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Functional Structure of the Promoter Regions for the Predominant Low Molecular Weight Isoforms of Tropomyosin in Human Kidney Cells

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ABSTRACT

High and low molecular weight (LMW) tropomyosin isoforms, by regulation of actin filaments, have a major role in the regulation of cell behaviour. They affect malignant transformation, motility, differentiation, metastasis and cell membrane protein presentation. Expression of LMW isoforms from the TPM1 and TPM3 genes have an important role in these effects but the regulation of their expression is unknown. Luciferase assays on a progressively truncated 1.7 kb fragment upstream of the exon 1b translation start site in the TPM1 and TPM3 genes in HEK-293 cells showed upstream activation sequences in TPM1 between −152 and −139 bp and in TPM3 between −154 and −102 bp. The effect of mutating candidate transcription factor binding sites identified an AML1-like transcription factor binding site in TPM1 and a cAMP response element in TPM3. Downstream from the primary activation sequence in TPM1 was a repressor region corresponding to two Sp/KLF family binding GC boxes. Band shift assays confirmed that deletion of these sites altered transcription factor binding and ChIP assays confirmed the presence of AML1 and CREB at the TPM1 and TPM3 activation sequences in the respective promoters. Expression of LMW isoforms from TPM1 and TPM3 genes is regulated very differently. This facilitates regulation of the many cell processes involving these proteins. In situations where these proteins have a critical role, such as cancer metastasis, it also facilitates specific intervention. J. Cell. Biochem. 113: 3576–3586, 2012.

KEY WORDS: TROPOMYOSIN GENE EXPRESSION; TPM1; TPM3; LMW ISOFORM PROMOTER; CREB; AML1

The actin cytoskeleton is central to the regulation of many cell processes [Parsons et al., 2010]. An important part of the regulation of the actin cytoskeleton is the transcription of genes encoding proteins that comprise actin filaments [Olson and Nordheim, 2010]. Tropomyosin, in its many isoforms, has a central role in determining the structure and behaviour of actin filaments [Lin et al., 2008]. Changes in expression of tropomyosin isoforms between high and low molecular weight forms have a major effect on cellular plasticity by altering actin filament function and adhesion complexes [Bach et al., 2009]. This affects cell functions such as motility, shape change, invasion and extracellular matrix degradation [Zheng et al., 2008]. Tropomyosin isoform expression is subject to extensive temporal and spatial regulation [Gunning et al., 2008].

In mammals, tropomyosins are encoded by four genes, TPM1-4. Three of these genes, TPM1, 3 and 4, can give rise to low as well as high molecular weight (HMV) isoforms whereas TPM2 encodes only HMV isoforms. Transcription of the HMW isoforms is initiated from a promoter upstream of exon 1a in all TPM genes. TPM 1, 3 and 4 have an additional internal promoter located in the intron upstream of exon 1b. This internal promoter drives transcription of the LMW isoforms. Tropomyosins function as coiled-coil dimers that bind either 6 (LMW isoforms) or 7 (HMW isoforms) actin molecules to stabilize actin filaments. Actin binding characteristics of different tropomyosin isoforms vary and tropomyosin influences the binding of other proteins to actin filaments. Thus tropomyosin isoforms are central to the structure and to the function of actin filaments.

The critical role for the expression of HMW tropomyosin isoforms in cell differentiation and neoplastic transformation is well documented [Helfman et al., 2008]. In this respect, the effects of HMW isoforms from the TPM2 gene are especially well reported [Mahadev et al., 2002; Raval et al., 2003]. The transcription of HMW

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isoforms from TPM1 has been described in *Xenopus* oocytes and is controlled by the ubiquitously expressed TEF-1 binding to the MCAT *cis*-element [Pasquet et al., 2006]. This element is present in the promoters of some muscle specific genes including human HMW tropomyosin. However, it is not found in promoters for human TPM low molecular weight (LMW) isoforms.

The fundamental role of LMW isoforms in cell structure and function has been shown in several cell types. In LLC-PK1 kidney epithelial cells TM-5b, a LMW isoform from TPM1, is only expressed when cell–cell contacts have been established and is found predominantly in the adhesion belt whereas HMW isoforms are only present in stress fibres [Temm-Grove et al., 1998]. In addition, B35 rat neuro-epithelial cells over-expressing Tm5NM1, a LMW isoform from the TPM3 gene, had large stress fibres with increased stability that were resistant to disruption by latrunculin A and cytochalasin D [Creed et al., 2008]. The expression of LMW tropomyosin isoforms, especially from the TPM1 and TPM3 genes, is vital for correct oogenesis [Gaillard et al., 1998] and embryonic development [Hughes et al., 2003; Hook et al., 2004]. The expression of LMW isoforms is essential for embryonic development and cell survival in mice [Hook et al., 2004]. In *Xenopus laevis* LMW isoforms are expressed strongly in early embryonic development but are down-regulated in mature tissues [Gaillard et al., 1998]. The LMW isoform, Tm5NM1, from the TPM3 gene has a critical role in focal adhesion disassembly and focal complex formation that is required for productive and directed mesenchymal cell migration [Bach et al., 2009]. Tm5NM1 is also required for the normal excitation–contraction coupling in skeletal muscle [Vlahovich et al., 2009].

LMW tropomyosins also have an important role in disease, including malignant transformation and metastasis [Miyado et al., 1996; Lees et al., 2011] and in inflammatory kidney disease [Nazeer et al., 2009]. LMW isoforms from the TPM1 gene are absolutely required for motility in metastasis and invasion of B16-F10 mouse melanoma cells [Miyado et al., 1996]. In anti-Thy-1 nephritis in rats, a model of glomerulonephritis in humans, glomeruli had decreased LMW isoforms Tm5a/5b from the TPM1 gene in the early mesangiolytic phase followed by increased levels of these isoforms as disease progressed to the late proliferative phase [Nazeer et al., 2009].

Despite the essential requirement for LMW tropomyosin isoforms in both normal and pathological cell processes, there have been no studies on the transcriptional control of LMW tropomyosin isoforms and the regulation of their expression is unknown. We have studied the expression of the major LMW isoforms, from the TPM1 and TPM3 genes, in a human embryonic kidney cell line. We have also determined the major regulatory sequences for these LMW isoforms in their respective promoter regions and show that they are unique for each gene and are unusual and complex.

MATERIALS AND METHODS

CELL CULTURE

Human embryonic kidney line 293 cells (European Collection of Cell Cultures) were cultured at 37°C, 5% CO_2 and 100% humidity. The growth medium was Minimum Essential Medium supplemented with Earle's basal salt solution, non-essential amino acids and pyruvate (Lonza Bioscience), 2 mM glutamine and 10% foetal bovine serum (Invitrogen).

PLASMID CONSTRUCTION

Primers used in the construction of the luciferase reporter plasmids are given in Table I.

