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Regulation of myosin light chain kinase and telokin expression in smooth muscle tissues

B. Paul Herring, Omar El-Mounayri, Patricia J. Gallagher, Feng Yin, and Jiliang Zhou

Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana

Abstract

The *mylk1* gene is a large gene spanning ~250 kb and comprising at least 31 exons. The *mylk1* gene encodes at least four protein products: two isoforms of the 220-kDa myosin light chain kinase (MLCK), a 130-kDa MLCK, and telokin. Transcripts encoding these products are derived from four independent promoters within the *mylk1* gene. The kinases expressed from the *mylk1* gene have been extensively characterized and function to regulate the activity of nonmuscle and smooth muscle myosin II. Activation of these myosin motors by MLCK modulates a variety of contractile processes, including smooth muscle contraction, cell adhesion, migration, and proliferation. Dysregulation of these processes contributes to a number of diseases. The noncatalytic gene product telokin also has been shown to modulate contraction in smooth muscle cells through its ability to inhibit myosin light chain phosphatase. Given the crucial role of the products of the *mylk1* gene in regulating numerous contractile processes, it seems intuitive that alterations in the transcriptional activity of the *mylk1* gene also will have a significant impact on many physiological and pathological processes. In this review we highlight some of the recent studies that have described the transcriptional regulation of *mylk1* gene products in smooth muscle tissues and discuss the implications of these findings for regulation of expression of other smooth muscle-specific genes.

Keywords

myocardin; serum response factor; GATA; gene expression; *Hox* genes

MYOSIN LIGHT CHAIN KINASES (MLCK) are encoded by at least two genes in mammals, the *mylk1* and *mylk2* genes (23,41,90,95).¹ The *mylk2* gene encodes a MLCK isoform that is exclusively expressed in skeletal muscle cells (95). In contrast, the products of the *mylk1* gene are widely expressed, being detectable in many, if not all, tissues and cell types, including skeletal and cardiac muscle (19,27). In this review we focus on the regulation of the *mylk1* gene, because there are no published studies on the regulation of the *mylk2* gene. Three protein products encoded by the *mylk1* gene have been described, and they include two different isoforms of MLCK and the noncatalytic gene product, called telokin (Fig. 1). The two kinases expressed from the *mylk1* gene have been extensively characterized and function to regulate the activity

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Address for reprint requests and other correspondence: B. P. Herring, Dept. of Cellular and Integrative Physiology, Indiana Univ. School of Medicine, Indianapolis, IN 46202-5120 (pherring@iupui.edu).

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¹In this review we have used the 220-kDa MLCK and 130-kDa MLCK terminology to describe the MLCK products of the *mylk* gene for simplicity. This terminology is derived from the apparent molecular masses of the mouse MLCK molecules determined by SDS-PAGE. The true molecular masses of these molecules predicted from their cDNA sequences are 213 and 214 kDa for the two forms of the 220-kDa MLCK and 114 kDa for the 130-kDa MLCK.

of nonmuscle and smooth muscle myosin II. Activation of these myosin motors by MLCK modulates a variety of contractile processes, including smooth muscle contraction, cell adhesion, migration, and proliferation (36,38), and dysregulation of these processes contributes to a number of diseases. For example, in the vascular system, alterations of these contractile processes directly contribute to the vascular pathologies that occur during the development of atherosclerosis, restenosis, and hypertension (62). Given the crucial role of MLCKs in regulating numerous contractile processes, it seems intuitive that alterations in the transcriptional activity of the *mylk1* gene also will have a significant impact on the pathogenesis of these diseases. In this review we highlight some of the recent studies that have investigated the transcriptional regulation of *mylk1* gene in smooth muscle tissues and discuss the implications of these findings for regulation of expression of other smooth muscle-specific genes.

