATGFP transgene was expressed in the early neurula stage embryo, with no restriction in cell specificity (Fig. 9C, d and data not shown). Later during development, the transgene is expressed in muscle and non-muscle cells of the embryo, a pattern that resembles the expression seen with transgenes driven by a ubiquitous promoter (Fig. 9C, h). Therefore mutation of the MCAT sequence and the resulting impairment of TEF-1 binding leads to a temporal and spatial deregulation of transgene expression. It is difficult to detect live GFP expression in smooth muscle tissues of the embryo because of the autofluoresence of these tissues. In order to know whether the GFP transgene was expressed in smooth muscle tissues, we let the transgenics develop further and undertook RT-PCR analysis from juvenile tissues. We analysed the GFP mRNA content of striated muscles (skeletal and heart), smooth muscle (stomach and intestine) and from the non-muscle tissue liver. In parallel we analysed the levels of striated muscle and smooth muscle α-Tm mRNA isoforms (10). As shown in Fig. 9D, pGL284GFP gene expression is restricted to all muscle tissues but absent from liver. In contrast, the pGLMCATGFP transgene containing a mutated version of MCAT sequence is expressed in all tissues, albeit at a lower level. Together, these data suggest that in vivo, an intact MCAT sequence is critical for a correct pattern of expression of the α-Tm gene in the three muscle lineages.

**DISCUSSION**

The goal of this study was to identify cis-elements and trans-acting factors that are important for the transcriptional regulation of the α-tropomyosin (α-Tm) gene in the different muscle cell types. Our previous work has shown that the Xenopus laevis α-Tm gene, like its mammalian orthologues is expressed in skeletal, cardiac and smooth muscle cells during development and in the adult (6,10). As yet, there have been no studies describing the elements involved in the activation of the gene in these different muscle types. In order to characterize such sequences, we have used in a first approach, an in vitro cell culture system with skeletal, cardiac and smooth muscle cells. From deletion analysis we determined that a minimal promoter containing the first 284-bp upstream from the transcription start site was sufficient for maximal expression in all muscle cells but was inactive in myoblasts and fibroblasts. We found that this promoter contains several known cis-elements that have been implicated in the regulation of numerous muscle genes i.e. four E box motifs, a GC-rich region, an A/T-rich region, a CArG-like element and one MCAT inverted sequence. Mutation analysis reveals that all these cis-sequences, with the exception of E-box4 mediate positive regulation of the α-Tm promoter. The decrease in promoter activity resulting from the different mutations is dependent on the muscle cell type. Regarding E-box sequences, we observed that E-box1 has a more pronounced positive effect in skeletal muscle while E-box2 and E-box3, although having identical core sequences, have positive effect in skeletal muscle and smooth muscle cells respectively. These data suggest, as it has been already established, the presence in muscle cells of distinct bHLH factors that can bind E-box elements and the combinatorial interaction between those factors and more generally expressed factors (15). It has been previously reported that the expression of the rat smooth α-actin promoter could be directed by E-box elements through distinct bHLH factors in

### Reference

1. [Cis-elements and trans-acting factors in muscle gene regulation (6,10)].

2. [Mutation analysis of α-Tm promoter](#).
skeletal versus smooth muscle cells (64). Similarly, we may hypothesize that distinct bHLH factors could participate in the activation of the gene in the different muscle lineages. Accordingly, when performing gel shift analysis with the distinct E-box probes we observed variations in the efficiency of competition between the different E-box sequences suggesting the existence between muscle tissues of proteins with different binding affinity (data not shown). The A/T-rich sequence of the 284-bp α-Tm promoter resembles, although not completely, a MEF-2 consensus site and, when mutated in the context of the promoter, a significant reduction of promoter activity is observed only in skeletal muscle. On the contrary, mutation of the GC-box impaired transcriptional activity in the three muscle cell types with a similar efficiency. The GC-box constitutes a DNA motif present in the promoter of a very large number of genes that is recognised by members of the Sp1 family. Toutant et al. have characterized an enhancer element in the chicken β-Tm gene that contains a stretch of 7 Cs whose mutation results in a decrease of myotube-specific transcriptional activity (13). The GC sequence of the frog α-Tm promoter also contains 7 Cs and could represent similar target sequence for Sp1 family members. Our in vitro analysis indicates that muscle restricted activity of the α-Tm gene is regulated by multiple positive regulatory elements that are active in different muscle cell types.