Plasmids containing progressive truncations of the TPM1 and TPM3 exon 1b promoter were amplified using Bio-X-Act DNA polymerase (Bioline). PCR products were digested with *Xho*I and *Sac*I and ligated into similarly digested pGL4.10 luciferase reporter plasmid (Promega) using the Quick Ligation Kit (New England Biolabs).

Plasmids containing discrete sequence deletions were made using the inverse PCR technique with either Bio-X-Act DNA polymerase or Phusion DNA polymerase (NEB). Template plasmids were either 1.WT or 3.WT. PCR products were digested with *Eco*RI and then ligated to form a single *Eco*RI restriction site in place of the deleted region. Constructs were validated by sequencing (Eurofins MWG Operon).

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAYS

At approximately 85% confluence HEK-293 cells were harvested and resuspended at 1×10^6 cells/ml. 3.75×10^5 cells with 1.5 pmol of test plasmid and 0.03 pmol of *Renilla* control plasmid, pGL4.73 (Promega), in 0.4 ml of growth medium were electroporated in a 2 mm gap cuvette using a 110 V, 25 ms, square wave pulse. After dilution with 0.6 ml pre-warmed medium, 200 μl of cell suspension was incubated in each well of a white 96-well plate for 18–24 h.

Luciferase activity was measured using the dual-luciferase system (Promega) on a Luminoskan Ascent luminometer (Thermo Scientific). Mean values with standard error were calculated. Transfection efficiency was adjusted to the co-transfected pGL4.73 plasmid. The promoter-less pGL4.10 vector was the negative control. The SV40 promoter containing pGL4.13 plasmid was the positive control (not shown). Assays were carried out at least in triplicate. *P* values were calculated using a one-way analysis of variance with Tukey's post hoc least significant difference test.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS

HEK-293 nuclear extracts were prepared using the NucBuster Protein Extraction Kit (Novagen) and assayed using the BCA Protein Assay Kit (Novagen) according to the manufacturer's instructions.

Double stranded oligonucleotide probes were produced by annealing complementary single stranded oligonucleotides (Invitrogen). The oligonucleotide sequences used are given in Table I. 500 pM of each complementary oligonucleotide were mixed in a final volume of 25 μl Oligo Annealing Buffer (50 mM NaCl, 10 mM Tris and 1 mM EDTA). Samples were then heated to 95°C and gradually cooled to 20°C over 60 min.

Mobility shift assays were carried out using the 2nd Generation DIG Gel Shift Kit (Roche). 3.85 pmol of double stranded oligonucleotide was labelled using terminal transferase according to the manufacturer's instructions. Either 50 or 100 fmol DIG-labelled DNA was incubated with 30–50 μg HEK-293 nuclear extract, 1 μg poly(deoxyinosinic-deoxycytidylic) acid (Sigma), and 3 μl 5× binding buffer (100 mM Hepes pH 7.6, 5 mM EDTA, 50 mM

TABLE I. Oligonucleotide Sequences

Template	Name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Luciferase plasmid generation			
TPM1	1.WT	ggggagctccccaaagttaggatgcacccaaagcc	gggctcgagatcgcgccggacggtaggcgggc
	1.F1	ggggagctcgactggattccaggagatctc	gggctcgagatcgcgccggacggtaggcgggc
	1.F2	ggggagctccagccagtcccgggatcc	gggctcgagatcgcgccggacggtaggcgggc
	1.F3	ggggagctcctcctggccgcaggggggc	gggctcgagatcgcgccggacggtaggcgggc
	1.F4	ggggagctcgccgcaggggggcgccgccat	gggctcgagatcgcgccggacggtaggcgggc
	1.F5	ggggagctcggggcgccgccatcgcacag	gggctcgagatcgcgccggacggtaggcgggc
	1.F6	ggggagctccgcacagagaggcctgggcg	gggctcgagatcgcgccggacggtaggcgggc
	1.F7	ggggagctcggtatttcagggcgtagc	gggctcgagatcgcgccggacggtaggcgggc
	1.D1	ggggaattcggcggaccggcgctcggcag	cccgaattcaggcctctctgtgcgatggc
	1.D2	ggggaattcaccggcgctgggcagccagg	cccgaattccgcccaggcctctctgtgcgatg
	1.D1:2	ggggaattcaccggcgctgggcagccagg	cccgaattcaggcctctctgtgcgatggc
	1.D3	ggggaattcgccgcagggggcgccgcc	cccgaattccagtccggaagtcgcagc
	1.D4	ggggaattcggggcgccgccatcgcac	cccgaattccaggagcagtccggaagtc
	1.D1:4	ggggaattccagccgggtccgcagggc	cccgaattccaaaccgcaggcgggcgc
TPM3	3.WT	ggggagctcccttcccggctcaagaaatc	gggctcgagatggtgcccacccagctactgc
	3.F1	ggggagctcggttaatttggtgcatgatgactctc	gggctcgagatggtgcccacccagctactgc
	3.F2	ggggagctctcgaagctgtagtgagttgtg	gggctcgagatggtgcccacccagctactgc
	3.F3	ggggagctctactggcccaaggtaaggcc	gggctcgagatggtgcccacccagctactgc
	3.F4	ggggagctccactccctgccgcggctc	gggctcgagatggtgcccacccagctactgc
	3.F5	ggggagctcggtgggagggcgcggcag	gggctcgagatggtgcccacccagctactgc
	3.F6	ggggagctcggtatttcagggcgtagc	gggctcgagatggtgcccacccagctactgc
	3.D1	ggggaattcgcgcggcagagggacgtcac	cccgaattcaccgccaggcaggcgggaag
	3.D2	ggggaattccagagggacgtcacatccgg	cccgaattccctcccaccgccaggcaggc
	3.D3	ggggaattccatccgggcgggttggtgag	cccgaattcccctctgccgcgccctccc
	3.D4	ggggaattcgttggtgagttccggtatttc	cccgaattcggatgtgacgtccctctgcc
	3.D1:4	ggggaattcgttggtgagttccggtatttc	cccgaattcaccgccaggcaggcgggaag
EMSA probes			
TPM1	AML1 WT	cgacttccggactgctcctggccgcagggggcgccgccatc	gatggcggcgcccccctgcggccaggagcagtccggaagtcg
	AML1 1.D3	cgacttccggactggccgcagggggcgccgccatc	gatggcggcgcccccctgcggccagtccggaagtcg
	AML1 1.D4	cgacttccggactgctcctgggggcgccgccatc	gatggcggcgcccccaggagcagtccggaagtcg
	GC-Box WT	cagagaggcctgggcggggcggaccggcgctgg	ccagcgccggtccgcccgcccaggcctctctg
	GC-Box 1.D1:2	cagagaggcctaccggcgctgg	ccagcgccggtaggcctctctg
TPM3	CRE WT	ggcagagggacgtcacatccgggc	gcccggatgtgacgtccctctgcc
	CRE 3.D3	ggcagagggcatccgggc	gcccggatgccctctgcc
RT-PCR			
RNA	Adapter		ggccacgcgtcgactagtacttttttttttttttttt
TPM1	1B F	gaccacgagaggaagctgag	
	9A R		tcctcgctgatggctttgtac
	9B R		atgcagagctcagagaggtg
	9C R		tgctcaagttgctggtagag
	9D R		acgaaagaatgtggtcgcagc
TPM3	1B F	gctgggatcaccaccatcgag	
	9A R		tagaggtcatgtcattgagg
	9B R		gcagatccagaacagagcag
	9C R		cagtcacacagatcatgcag
	9D R		atctcattcaggtcaagcag
ChIP assays			
TPM1	AML1	cctggaggctgcgacttc	agggctgcagtgggagag
TPM3	CRE	cctccagtggactgccttc	cctgctacgccctgaaatac