MLCK FUNCTION

MLCK phosphorylates the 20-kDa myosin regulatory light chain (RLC) of smooth and nonmuscle myosin II in the presence of Ca^{2+} and calmodulin, which facilitates myosin interaction with actin filaments (20). In most, if not all, cell types, RLC phosphorylation induced by the calcium/calmodulin-dependent activation of MLCK is important for regulating actomyosin-based cytoskeletal functions such as focal adhesion and stress fiber formation, secretion, ion exchange, cytokinesis, neurite growth cone advancement, cell spreading, endothelial and epithelial barrier formation, and cell migration (36). In smooth muscle, phosphorylation of the myosin RLC is also known to be an obligatory step involved in the initiation of contraction. Given the pivotal role of MLCK in regulating numerous functions in nonmuscle cells and for smooth muscle contractile activity, it is likely that changes in the expression of MLCK will have profound effects on the physiological functions of cells. For example, changes in MLCK expression in the vascular system will affect barrier function in endothelial cells and tone in vascular smooth muscle cells, thereby affecting blood pressure. Consistent with this concept, several recent studies have described changes in MLCK expression during development (13,17,27) and under various pathological conditions, including vascular injury (21), intestinal inflammation (3,11,47,82), airway hypersensitivity (35,72), and dedifferentiation of smooth muscle following culture in vitro (48).

Although the 220- and 130-kDa MLCKs have distinct intracellular localizations and cell-specific expression patterns (4), both of the MLCK products of the *mylk1* gene have an identical catalytic domain. It remains unclear whether each of the MLCK isoforms have distinct or overlapping functions (Fig. 1). Mice harboring a specific knockout of the 220-kDa MLCK (both isoforms shown in Fig. 1) exhibited no gross morphological abnormalities, were viable and fertile, and exhibited no major cardiovascular defects (61,79). However, the 220-kDa MLCK knockout mice exhibited decreased susceptibility to lung injury and an impairment of shear stress-induced vasodilatation (61,79). These data suggest that the 220- and 130-kDa MLCKs exhibit at least some nonredundant functions. As a result of the overlapping structures of the *mylk1* gene products, to generate specific knockouts of the 130-kDa MLCK and telokin without altering expression of other products of the *mylk1* gene, it is necessary to delete key promoter elements responsible for their expression. This approach was recently used to generate telokin knockout mice (38).

TELOKIN FUNCTION

Telokin is a 17-kDa acidic protein that is expressed exclusively in smooth muscle tissues and cells (29,34). Telokin is expressed at very high levels in intestinal, urinary, and reproductive tract smooth muscle, at lower levels in vascular smooth muscle, and at undetectable levels in skeletal or cardiac muscle or nonmuscle tissues (10,18,29). Recent studies have begun to

resolve the physiological function of telokin. Telokin previously has been shown to stabilize myosin filaments in vitro and to inhibit MLCK activity toward intact myosin substrates (37, 59,68). Telokin also has been shown to activate myosin light chain phosphatase and to be important for cGMP-mediated calcium desensitization of phasic smooth muscle tissues (10, 38,40,80,87). Telokin knockout mice, generated by deleting key regulatory elements from the core of the telokin promoter, exhibit decreased myosin phosphatase activity, resulting in a leftward shift in the calcium-force relationship in visceral but not vascular smooth muscle tissues (38). The higher level of expression of telokin in visceral compared with vascular and smooth muscle cells can thus account for the lower calcium sensitivity of contraction in visceral compared with vascular tissues (10). These findings demonstrate that regulation of the expression of telokin in smooth muscle tissues will thus modulate its contractile properties.

The biochemical and physiological functions of MLCK and telokin in regulation of contractile processes have been studied extensively, and numerous reports have highlighted changes in the activity of the *mylk1* gene in pathological processes. The mechanisms responsible for mediating the changes in *mylk1* gene expression, however, have only recently begun to be scrutinized.