One major finding of our analysis is that among the different cis-elements present in the 284-bp promoter, the mutation of the MCAT sequence leads to the most striking reduction of promoter activity in all muscle cell types. This suggests that this sequence is a critical element for the regulation of the α-TM gene in muscle cells and additional lines of evidence support this. The MCAT element (5′-CATTCCCT-3′), originally described as an essential element for the promoter activity of the chicken TnT gene in skeletal muscle, has now been firmly established as an important regulatory element for the activation of several skeletal and cardiac genes (18). MCAT sequence has also been showed to be involved in the regulation of the mammalian smooth muscle α-actin gene (37,38). Although the MCAT element of the α-Tm promoter is in an inverse orientation (5′-AGGAATG-3′) it is present in all vertebrate α-Tm gene promoters in this same orientation and at a conserved position relatively to the transcription initiation site. Moreover the MCAT sequence is embedded in a highly conserved 30-bp sequence that shows only 3 divergent nucleotides between all vertebrate promoters. Outside this region there is no obvious sequence homology suggesting a conserved role of this region between distant species.

We have therefore focused our work on the MCAT sequence because of its conservation and its critical involvement in the activity of the promoter in the three muscle types. Results from EMSA analysis demonstrated that the MCAT sequence of the α-Tm promoter was bound by TEF-1 protein present in nuclear extracts of cardiac, skeletal and smooth muscle cells. With both muscle and non-muscle nuclear extracts, we reproducibly observed three different shift complexes with varying intensities. This pattern is in agreement with previous reports and is related to the multiple isoforms produced by the four TEF-1 vertebrates genes that all can bind the MCAT sequence. It is therefore difficult to identify whether one isoform is more specifically involved in the regulation of the α-Tm gene in muscle cells (24,38,49). Azakie et al. have shown that the three mobility shift complexes formed with cardiac nuclear extracts can be supershifted by a DTEF-1 rabbit antiserum and contain three polypeptides of 52, 54 and 57 kDa proteins identified in previous Western blot analysis described by Farrance et al. (24,49).

Our data shows that only the highest mobility complex formed in all cell types can be supershifted by a monoclonal TEF-1 antibody. Using Western blot analysis with this antibody, we have shown that the proteins eluted from all three complexes formed in cardiomyocytes and smooth muscle cells contain a 52 kDa TEF-1 polypeptide. This polypeptide is highly enriched in the highest mobility complex when compared to lower mobility complexes. These different observations confirm the presence of multiple and antigenically distinct TEF-1 isoforms able to interact with MCAT sequence in muscle and non-muscle cells.

We used ChIP assay to demonstrate that TEF-1 binds the α-Tm promoter within the context of chromatin in vivo. In those experiments, we used two different antibodies (BD Transduction Laboratories and AnaSpec) and we reproducibly found that they both immunoprecipitate TEF-1 proteins bound to the α-Tm promoter. One important and new finding is that TEF-1 binds the α-Tm promoter only in differentiated smooth
muscle cells (in serum deprived) or in skeletal muscle cells (myotubes). We found no evidence of TEF-1 binding to the promoter in myoblasts or in serum fed smooth muscle cells. Very few reports have described in vivo binding of TEF-1 to gene promoters. Azakie et al. have shown that DTEF-1 interacts with the cardiac troponin T (cTnT) promoter in vivo in cardiac cells (24). Shie et al. used a RTEF-1 antibody (Genemed Synthesis, Inc.) to show the binding of RTEF-1 to the VEGF promoter in endothelial cells (65). In this latter case, cells were previously transfected with RTEF-1 expression vector plasmid.