Primer sequences used during production of the truncation and deletion plasmids for luciferase assays. Oligonucleotide sequences used as double stranded probes for mobility shift assays. Primers used in the production and amplification of cDNA to determine exon 9 isoform specificity. Primers for amplification of TPM1 and TPM3 promoters regions used in ChIP assays.

$(NH_4)_2SO_4$, 5 mM DTT, 1% Tween 20 and 150 mM KCl) in a 15 μl final volume. Competitor DNA or antibody was added as indicated. Antibodies used were RUNX1 (AML1) (N-20), CREB-1 (C-20), Sp1 (PEP-2) and Sp3 (D-20) (sc-8563X, sc-186X, sc-59X and sc-644X, respectively, Santa Cruz Biotechnology). Reactions were incubated at room temperature for 15 min, with supershifts performed by pre-incubating the antibody and protein extract on ice for 30 min. After incubation, 3 μl loading buffer (0.25× TBE, 60% glycerol) was added, the reactions loaded on a 6% 28:1 non-denaturing 1× TBE polyacrylamide gel and run at 150 V. Following electrophoresis the DNA–protein complexes were transferred to positively charged nylon membrane (Roche) by semi-dry electro-blotting and UV crosslinked to the membrane for 3 min.

Immunological detection of the labelled DNA was performed by washing the membrane for 1 min in electrophoretic mobility shift assay (EMSA) wash buffer (0.1 M Maleic acid, 0.15 M NaCl pH 7.5 and 0.3% v/v Tween-20), followed by a 30 min incubation in 100 ml blocking solution, 30 min in 20 ml antibody solution and washing twice for 15 min each in 100 ml EMSA wash buffer. The membrane was then equilibrated for 5 min in 20 ml detection buffer (0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5). The membrane was placed in a development folder (Novagen) with 1 ml disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) and incubated for 10 min at 37°C. Excess CSPD was removed and an image of the membrane was obtained using a Fusion FX7 (Peqlab). Each EMSA was repeated a minimum of four times, with the results given being typical examples.

CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express Enzymatic kit (Active Motif) according

to the manufacturer's instructions. Chromatin was produced from a 175 cm^2 flask of 80–85% confluent HEK-293 cells. Cells were fixed for 10 min and sheared with 100 strokes in a dounce homogeniser and incubated for 10 min in the enzymatic shearing solution. Immunoprecipitation reactions were performed using 35 μg prepared chromatin and 3 μg antibody in a 200 μl reaction volume. Phusion polymerase reagents and buffers (NEB) were used for the end-point PCR analysis with the primer sequences given in Table I. 2 μl of column-purified immunoprecipitated chromatin was used per reaction. TPM1 touchdown cycling conditions were an initial denaturation at 98°C for 30 s, then 20 cycles of 98°C for 10 s, 69.4°C (decremented by 0.5°C per cycle) for 20 s and extension at 72°C for 5 s, followed by 21 further cycles of 98°C for 10 s, 64.4°C for 20 s and 72°C for 10 s, with a final extension at 72°C for 10 min. TPM3 amplification was at 98°C for 30 s, then 35 cycles at 98°C for 10 s, 62°C for 20 s and 72°C for 5 s, with a final 10 min at 72°C. The RNA Polymerase II and total IgG positive and negative controls were supplied with the ChIP-IT Control Kit—Human (Active Motif). Specific antibodies used were RUNX1 (N-20) and CREB-1 (C-21). Each ChIP assay was repeated three times, with typical results being shown.

RNA EXTRACTION

RNA extraction was performed using the QIAamp Blood Mini kit (QIAgen) following the manufacturer's instructions for cultured cells. Approximately 1×10^7 cells were used per extraction and RNA yield and quality was assessed via A_{260}/A_{280} and A_{260}/A_{230} ratios and gel electrophoresis.

RT-PCR REACTIONS

First strand cDNA synthesis was performed by mixing 2 μg total RNA with 1 μl of the supplied adapter primer and heating to 70°C for 10 min and then cooling to 4°C. After equilibration to 42°C, 1 μl superscript II reverse transcriptase (Invitrogen) was added and the reaction incubated at 42°C for 50 min then 70°C for 10 min. Finally 1 μl (2U) RNAse-H was added to the reaction. PCR reactions were performed using the primers listed in Table I using Bio-X-Act polymerase according to the manufacturer's instructions.

RESULTS

TROPOMYOSIN ISOFORMS IN HUMAN EMBRYONIC KIDNEY CELLS

HEK-293 cells express LMW tropomyosin isoforms from the TPM1, 3 and 4 genes. A total of five LMW isoforms incorporating exons 9a, 9b, 9c and 9d are expressed from TPM1, with four expressed from TPM3 (Fig. 1). DNA sequencing showed all isoforms incorporated the exon 6a internal splice variant. Two distinct isoforms incorporated TPM1 exon 1b. The 636 bp product contained a single 9b exon, whilst the 762 bp product corresponded to an isoform containing both exons 9a and 9b. The single isoform observed from TPM3 exon 9b also contained exon 9a. A single TPM4 LMW isoform containing exon 9a was detected, although this was expressed at a lower level than the TPM 1 and TPM3 isoforms (data not shown).

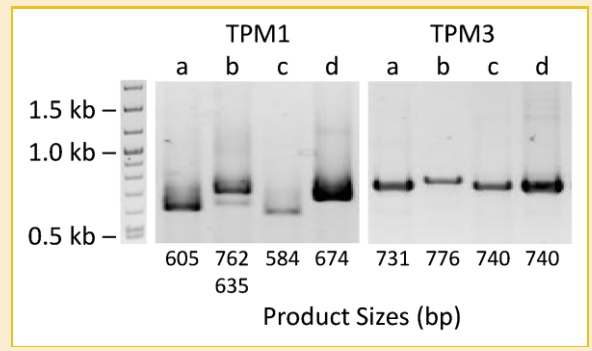

Fig. 1. RT-PCR of TPM1 and TPM3 mRNA from HEK-293 cells. Primer sequences are given in Table I. Forward primer binds in exon 1b and reverse primer for exon 9a, 9b, 9c or 9d primer as indicated. Expected product sizes are given below each lane.