MYLK1 GENE STRUCTURE

Comparison of MLCK and telokin cDNA sequences, obtained from our laboratory and available databases, with the mouse genome sequence facilitated mapping of the entire mouse *mylk1* gene that encodes these proteins (Fig. 1). A similar structure also has been reported based on the sequence of a BAC clone and homology to the human genome (23). The *mylk1* gene is a large gene spanning ~250 kb and comprising at least 31 exons, located on mouse chromosome 16. Sequence analysis, together with RNA and protein analysis, suggests that the gene encodes at least four protein products: two isoforms of the 220-kDa MLCK [also known as endothelial (22), long (36), or nonmuscle MLCK (70)], a 130-kDa MLCK [also known as short (36) or smooth muscle MLCK (19)], and telokin [also known as kinase-related protein (85)]. Transcripts encoding these products are derived from four independent promoters within the gene (Figs. 1 and 2). The published mouse 220-kDa MLCK cDNA begins in exon 1*; however, a number of mouse EST clones skip this exon and include a more 5' exon 1 instead, suggesting that there are two distinct isoforms of the 220-kDa MLCK. The genomic sequences immediately 5' of exon 1 and exon 1* both exhibit promoter activity, suggesting that the two 220-kDa MLCK isoforms likely arise from alternative promoter usage rather than alternative splicing (Fig. 2). Recently, work in our laboratory (90) showed that intron 14 of the *mylk1* gene contains the proximal promoter that drives expression of the 130-kDa MLCK. It also has been shown that telokin mRNA is transcribed from a promoter, located in intron 28 of the *mylk1* gene, that interrupts exons encoding the calmodulin-binding domain of the kinase (Fig. 1 and Refs. 12,18,31,94). In addition to the mouse MLCK transcripts produced by the independent promoters, it is likely that additional transcripts are derived by alternative splicing within the *mylk1* gene, as described for the human *MYLK1* locus (41). Each of the promoters within the *mylk1* gene exhibits cell-specific differences in their activity. For example, in 10T1/2 fibroblasts, the 220-kDa MLCK E1 promoter exhibits the highest level of activity, whereas in A10 smooth muscle cells, the telokin promoter has the highest levels of activity (Fig. 2). In the current review, we summarize results from studies aimed at unraveling the transcription regulatory network that controls the activity of the *mylk1* gene, and we highlight how these studies have provided important paradigms central to our understanding of the general mechanisms that regulate gene expression in smooth muscle cells.

AN AT-RICH ELEMENT AND CA_RG BOX ARE CRITICAL FOR TELOKIN PROMOTER ACTIVITY

A 370-bp (−190 to +180 bp) mouse telokin promoter has been shown to be sufficient to mediate cell-specific expression of a reporter gene in vitro and to direct smooth muscle-specific expression in vivo in adult transgenic mice (33). Within this promoter, a CA_RG element that binds to serum response factor (SRF) and an adjacent AT-rich element were found to be essential for promoter activity in vitro (30,33) and in vivo (38). In this latter study, the AT/CA_RG core of the endogenous telokin promoter was deleted using a Cre-based knockout strategy. After Cre-mediated recombination, a 30-bp region including the AT/CA_RG motif was deleted and replaced by a single loxP site. Mice homozygous for the 30-bp deletion within the telokin promoter revealed an almost complete loss of telokin protein and mRNA expression with only minimal effects on MLCK expression (Ref. 38 and Fig. 3). These results unequivocally indicate that in the context of the native *mylk1* gene, the AT-CA_RG region of the telokin promoter is critical for endogenous promoter activity. This study represents one of the first examples in which regulatory elements have been demonstrated to be important for regulating smooth muscle-specific gene expression in their native chromatin context in vivo. Analysis of regulatory elements in their native chromatin context is likely to be particularly important for a complex gene such as the *mylk1* gene, in which a single gene contains a number of overlapping transcription units. Although the telokin promoter is only active in smooth muscle cells, the other MLCK promoters are likely to be active in most cell types. Hence, the *mylk1* gene is likely to be transcribing through the telokin exons in many cell types in which the telokin promoter itself is inactive. Thus, although the telokin promoter is not active in nonmuscle cells, the body of the telokin gene is likely to be in a relaxed, transcriptionally favorable conformation because of chromatin remodeling induced by the MLCK promoters. A detailed analysis of the chromatin structure throughout the *mylk1* locus, in different cell types, is required to understand how chromatin structure regulates expression of the *mylk1* transcripts.