Using three different approaches (EMSA with supershift, EMSA combined with Western blot and ChIP), we have shown for the first time the binding of TEF-1 to MCAT sequence in smooth muscle cells and myotubes. The smooth α-actin gene is the only smooth muscle gene defined to date that has been shown to be regulated through MCAT element. The promoter contains two MCAT sequences that are highly conserved across mammalian species (51). Owens and co-workers have demonstrated the interaction of TEF-1 related proteins to these MCAT sequences and reported that mutation of these motifs resulted in increased transcriptional activity in smooth muscle cells (but not in myotubes or endothelial cells) (38). A recent study reported a more complex situation regarding the activity of the smooth muscle α-actin gene (37). This study showed regulation by a complex set of interactions involving single strand DNA-binding repressors and a cooperation between TEF-1 and SRF (37). We show here for the first time that TEF-1 binds in vivo to the MCAT sequence of α-Tm and the smooth α-actin genes in smooth muscle cells. However, one striking difference is that for the α-Tm gene, TEF-1 binding is strictly correlated to the differentiated status of the smooth muscle cells while, in the case of the α-smooth actin promoter, TEF-1 is already bound to the promoter in smooth muscle cells cultured in 10% serum. One unexpected finding from this study was the observation that TEF-1 binds to the MCAT sequence of the smooth actin gene promoter in myotubes. However this is consistent with a previous report showing immunodetection of smooth α-actin during the time course of myotube differentiation (58).

Our study demonstrates that the α-Tm gene constitutes an additional muscle gene regulated through an MCAT element that can bind TEF-1 protein present in differentiated smooth muscle cells. We show here that the α-Tm promoter activity is maximal in smooth muscle cells under conditions that favour differentiation. As we previously showed, the differentiated phenotype corresponds to cells cultured in serum free medium (40). In those conditions, the activity of the α-Tm promoter depends on an intact MCAT sequence. Moreover, we found that the nuclear localisation of TEF-1 is correlated with the activity of the promoter. In serum free cultured cells TEF-1 is mostly nuclear while it is partially relocalised in the cytoplasm in the presence of serum. This differential subcellular localisation observed in smooth muscle constitutes a novel pathway controlling TEF-1 transcription-promoting activity. It would be interesting to know whether the nuclear localization of TEF-1 is changed during the differentiation of myoblasts to myotubes.

TEF-1 proteins appeared to require transcriptional co-activators such as the ubiquitous YAP65 protein (66). It has also been shown that in a number of skeletal- and cardiac-specific gene promoters, the function of MCAT element is dependent on interaction with one or more cis-binding factors, including SRF, SP1 and PARP (30,60,67-69). Cooperativity has been observed in the case of the skeletal α-actin promoter between SRF and TEF-1 where they appear to function in concert to mediate TGFβ1-adrenergic stimulation in cardiac myocytes (70). The 284-bp α-Tm promoter does not contain a bona fide CArG box 5'-CC(A/T)GG-3' but instead a CArG-like element 5'-CC(A/T)GT-3' which is invariably located at a distance of 13-bp from the MCAT sequence in all known vertebrate α-Tm genes. Mutation of this sequence leads to a weak decrease in activity of the promoter and only in skeletal muscle cells, suggesting a minor role of this sequence in the activation of the gene in muscle cells. Moreover, we found no evidence of binding activity to this CArG like element when using nuclear extracts from muscle cells and the in vitro synthesized SRF has a very low affinity for this sequence. However, we observe, in the presence of in vitro translated SRF, an increase in the TEF-1 MCAT complex. Together, this suggests that at least in vitro, SRF can help TEF-1 to bind to its sequence or prevent its dissociation from its binding site.
Although \textit{in vitro} studies have limitations inherent to cell cultures, our data clearly indicate that the expression of the $\alpha$-TM gene in all muscle cell types is dependent on an evolutionary conserved MCAT site that can be bound with high affinity by TEF-1. Analysis of the activation of MCAT-dependent muscle promoters by TEF-1 has been limited (24). However, we report here the ability of TEF-1 to trans-activate $\alpha$-Tm promoter when overexpressed in \textit{Xenopus} embryonic cells. In addition, this activation could occur through the MCAT site present in 284-bp proximal promoter of the gene. No previous studies have addressed the \textit{in vivo} regulation of a vertebrate $\alpha$-TM gene promoter and we show here that a minimal promoter containing 284-bp of upstream sequences is sufficient to direct a temporal and spatial regulation of the transgene in the \textit{Xenopus} embryo. The expression of the transgene in the three muscle cells types is confirmed in juvenile frog tissues. Moreover when the MCAT sequence is mutated in the context of the minimal promoter, the transgene shows deregulated expression with a loss of muscle specificity in the embryo and the juvenile frog. This indicates an absolute requirement of TEF1 binding for a correct developmental muscle gene expression of the $\alpha$-Tm transgene. Similar observations have been made that highlight the importance of \textit{cis}-regulatory sequences in the correct developmental expression of muscle genes in the \textit{Xenopus} embryo. For instance, it has been showed that mutation of \textit{cis}-regulatory sequences YY1, Nkx2-5 or GATA result in the ectopic expression in transgenic \textit{Xenopus} embryo of the cardiac Myosin Light Chain 2 (MLC2) and Atrial Natriuretic Factor (ANF) genes (71,72). In those cases, these regulatory sequences may act as repressors of ectopic expression although they might have also positive role.