FUNCTIONAL STRUCTURE OF THE PROMOTER REGION FOR LMW ISOFORMS IN THE TPM1 GENE

To localise any upstream activation or repression sequences located in the first 1,770 bp of TPM1 promoter a series of luciferase reporter plasmids containing progressive TPM1-promoter truncations were constructed (Fig. 2a). The results of luciferase assays performed on these plasmids are given in Figure 2b. The largest promoter fragment tested (Fig. 2, 1.WT) contained 1,770 bp of promoter immediately upstream of the TPM1 LMW isoform translation start site. Expression of the 1.WT plasmid was set to 1.00 and all other luciferase measurements are given relative to this plasmid with the base pair positions referring to the translation start site. Luciferase expression from plasmid 1.F1 was 1.30× higher than 1.WT, indicating the presence of an upstream repressor sequence (URS) located between −1770 and −1026. Plasmid 1.F2 expression was reduced to 0.88 that of 1.WT, demonstrating the presence of an upstream activator sequence (UAS) in the region −1026 to −249. Further truncation of the promoter in plasmid 1.F3 increased expression to 1.41, suggesting an additional URS located between −249 and −152. Relative luciferase expression from 1.F4 was 0.64 and from 1.F5 was 0.23, indicating one or more UASs located between −152 and −139. Truncations in plasmids 1.F6 and 1.F7 gave activities of 0.24 and 0.23, respectively, showing that there were no additional UASs downstream of −139. Relative luciferase expression from the empty vector was 0.15. This suggests that the primary UAS controlling transcription is located between −152 and −139.

MODIFICATION OF CANDIDATE PRIMARY ACTIVATION SITES IN THE PROMOTER REGION FOR LMW ISOFORMS IN THE TPM1 GENE

The identification of key activation and repression sequences was confirmed by the use of inverse-PCR to delete small regions of the TPM1 LMW isoform promoter. The deletions are shown in Figure 2a and relative luciferase activities are given in Figure 2c. Deletion of the promoter between −187 and −71 (plasmid 1.D1:4) reduced relative luciferase expression to 0.57 compared to 1.WT, despite promoter truncation to −99 (1.F7) causing expression levels similar to that observed in the empty vector. Deletion from −152 to −147 (1.D1) reduced expression to

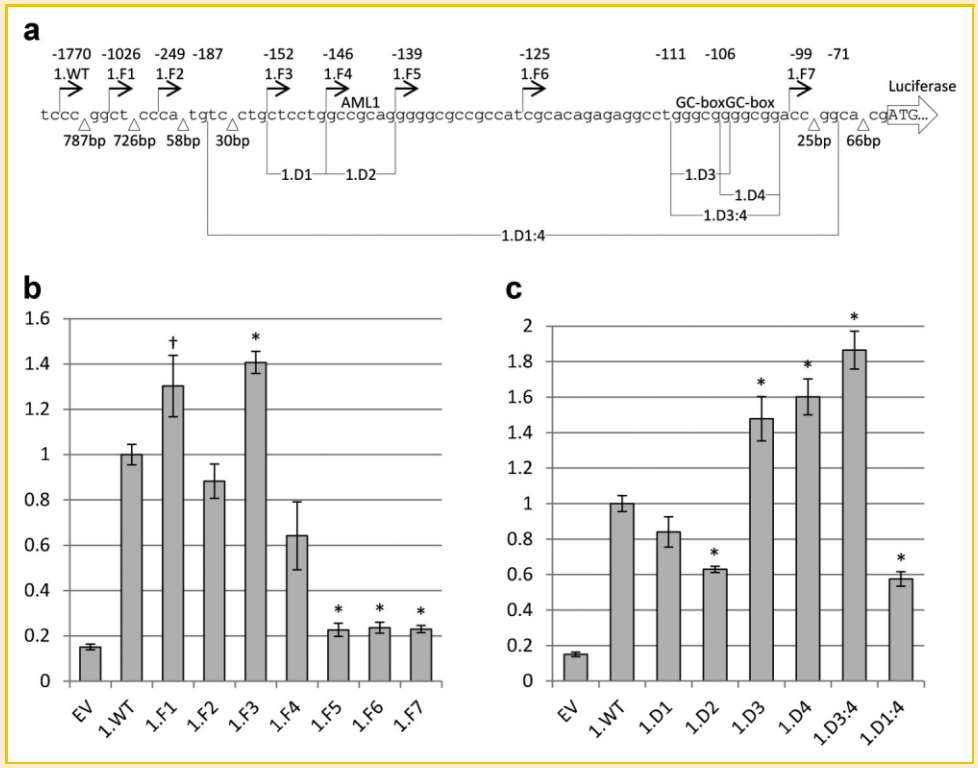

Fig. 2. TPM1 exon 1b promoter truncation and deletion fragments were cloned into the pGL4.10 luciferase vector. Promoter amplification primers are given in Table I. HEK-293 cells were transiently transfected with the pGL4.73 control plasmid and the indicated plasmid. Luciferase activity was assayed after 24 h. The luciferase activity of 1.WT was set to be 1.0 and values given are the mean ± standard error. $^{\dagger}P < 0.005$, $^{*}P < 0.001$ compared to 1.WT. a: Schematic representation of the promoter fragment. The top line gives base positions relative to the translation start site. Wedges separating the promoter sequence represent sections of DNA sequence that have been removed from the figure, with the number of bases removed indicated below each wedge. The position and name of each truncation fragment is indicated above a right-facing arrow. Internal deletions are shown below the promoter sequence. The name and location of the region replaced by an *Eco*RI restriction site is indicated. b: Truncation plasmids. EV is the empty vector, and 1.WT is the 1,770 bp TPM1 wild-type promoter fragment. c: TPM1 exon 1b promoter deletion fragments were produced in the 1,770 bp vector using inverse-PCR. EV is the empty vector, and 1.WT is the 1,770 bp wild-type promoter fragment.

0.84 whilst deletion from −146 to −140 (1.D2) reduced expression to 0.63, equivalent to the reduced activity caused by the much larger 1.D1:4 deletion. This indicates that the region between −146 and −140 contained a critical UAS. This region shows some similarity to an acute myeloid leukaemia 1 protein (AML1) binding site. AML1, also known as the Runt-related transcription factor 1 (RUNX1), binds to the sequence 5′-TGT/CGGT-3′ [Meyers et al., 1993]. In the reverse orientation, the region deleted from the 1.D2 construct had the sequence -140-CTGCGGC-146. Additionally, luciferase expression from the 1.D3 and 1.D4 plasmids increased to 1.48 and 1.60, respectively. 1.D3 was deleted between −111 and −106 and 1.D4 was deleted between −107 and −101. Deletion of the region from −111 to −101 (1.D3:4) increased expression to 1.86. This suggests the presence of two further repressor sequences located between −111 and −101. This region contains two overlapping GC-boxes, which are the binding sites of Sp/KLF family transcription factors [Thiesen and Bach, 1990].