TELOKIN AT/CA_RG MOTIF ACTS AS A BLADDER SMOOTH MUSCLE CELL-RESTRICTED ENHANCER

Addition of the AT-rich region/CA_RG box from the telokin promoter (−49 to −94 bp) to the SM22 α promoter (−475 to +61 bp) increased the activity of the SM22 α promoter in visceral smooth muscle cells (33). This fragment of the SM22 α gene normally directs transgene expression exclusively to arterial smooth muscle (33,39,42). Of the 10 AT-CA_RG/SM22 α promoter transgenic lines that transmitted and expressed β -galactosidase activity, all exhibited staining in bladder smooth muscle cells in addition to vascular smooth muscle cells (33). Interestingly, this chimeric promoter fragment did not increase transgene expression in intestinal or reproductive tract smooth muscle cells. These data suggest that other elements, outside the −49 to −94-bp region of the telokin promoter, are required for transcription in intestinal and reproductive tract smooth muscle cells (Fig. 4A). These findings also highlight an important paradigm in which a single promoter uses distinct *cis*-acting regulatory elements to drive expression in different smooth muscle tissues. This raises the exciting possibility that it may be possible to develop reagents that specifically alter expression of a protein, in a single smooth muscle cell type, through targeting the tissue-specific elements.

SERUM RESPONSE FACTOR PLAYS A CENTRAL ROLE IN REGULATING EXPRESSION OF *MYLK1* GENE

Similar to the telokin promoter, the proximal promoter of the 130-kDa MLCK contains a CA_RG element that has been shown to be critical for transcriptional activity. In addition, a highly

conserved CArG element within 130-kDa MLCK intron 1 (intron 15 of the *mylk1* gene) has been proposed to be important for promoter activity (Fig. 5) (90). Each of these CArG boxes within the *mylk1* gene has been shown to bind to serum response factor (SRF) both in vitro and in vivo in native chromatin (30,90,98). SRF and SRF-associated factors play a central role in the expression of many different smooth muscle-specific genes, including smooth muscle myosin heavy chain (smMHC), smooth muscle α - and γ -actin, SM22 α , and calponin, in addition to 130-kDa MLCK and telokin (53,90). SRF is an evolutionarily conserved MADS (MDM1, agamous, deficiens, SRF) domain-containing protein that is required for specification of smooth, cardiac, and skeletal muscle lineages from early mesoderm (2). SRF is a multifunctional protein that binds a highly conserved *cis*-regulatory element, CC(A/T)₆GG, termed a CArG box, found in the promoters of most cardiac and smooth muscle-specific genes. SRF also provides docking surface, within the conserved core MADS domain, for interaction with a wide variety of accessory cofactors. Although SRF expression is greatest in muscle tissues and plays an important role in the differentiation of smooth, cardiac, and skeletal muscle tissues, it is expressed in all tissues, where it is important for the growth factor-mediated induction of immediate-early genes (53). To perform this myriad of functions, SRF associates with various cell-restricted and/or signal-dependent accessory factors that confer coactivator or corepressor activity via ternary complex formation. A number of different SRF-complexes have been shown to regulate the activity of smooth muscle-specific genes. For example, Nkx3.1 and Nkx2.3 are tissue-restricted homeodomain-containing proteins that have been shown to interact with SRF and cooperatively activate the smooth muscle γ -actin promoter (6,64). Several other homeodomain transcription factors, including Barx1b, Barx2b, and Prx (MHox), have been shown to enhance the DNA binding activity of SRF (26,28,57,91). An architectural transcription factor, HMG(Y), also has been reported to interact with SRF and activate the SM22 α promoter (9). In addition, a trimeric complex of SRF-GATA6-CRP2 has been reported to be required for the induction of smooth muscle differentiation in cells from the proepicardial organ, which give rise to coronary artery smooth muscle cells (7), and a complex of Nkx3.2/SRF/GATA-6 has been shown to cooperatively activate the SM22 α promoter (58). However, none of these trimeric complexes has yet been shown to regulate either MLCK or telokin promoters. On the other hand, GATA-6 has been shown to repress the activity of the 130-kDa MLCK and telokin promoters, possibly through disruption of SRF-myocardin complexes (Figs. 4 and 5) (90). The myocardin family of SRF-associated proteins are very powerful activators of many smooth muscle-specific promoters, including both the 130-kDa MLCK and telokin promoters (Figs. 4A and 5A) (8,14,15,43,⁴⁵,⁸⁴,90,92,93,96).