Why does mutation of the MCAT motif in the 284-bp $\alpha$-Tm gene promoter result in ubiquitous transgene expression in the early embryo? One possible interpretation is that, in the early embryo, the MCAT site mediates repression of the gene when bound by TEF-1 either alone or in conjunction with a co-repressor. Later during development, the co-repressor is absent from muscle cells and allows the expression of the gene. Alternatively, a co-activator whose expression is restricted to muscle replaces it. For instance the co-activators TONDU and PARP have been described to interact with TEF-1 (69,73,74). In the latter case, the co-activator PARP has been shown to be essential for the the correct \textit{in vivo} expression of the cardiac TnT gene in zebrafish embryo (75). The potential involvement of such co-activators in the activation of the $\alpha$-Tm gene awaits additional experiments.

Very few genes have been showed to be regulated \textit{in vivo} in the three muscle lineages through a unique \textit{cis}-regulatory sequence. One of these genes is the HRC gene which encodes the histidine-rich calcium-binding protein and whose \textit{in vivo} expression in cardiac, skeletal and arterial smooth muscle relies on a MEF2 site (76). Our studies suggest that the MCAT site of the $\alpha$-Tm promoter could be another \textit{cis}-regulatory sequence necessary for a correct spatial-temporal expression of a gene in the three muscle lineages. To our knowledge this is the first report describing the transcriptional control analysis of the $\alpha$-Tm gene in muscle and the \textit{in vivo} requirement of an MCAT sequence in the activation of a muscle gene during development and in the three muscle cell types.
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FOOTNOTES

1 Supported by a PhD fellowship awarded by the MENRT. 2 These authors made equal contributions to this work. The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL data bank unit with accession number DQ871277.

The abbreviations used are: bp, base pair; ChiP, chromatin immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; MCAT, muscle CAT; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pLUC, plasmid luciferase reporter vector; SMC, smooth muscle cell; SM-MHC, smooth muscle myosin heavy chain; SRF, serum response factor; Tm, tropomyosin; TnT, troponin; cTnT, cardiac troponin; TEF-1, transcription enhancer factor 1.
Fig. 1. A 284-bp minimal promoter of the α-Tm gene confers maximal activity to a luciferase reporter gene in muscle cells. U8A4 smooth muscle cells (SMC), cardiomyocytes or myotubes were transiently transfected with the indicated α-Tm-LUC reporter constructs. Negative numbers to the left represent the 5’ end of the various deletions constructs. Luciferase activities (light units) were corrected for variations in transfection efficiencies as determined by β-galactosidase activities. Bars show the mean corrected luciferase value and standard deviation for each construct.

Fig. 2. Cis-sequence mutation analyses of the 284-bp α-Tm gene promoter identifies a critical MCAT sequence required for maximal activity in muscle cells. A, Sequence of the 284-bp minimal promoter of the α-Tm gene. The +1 corresponds to the transcription initiation site previously characterized. The cis-sequences that have been mutated in the α-Tm-LUC reporters are in bold type and underlined. B, U8A4 smooth muscle cells (SMC), cardiomyocytes or myotubes were transiently transfected with the indicated α-Tm-LUC reporters. The various cis-sequences that were mutated in the context of the pGL284LUC promoter construct are shown by black boxes. Luciferase activities (light units) were corrected for variations in transfection efficiencies as determined by β-galactosidase activities and the activity of the wild type pGL284LUC promoter set to 100%. Bars show the mean corrected luciferase value and standard deviation for each construct.