FUNCTIONAL STRUCTURE OF THE PROMOTER REGION FOR LMW ISOFORMS IN THE TPM3 GENE

Luciferase reporter plasmids containing progressive truncations of the first 1,725 bp of TPM3 LMW isoform promoter (Fig. 3a) were assayed to locate any upstream activation or repression sequences (Fig. 3b). The plasmid containing the longest TPM3 LMW isoform promoter fragment (3.WT) gave approximately 10× the level of luciferase expression compared to the corresponding TPM1 promoter. The progressively truncated promoter fragments 3.F1, 3.F2, 3.F3 and 3.F4 showed no significant change in luciferase expression, compared to 3.WT. Plasmid 3.F5 showed an increase in expression to 1.43, indicating that an URS is located between −206 and −154. Further truncation to −102 reduced expression to 0.065 (3.F6). This suggests that the primary UAS controlling transcription is located between −154 and −102.

MODIFICATION OF CANDIDATE PRIMARY ACTIVATION SITES IN THE PROMOTER REGION FOR LMW ISOFORMS IN THE TPM3 GENE

The promoter segment between −102 and −154 contains several transcription factor binding motifs including sites for the Sp/KLF family (GC-box), early growth response/nerve growth factor induced family (EGRF) [Swirnoff and Milbrandt, 1995], transcription factor II-B recognition element (BRE) [Thomas and Chiang, 2006] and a cAMP response element (CRE). Inverse PCR was used to remove these binding sites (Fig. 3a) and the effect on luciferase activity was measured (Fig. 3c). Deletion of the EGRF site between

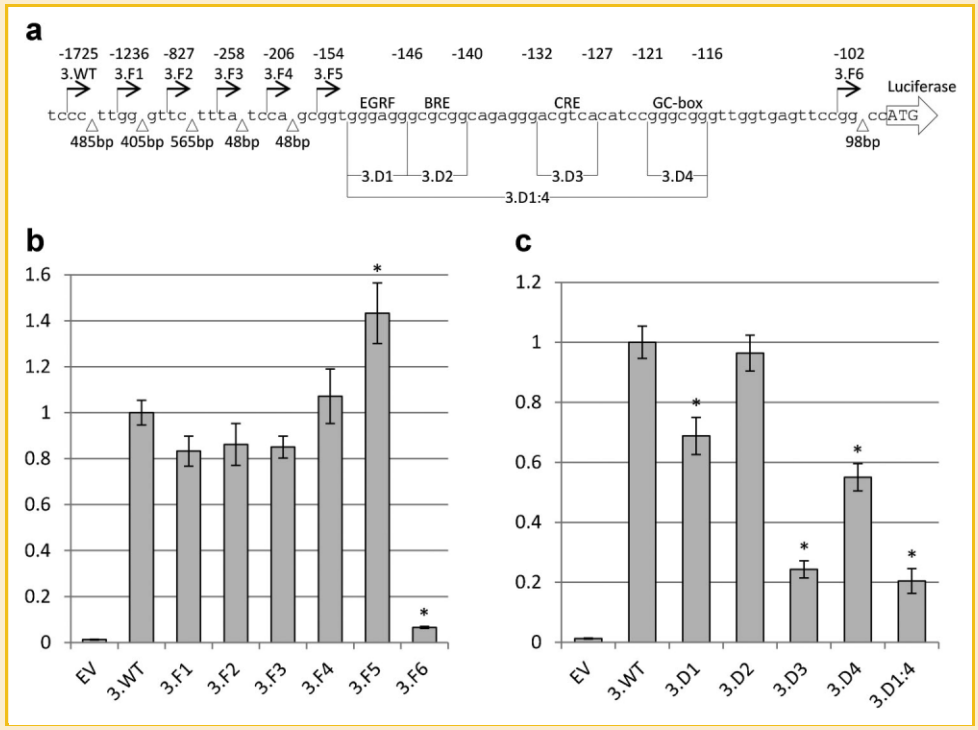

Fig. 3. TPM3 exon 1b promoter fragments were cloned into the pGL4.10 luciferase vector. Promoter amplification primers are given in Table I. HEK-293 cells were transiently transfected with the pGL4.73 control plasmid and the indicated plasmid. Luciferase activity was assayed after 24 h. A schematic representation of the cloned region is shown. The luciferase activity of the longest 1,725 bp fragment, 3.WT, was set to be 1.0. The values given are the mean ± standard error. $^{*}P < 0.001$ compared to 3.WT. a: Schematic representation of the promoter fragment. The top line gives base positions relative to the translation start site. Wedges separating the promoter sequence represent sections of DNA sequence that have been removed from the figure, with the number of bases removed indicated below each wedge. The position and name of each truncation fragment is indicated above a right-facing arrow. Internal deletions are shown below the promoter sequence. The name and location of the region replaced by an *Eco*RI restriction site is indicated. b: TPM3 exon 1b promoter truncation fragments. c: Promoter deletion fragments were produced in the 3.WT vector using inverse-PCR.

−151 and −146 (3.D1) reduced expression to 0.69 that seen in plasmid 3.WT. Plasmid 3.D2 showed no significant change in expression, suggesting that the BRE site located between −145 and −140 is non-functional. Deletion between −132 and −127 from plasmid 3.D3 produced a reduction in expression to 0.24. This indicates that the cAMP response element located in this region is an important activation sequence. Deletion of the GC-box located between −121 and −116 from plasmid 3.D4 reduced expression to 0.55. The larger deletion from −151 to −116 in construct 3.D1:4 reduced expression to 0.20.

BAND-SHIFT EFFECTS WITH PRIMARY ACTIVATION SITE SEQUENCES

The native promoter sequence from −166 to −126 bp upstream from the translation start site in the TPM1 gene, when mixed with nuclear protein extract, showed three retarded bands on electrophoresis (Fig. 4a, bands A–C). Competition studies using 40-fold excess unlabelled probe showed that two complexes (Fig. 4a, lane 1, bands A and B) were sequence specific. Pre-incubation of the nuclear extract with 6 μg of Runx1 antibody prevented formation of the highest weight complex (Fig. 4a, lane 3, band A).