Fig. 3. Sequence conservation across vertebrate α-Tm genes promoters of a 30-bp domain encompassing a CArG-like element and an MCAT sequence. Sequence alignments of a 30-bp domain covering the CArG-like element and the MCAT sequence (in an inverse orientation) within the Xenopus laevis α-Tm gene promoter and similar domain identified in avian and mammals α-Tm gene promoters. Flanking nucleotides on each side of the module are shown in lower-case letters. Numbers to the right indicate the position of each module within its respective promoter relative to the transcription start site when characterized. The CArG-like element and the MCAT sequence are boxed. DNA Sequences obtained from the data bank are as follows: xenopus: DQ871277, quail: X16230; chicken: M69140; mouse: AC160341; rat: J05467 and M16432; dog: XM_860214; human: AC087612.

Fig. 4. The MCAT site of the α-Tm gene promoter binds TEF-1 in muscle cells. A, Radiolabelled 24-bp oligonucleotide probe encompassing the MCAT sequence was incubated with in vitro translated chicken TEF-1 and the complexes formed were analysed on a non-denaturing polyacrylamide gel (lanes 1-5). Specificity of the binding was tested by adding 100 or 200-fold molar excess of cold competitor probe (lanes 2 and 3), 200-fold of the consensus MCAT site from the chicken cardiac TnT gene (lane 4), or 200-fold of the mutated cold probe (lane 5). B-D, Nuclear extracts from cardiomyocytes (B), U8A4 smooth muscle cells (C) and C2/7 myotubes were incubated with the radiolabelled MCAT probe and the complexes formed were analysed on a non-denaturing polyacrylamide gel (lanes 1-6). The specificity of the binding was tested by adding 100-fold molar excess of cold competitor probe (lane 2), the consensus MCAT sequence from the chicken TnT gene (lane 3) or the mutated cold probe (lane 4). The addition of TEF-1 antiserum produced a supershift (lane 5, position indicated by an arrow), whereas preimmune serum did not (lane 6). E, Nuclear extracts from C2/7 myoblasts (lanes 1-3) or NIH-3T3 fibroblasts (lanes 4-6) were incubated with the radiolabelled MCAT probe and the complexes formed were analysed on a non-denaturing polyacrylamide gel. The specificity of the binding was tested by adding 100-fold molar excess of cold competitor probes (lanes 2 and 5). The addition of TEF-1 antiserum produced a supershift (lane 3 and 6, position indicated by an arrow). FP indicates position of free probe and the position of the three principle nucleoprotein/DNA complexes C1, C2 and C3 is indicated (B-E). F, Localization by autoradiography of the complexes C1, C2 and C3 from cardiomyocytes (lane 1) or U8A4 smooth muscle cells (lane 2). The complexes were cut from the gel and the proteins within the complexes were subjected to SDS-PAGE and Western blot analyses using a TEF-1 antiserum (BD Transduction Laboratories) (G). G, Western blot analysis of complexes C1 (lanes 1 and 4), C2 (lanes 2 and 5) and C3 (lanes 3 and 6) from cardiomyocytes (lanes 1-3) or U8A4 smooth muscle cells (lanes 4-6). A 52
kDa polypeptide is present in the three complexes. When no MCAT probe was included in EMSA, no TEF-1 protein was detected in the region of the gel containing complexes C1, C2, or C3. Control nuclear extracts from cardiomyocytes (lane 7) and U8A4 smooth muscle cells (lane 8) were analysed in parallel. H, Western blot analysis of nuclear extracts from cardiomyocytes (lane 1), U8A4 smooth muscle cells (lane 2), C2/7 myotubes (lane 3), C2/7 myoblasts (lane 4) and NIH-3T3 fibroblasts (lane 5) with a TEF-1 antiserum (BD Bioscience) identifies a 52 kDa polypeptide present in all extracts.