Mobility shift assays were performed on the TPM1 promoter sequence from −132 to −88. When incubated with nuclear extract the wild-type sequence produced two shifted bands (Fig. 4b, lane 1, bands A and B), one of which was sequence specific (Fig. 4b, lane 3, band A). Incubation using a mutant promoter fragment lacking the dual GC-box motif from −114 to −104, equivalent to the deletion in the 1.D3:4 construct, abolished binding of this sequence specific complex (Fig. 4b, lane 2, band A). Extract pre-incubation with Sp1 or Sp3 antibody did not alter the mobility of any complexes (not shown).

The native LMW TPM3 promoter sequence from −141 to −118, when mixed with nuclear extract, showed three heavily retarded bands on electrophoresis (Fig. 4c, lane 1, bands A–C). Bands A and B were absent when the promoter with the CRE site deletion (−132 to −127) was used (Fig. 4c, lane 2). This deletion corresponds to the 3.D3 construct. When a 75-fold excess of unlabelled WT fragment was used as a specific competitor, complexes A and B were no longer present (Fig. 4c, lane 3). However, these complexes were observed when a 75-fold excess of the 3.D3 deletion fragment was used as the unlabelled competitor (Fig. 4c, lane 4). The formation of complexes A and B was also prevented by pre-incubation of the nuclear extract with 6 μg CREB-1 antibody (Fig. 4c, lane 5).

IN VIVO TRANSCRIPTION FACTOR BINDING AT THE TPM1 AND TPM3 PROMOTERS

Chromatin immunoprecipitation was used to assess the interaction between specific transcription factors and the TPM1 and TPM3 LMW

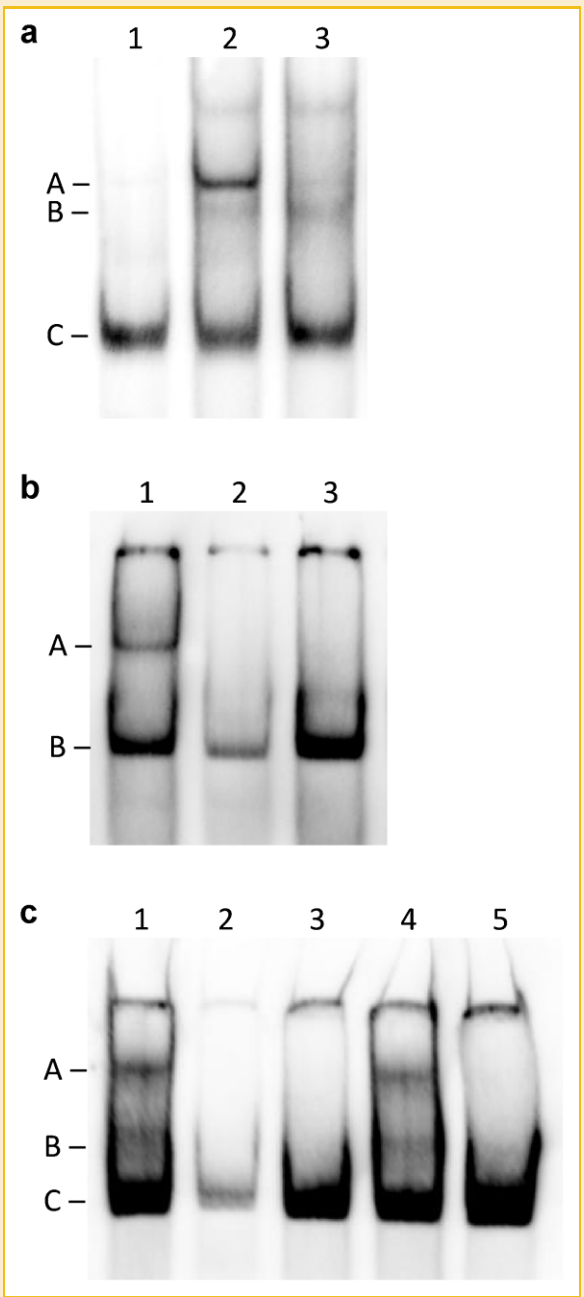

Fig. 4. a: Mobility shift assay of the −166/−126 region of the TPM1 exon 1b promoter. All lanes contain 100 fmol labelled DNA, 50 μg HEK-293 nuclear extract, 1× binding buffer and 1 μg poly(dI:dC). Lane 1: as lane 2, with the addition of 40-fold excess unlabelled WT DNA; lane 2: WT DNA; lane 3: as lane 2 with the addition of 6 μg Runx1 antibody. (b) Mobility shift assay of the −122/−90 region of the TPM1 exon 1b promoter. All lanes contain 50 fmol labelled DNA, 30 μg HEK-293 nuclear extract, 1× binding buffer and 1 μg poly(dI:dC). Lane 1: WT DNA; lane 2: 1.D3:4 deletion DNA; lane 3: as lane 1 with the addition of a 150-fold excess unlabelled WT probe fragment. c: Mobility shift assay of the −141/−118 region of the TPM3 exon 1b promoter. All lanes contain 100 fmol labelled DNA, 50 μg HEK-293 nuclear extract, 1× binding buffer and 1 μg poly(dI:dC). Lane 1: WT DNA; lane 2: 3.D3 deletion; lanes 3–4: as lane 1 with the addition of a 75-fold excess of, respectively, unlabelled WT or 3.D3 deletion fragments and lane 5: as lane 1 with the addition of 6 μg CREB-1 antibody.

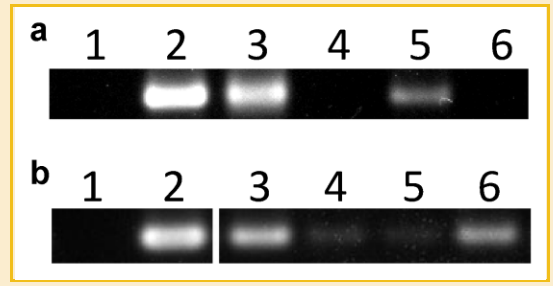

Fig. 5. a: TPM1 exon 1b promoter chromatin immunoprecipitation. Primers amplify the region from −177 to −25. b: TPM3 exon 1b promoter chromatin immunoprecipitation. Primers amplify the region from −186 to −82. Lane 1: no template control; lane 2: ChIP input DNA; lane 3: ChIP performed using RNA polymerase II mouse monoclonal IgG; lane 4: ChIP using mouse total IgG; lane 5: ChIP using Runx1 IgG and lane 6: ChIP using CREB-1 IgG.

isoform promoters in vivo in the HEK-293 cell line. Primers were used to amplify a 153 bp fragment, from −177 to −25, of the TPM1 promoter (Fig. 5a). This fragment contains the transcription start site and the putative AML1 binding site, which corresponds to the region deleted from the 1.D2 construct. Amplification of this fragment was observed using ChIP input DNA (lane 2), and after enrichment by immunoprecipitation with antibodies to both RNA polymerase II (lane 3) and Runx1 (lane 5). No amplification was observed without the presence of template DNA (lane 1) or with total IgG (lane 4) or CREB antibody (lane 6).