Fig. 5. Chromatin immunoprecipitation assay showing that TEF-1 protein binds the MCAT sequence of the α-Tm gene promoter in differentiated muscle cells. Occupancy of the MCAT element within the α-Tm (α-Tm) and smooth α-actin (α-actin) promoters by TEF-1 was measured using ChIP analysis. A, Soluble chromatin from PAC1 smooth muscle cells (SMC) (lanes 1-6) cultured in 10% or 1% serum and from C2C12 myoblasts or myotubes (lanes 7-12) was immunoprecipitated with an antibody against TEF-1 (BD Transduction Laboratories) (+, lanes 3, 6, 9, 12) or without antibody (-, lanes 2, 5, 8, 11). The final DNA was amplified using pairs of primers that span the regions of the MCAT sequence of the promoters as indicated. PCR amplification of the same promoter regions from chromatin fragments prior to immunoprecipitation (Input DNA, In, lanes 1, 4, 7, 10) was used as positive control.

Fig. 6. SRF binds very weakly to the CArG-like element of the 30-bp α-Tm module but is associated to an increase in TEF-1 binding to the MCAT site. A, Radiolabelled 20-bp oligonucleotide probe corresponding to the CArG1 box sequence of the SM-MHC gene promoter was incubated with in vitro translated Xenopus SRF and the complexes formed were analysed on non-denaturing polyacrylamide gel (lanes 1-6). Competition experiments were performed with a 200-fold molar excess of either cold probe (lane 2), CArG-like element of the α-Tm gene (lane 3), CArG4 box of the mouse desmin gene (lane 4), SRE of the human c-fos gene (lane 5) or A/T rich sequence of the α-Tm gene (NS). B, Radiolabelled 20-bp oligonucleotide probe corresponding to the CArG4 box sequence of the desmin gene promoter was incubated with in vitro translated SRF and the complexes formed were analysed on a non-denaturing polyacrylamide gel (lanes 1-14). Competition experiments were performed with a 25-100-fold molar excess of either cold probe (lanes 2-4), CArG-like element of the α-Tm gene (lane 5-7), CarG1 box of the SM-MHC gene (lanes 8-10), SRE of the c-fos gene (lanes 11-13) or 100 fold molar excess of A/T rich sequence of the α-Tm gene (NS) (lane 14). C, Radiolabelled 42-bp oligonucleotide probe called CArG-MCAT Tm encompassing the CArG-like element and the MCAT site of the α-Tm gene promoter was incubated with 1µl or 2µl of fixed amounts of in vitro translated TEF-1 and increasing amounts (0.5, 1 and 2µl) of in vitro translated SRF (lanes 1-9) or with 2µl of unprogrammed lysate reticulocyte (L, lane 10).

Fig. 7. Serum down-regulates the α-Tm promoter activity in smooth muscle cells through the MCAT sequence and favours cytoplasmic redistribution of TEF-1. U8A4 smooth muscle cells were cultured either in the absence of serum (serum deprived) or in 10% serum (serum fed). A, U8A4 smooth muscle cells were transiently transfected with the indicated luciferase reporters. Luciferase activities (light units) were corrected for variations in transfection efficiencies as determined by β-galactosidase activities and the activity of the pGL3-Control vector set to 100%. Bars show the mean corrected luciferase value and standard deviation for each construct. B, Western blot analysis of total proteins extracts from U8A4 smooth muscle cells using a TEF-1 antiserum (Gift of Dr I. Farrance). A 52 kDa polypeptide is detected by the TEF-1 antiserum in all extracts. As a control the extracts were probed with a Tm antiserum that recognizes 36-39 kDa ubiquitously expressed non-muscle Tm. C, TEF-1 and SRF show differential expression in nuclear versus cytoplasmic compartments in U8A4 smooth muscle cells. Immunofluorescence localization of TEF-1 (Gift of Dr I. Farrance) or SRF antibodies (Santa Cruz, sc335X clone) and subsequently detected with a FITC fluorescence in green. Nuclei were identified (b, d, f, h) using propidium iodide and shown as a red fluorescence. The merge staining is shown in yellow (b, d, f, h). Scale bar is 20 µm.
Fig. 8. TEF-1 mRNA overexpression induces endogenous and exogenous α-Tm gene expression in embryonic cells. A, RT-PCR analysis of α-Tm gene expression in animal cap cells derived from embryos injected either with 1 ng of TEF-1 mRNA (+ TEF-1), or non-injected (Control). EF1α mRNA detection served as positive control. Two independent experiments are shown. B, Induction of luciferase activity by TEF-1 mRNA in embryos. 50 pg of pGL284LUC or pGLMCATLUC DNA constructs were injected in two-cell stage embryos either alone or co-injected with 100 pg of TEF-1 mRNA. Embryos were collected at stage 12 for luciferase reporter assay as described in Material and Methods. Background signal from control embryos not injected with a reporter plasmid is also indicated (Control).

Fig. 9. An intact MCAT binding site is required for correct in vivo spatio-temporal expression of the α-Tm transgene in Xenopus embryo and juvenile. A, Whole-mount in situ hybridization analysis of α-Tm striated muscle transcripts in late neurula (a) and tadpole stages (b,c) in wild type embryos. B,C, The expression of a wild type (pGL284GFP) (B) or a mutated version in the MCAT site (pGLMCATGFP) (C) of the α-Tm-GFP transgene was assayed by fluorescence in neurula (a-d) and tadpole stage (e-h) embryos. An arrow indicates the embryonic heart. D,E, The expression of a wild type (pGL284GFP) (D) or a mutated version in the MCAT site (pGLMCATGFP) (E) of the α-Tm-GFP transgene was assayed using RT-PCR analysis in juvenile tissues. Skeletal muscle (Sk. muscle), heart, stomach, intestine or liver were analysed for their content in GFP mRNA, striated muscle α-Tm mRNA or smooth muscle Tm mRNA. Ornithine Decarboxylase (ODC), an ubiquitously expressed mRNA, was used as a control. -RT, no RNA was added in the reverse transription reaction.
Figure 2

A

E-box4
-284 GAGCTCCCTT CCCAGATCA TGCCCTGAAG TTTAGAAATA GGAACACGCC

E-box3
-234 GCTCTCCCCC GCAGCAGCAGC CCCACCCCCA GCAGCTCTTG CCCATACACAC

E-box2
-184 CCCTTATATA GTCACAATACGCA GTCAAAATGCT GTGCGTCAGTG CGGAGCAGCAG CAAATGGAGA

A/T-rich
-134 AGCCATCCCTT AAAAAAAAGCC AGCAGTGGAAG CAGAAAGAGCA AGGCTTCGCAA

CARG-like
-84 AAAAGTTATTG GGTGTCGGGCA AGGATGTGTG TGGCCCCGGCT CGCTGCTCTTC

MCAT
GC-box
E-box1
-34 CCCACTACAT ATTTGTGAGG CTGGAACAGC TGAGATTTG CACTTCAGCC

+17 CAGGCTCAC ACTAAGAGG ATCC

B

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Relative luciferase activity
Figure 3

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30-bp module
Figure 4

A

B

C

D

E

F

G

H
Figure 5

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Figure 7

A

B

C

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- TEF-1: 55kD, 40kD, 33kD
Figure 8

A

B

Luciferase activity

Control  pGL284LUC  pGLMCATLUC  pGLMCATLUC + TEF-1

0  100  200  300  400
Figure 9

A  Wild Type embryos

B  pGL284GFP

C  pGLMCATGFP

D  pGL284GFP

Sk. muscle  Heart  Stomach  Intestine  Liver

E  pGLMCATGFP

Sk. muscle  Heart  Stomach  Intestine  Liver

GFP  Striated $\alpha$-Tm  Smooth $\alpha$-Tm  ODC -RT
TEF-1 dependent expression of the alpha-tropomyosin gene in the three muscle cell types
Stéphanie Pasquet, François Naye, Corinne Faucheux, Odile Bronchain, Albert Chesneau, Pierre Thiébaud and Nadine Thézé

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