A 105 bp fragment, from −186 to −82 and containing TATA box and the putative cAMP response element, was amplified from the TPM3 promoter (Fig. 5b). A substantial product was obtained after amplification with ChIP input DNA (lane 2) and DNA chromatin immunoprecipitated using RNA polymerase II (lane 3) and CREB (lane 6) antibodies. No amplification was obtained from the no template control (lane 1) and only minimal amplification from the total IgG (lane 4) and Runx1 (lane 5) ChIP reactions.

DISCUSSION

TROPOMYOSIN ISOFORMS IN HUMAN EMBRYONIC KIDNEY CELLS

The array of tropomyosin isoforms in a cell are a major determinant of actin filament properties and hence of the structure and responses of that cell. In HEK-293 cells the expression of LMW tropomyosin isoforms from the TPM1 and TPM3 genes is responsible for almost all LMW isoforms in these cells, with just a single TPM4 LMW isoform being detected at a low level. Each of the four possible single-exon 9 splice variants expressed from TPM1 and three single-exon 9 splice variants were detected from TPM3. Additionally, both TPM1 and TPM3 expressed a splice variant containing both exons 9a and 9b. However, the functional organisation of the promoter regions and the regulation of the expression of these tropomyosin isoforms has not previously been investigated.

FUNCTIONAL IMPORTANCE OF REGULATION OF EXPRESSION OF LOW MOLECULAR WEIGHT TROPOMYOSIN ISOFORMS

The tropomyosin genes are believed to have arisen by gene duplication [Vrhovski et al., 2008]. However, the evolutionary

divergence in the promoter regions is clearly essential for independent control of expression. The functional organisation of the promoter region for LMW isoforms from the TPM1 and TPM3 genes is completely different and involves different transcription factors. The TPM1 LMW promoter is TATA-less whilst the TPM3 LMW promoter does have a TATA box. TATA boxes bind the TATA-binding protein and serve as the initial nucleation site of the transcription pre-initiation complex [Thomas and Chiang, 2006]. Whilst they are the best characterised eukaryotic core promoter element, TATA boxes are found in just 11 [Bajic et al., 2004] to 30% [Suzuki et al., 2001] of human promoters. Transcription initiation from TATA-less promoters requires one or more alternative core promoter sequence elements, such as BRE, the initiator element, downstream core element or the downstream promoter element [Baumann et al., 2010]. The TPM1 LMW promoter does not appear to contain any such sequences, suggesting it may be one of the 25% of human promoters lacking any known core promoter elements [Gross and Oelgeschlager, 2006].

The actin cytoskeleton is a fast response system involved in many aspects of cell function. However, the response to any stimulus will depend on the structure of actin filaments for which the correct relative abundance and type of tropomyosin isoforms is vital. cAMP, mediated by protein kinase-A (PKA), has an important role in transendothelial migration of leukocytes [Lorenowicz et al., 2007] which is dependent on the dynamics of F-actin rich membrane domains [Millan et al., 2006]. cAMP is also involved in the metastasis and invasion of melanoma and breast cancer cells [Voura et al., 1998]. It is feasible that cAMP may influence the tropomyosin isoform complement of actin filaments to optimise its effect. Similarly, the actin cytoskeleton is a critical factor for the membrane organisation of matrix metaloproteinases [Ogier et al., 2006] in basement membrane degradation for cell invasion. AML1 is associated with MMP2 and MMP9 cell membrane presentation in endometrial and ovarian carcinoma invasion [Planaguma et al., 2011] where its membrane presentation is increased [Planaguma et al., 2004] correlating with the initial steps of myometrial infiltration. An indication that AML1 affects tropomyosin isoform expression is provided by overexpression of AML1 in the fibroblast line NIH3T3, which causes transformation. This also indicates that AML1 plays a role in cellular proliferation in addition to myeloid cell differentiation [Kurokawa et al., 1996].

TRANSCRIPTION OF LOW MOLECULAR WEIGHT ISOFORMS FROM TPM1 AND TPM3

The regulation of TPM1 and TPM3 expression, as revealed by truncation and deletion analysis, is both complex and distinct. The TPM1 promoter contains at least three repressor and two activator sequences, whilst the TPM3 promoter contains one repressor and three activator sequences.

Although truncation analysis of the TPM1 promoter clearly identified the region between −146 and −140 as being the most critical activator site, deletion of a 117 bp segment (1.D1:4) containing this region reduced expression by only 43%. This may indicate a role for additional upstream *cis*-acting elements in regulation of TPM1. The sequence in the −146/−140 TPM1 promoter does not precisely match any ideal transcription factor binding sequence. However, of interest from the AML1 effects discussed above, the closest match is in the complementary strand to the AML1 binding sequence. This region differs from the AML1 consensus sequence by only a single base. The AML1 consensus sequence of TGT/CGGT and the −140 to −146 region of TGCGGC differ only at the final nucleotide. The 3′ C has, however, also been shown to be present in functional AML1 binding sites with a frequency of 0.05. Truncation of the TPM1 promoter to −146 (1.F4) and deletion of the region between −152 and −146 (1.D1) both caused a decrease in expression compared to the full-length promoter. The presence of an additional UAS located between −152 and −146 cannot therefore be ruled out. However, the −152/−146 region does not resemble any known transcription factor binding site. This, combined with the relatively modest reduction in expression and the adjacent location of the critical UAS, suggests that truncation to −146 has removed a peripheral segment of the AML1 binding site, sufficient to weaken but not abolish transcription activation.

In addition to the UAS, two overlapping repressor regions were detected between −101 and −111 bp upstream of the translation start site with the sequence GGGCGG. Deletion of either GC box individually caused a significant increase in expression. Deletion of both GC boxes simultaneously produced even higher expression levels, suggesting a combinatorial system of repression. GC boxes are commonly found within TATA-less promoters and bind transcription factors from the Sp/KLF family. This sequence is conserved in the TPM3 promoter, 118 bp upstream from the translation start site, and is the only regulatory sequence shared between both promoters. It functions very differently in each, however. Deletion of the GC-box region significantly increased transcription from TPM1 (1.D3:4), but significantly reduced transcription from TPM3 (3.D4).

Mutation of candidate transcription factor binding sites in the primary activation sequence of the TPM3 gene clearly identified a site between −127 and −132 bp upstream of the translation start site. This UAS corresponded to the cAMP-response element (CRE). The cAMP response element binding protein (CREB) is able to promote transcription in response to cAMP via protein kinase-A mediated phosphorylation and growth factors.

There are also multiple promoter regions upstream of the primary activation sites in both TPM1 and TPM3 that have significant effects on transcription of these LMW isoforms. These additional sites appear to modify expression driven from the primary UASs, rather than promote transcription independently, as deletion of these upstream sites never reduced transcription to a level below that observed upon deletion of either the TPM1 −146/−139 region or the TPM3 −132/−127 region. However, these additional sites do contribute to the complex regulatory system that controls TPM expression.

TRANSCRIPTION FACTOR BINDING TO PRIMARY ACTIVATION SITES IN THE PROMOTERS FOR LOW MOLECULAR WEIGHT ISOFORMS FROM TPM1 AND TPM3 GENES

Confirmation of transcription factor binding to promoter sequences that affected gene expression in luciferase assays was obtained using the band-shift technique with nuclear protein extract from HEK-293

cells. DNA-binding of the candidate was established using chromatin immunoprecipitation. The TPM3 promoter fragment showed two band-shifts that were abolished by mutation of the CRE sequence. This indicates that the binding of multiple proteins, possibly the transcription complex, is dependent upon the CRE binding site. These two complexes were also abolished by the presence of CREB antibody in the reaction, indicating that CREB forms an essential component of the shifted complexes. The amplification enrichment seen when using CREB antibody in a ChIP assay shows that CREB must be present on the TPM3 promoter in vivo. CREB mediates effects of cAMP on gene transcription and cAMP is an important regulator of the actin cytoskeleton in several cell types both in acute and longer term structural effects, where altered gene expression is involved. In HEK-293 cells less than 2% of CREB occupied genes are responsive to cAMP [Zhang et al., 2005]. However, mutation of the CRE binding site abolishes expression of LMW isoforms from the TPM3 gene clearly showing that in this case CREB is functional.

Therefore, it appears that cAMP not only has acute effects on the proteins that regulate actin filament structure, but also influences the underlying composition of the actin filaments by an effect on tropomyosin isoform expression. It is likely that the CRE regulated expression of LMW isoforms from the TPM3 gene has an important role in the effects of cAMP.

Since mutation of the strongest candidate primary activation sequence in the TPM1 promoter gave only a 43% decrease in gene expression, a clear band-shift change may not occur. However, three shifted bands were observed with the native promoter region. Two of these were abolished by competition with unlabelled WT DNA, and one was also abolished by the addition of AML1 antibody. A ChIP assay using AML1 antibody also shows that AML1 is present on the TPM1 promoter in HEK-293 cells. Therefore, this confirms the role of the AML1 transcription factor in the regulation of LMW isoforms from TPM1 although it has not previously been directly linked to cytoskeletal regulation. From the evidence discussed above, some cancer related effects of AML1 may be related to its effect on LMW tropomyosin expression. LMW isoforms from the TPM1 gene are increased in tumorigenic variants of the rat fibroblast cell line 3Y1 [Miyado et al., 1997].

Band shifts performed using the region containing the two GC-boxes produced two clear shifted complexes, one of which was sequence specific. This specific complex was unable to bind the mutated fragment lacking the GC-box. The 24-member Sp/KLF family recognise the GC-box, with some factors functioning as both transcriptional activators and repressors [Dennig et al., 1996]. Although supershift assays were attempted using antibodies to Sp1 and Sp3 no alterations in complex formation were observed. This suggests that the factors observed binding to the dual GC-box region could be other members of the Sp/KLF family, which are not recognised by these antibodies. Sp/KLF family transcription factors regulate cell proliferation and tumorigenesis [Abdelrahim et al., 2004] and control expression of other cytoskeletal proteins.

CROSS-SPECIES HOMOLOGY IN THE PROMOTERS FOR LOW MOLECULAR WEIGHT ISOFORMS FROM TPM1 AND TPM3 GENES

It is likely that sequences of major importance for the regulation of gene expression will be relatively better conserved between species. The CRE site in the TPM3 promoter is highly conserved across species, which is consistent with its importance. In primates the 1 kb sequence upstream from the translation start site in the promoters for LMW isoforms from the TPM1 (Table IIa) and TPM3 (Table IIb) genes are generally well conserved. However, sequence conservation is much less in other mammals at about 50%, but in TPM3 in *Canis lupus* this is only 13%. In the latter, the sequence is conserved for 100 bases upstream from the translation start site but diverges after the CRE binding site. In other mammals examined, this region is generally well conserved. The regions of promoter that we have identified as critical for gene expression in TPM1, the two GC boxes that gave repression and the AML-1 like region that gave primary activation, were well conserved across species although there was considerable both down- and up-stream variation of sequence.

CONTRASTING REGULATION OF TRANSCRIPTION OF LOW MOLECULAR WEIGHT ISOFORMS FROM TPM1 AND TPM3

Expression from the TPM3 promoter appears to be regulated primarily by CREB, possibly modulated by EGR- and Sp/KLF-family members. In contrast, expression from the TPM1 promoter is activated by AML1 and modulated by Sp/KLF-family factors and

TABLE II. Percentage Sequence Identity Between the Human TPM1 LMW Isoform Promoter (a) and the Human TPM3 LMW Isoform Promoter (b) Compared to Six Other Mammalian Species

Species	−1 to −1000	−1 to −100	−101 to −200	−201 to −300	−301 to −400	−401 to −500	−501 to −600	−601 to −700	−701 to −800	−801 to −900	−901 to −1000
(a) TPM1											
Pan troglodytes	98	100	97	100	99	99	99	100	96	99	100
Macaca mulatta	88	96	95	71	77	77	59	45	45	46	43
Bos taurus	55	61	79	48	46	56	48	31	40	10	11
Canis lupus	66	58	66	52	19	38	8	38	41	33	43
Rattus norvegicus	58	27	67	53	29	39	11	16	10	14	19
Mus musculus	59	47	64	56	27	29	10	11	7	17	15
(b) TPM3											
Pan troglodytes	98	100	99	99	98	99	100	100	88	86	87
Macaca mulatta	93	100	98	93	92	77	76	78	77	86	86
Bos taurus	55	89	89	65	44	51	55	46	8	14	7
Canis lupus	13	88	43	36	37	8	11	18	10	20	17
Rattus norvegicus	42	85	79	57	67	42	56	15	8	10	21
Mus musculus	42	83	68	68	68	37	60	14	9	9	10

Produced using ClustalW2 by aligning 1,000 bases immediately upstream of the translation start site.

additional factors located further upstream. This suggests that expression of these LMW isoforms is subject to control through a variety of independent pathways. This further suggests that the roles of these two groups of isoforms are specific and different. Tropomyosins have an important role in cell behaviour with effects on cell motility, differentiation and exposure of surface molecules such as adhesion molecules and enzymes. It appears that the regulation of the expression of the many isoforms from TPM genes is correspondingly complex. However, this provides for modulation of the actin cytoskeleton to facilitate responses of cells in varying environments. The manipulation of tropomyosin isoform expression may offer therapeutic opportunities in the future.

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