MYOCARDIN, AN SRF-DEPENDENT COACTIVATOR, IS IMPORTANT FOR MLCK AND TELOKIN GENE EXPRESSION

Myocardin is a SAP (SAF-A/B, acinus, PIAS) domain-containing protein that was identified by an in silico screen of cardiac-restricted cDNAs (81). Myocardin contains a basic domain, a polyglutamine-rich domain (Q-rich), and a SAP domain in the NH₂-terminal portion of the protein and a strong transcriptional activation domain at the COOH terminus (81). Myocardin is restricted to cardiac and smooth muscle lineages in developing mouse embryos. It is an extraordinarily potent activator of CArG box-containing cardiac and smooth muscle promoter-reporter gene constructs, in a manner that is strictly dependent on its association with SRF (81). Overexpression of myocardin in a number of different cell types leads to the induction of many smooth muscle-restricted proteins, including the 130-kDa MLCK and telokin (8,15, 75,76,⁸¹,84,90,92,96). Myocardin null mice die around embryonic day 10.5 due to defects in the placental blood vessels (84). These mice also display no detectable vascular smooth muscle cells in the developing aorta, suggesting that myocardin is required for the development of vascular smooth muscle cells. Unfortunately, because myocardin null embryos die before the normal onset of smooth muscle differentiation in visceral smooth muscle tissues, it has not yet

been possible to determine its role in smooth muscle differentiation in these tissues. Intriguingly, it has recently been reported that myocardin null embryonic stem cells can give rise to smooth muscle cells that express smooth muscle-specific markers in vitro, suggesting that not all smooth muscle lineages are dependent on myocardin (65). Myocardin downregulation in cultured smooth muscle cell lines correlates with the decreased expression of smooth muscle-specific contractile proteins (8). These data suggest that myocardin may function not only in the initial steps of smooth muscle cell differentiation but also in the maintenance of differentiation (15,93). Myocardin specifically activates the promoters of SRF-dependent cardiac- and smooth muscle-restricted genes without affecting other SRF-dependent genes such as *c-fos* or *egr1* (96). It has been proposed that the number of CArG boxes within a gene is critical for myocardin to distinguish between different SRF-dependent genes, with myocardin-dependent genes having two or more CArG boxes and myocardin-independent genes having only a single CArG box (84). Although two CArG boxes are important for the activity of many smooth muscle-specific genes, this mechanism is not sufficient to explain the gene-specific effects of myocardin. For example, some myocardin-independent promoters, such as the *Egr1* promoter, contain multiple CArG boxes, and the smooth muscle-specific myocardin-dependent telokin promoter contains only one CArG box (96). In addition, analysis of the smooth muscle α -actin promoter in transgenic mice demonstrated that a promoter fragment containing two CArG boxes was not sufficient to drive smooth muscle cell-specific transgene expression (49).

Myocardin differentially affects the promoters within the *mylk1* gene and is able to induce expression of the 130-kDa MLCK and telokin in many cell types without altering expression of the 220-kDa MLCK (90,96). Although the telokin promoter, which contains only a single CArG box, is activated several hundredfold by myocardin (Fig. 3), the proximal 130-kDa MLCK promoter, which also contains a single CArG box, is only activated two- to threefold (90). In addition, depletion of myocardin in A10 smooth muscle cells by short hairpin RNA significantly decreased telokin expression but did not affect 130-kDa MLCK expression (96). These data could simply reflect a longer half-life of the 130-kDa MLCK protein compared with telokin. Alternatively, it is more likely that telokin is more dependent on myocardin for expression in A10 smooth muscle cells compared with 130-kDa MLCK. In fact, the broad tissue distribution of 130-kDa MLCK compared with telokin would suggest that myocardin-independent pathways must exist for activating this promoter in other cell types (Fig. 5A). Nevertheless, the ability of myocardin to induce 130-kDa MLCK expression in 10T1/2 fibroblasts without significantly activating the promoter in transient reporter assays suggests that the elements required for myocardin to activate this promoter may be located a large distance from the promoter (>6 kb). This arrangement would be unique among myocardin-stimulated genes. Alternatively, the 130-kDa MLCK promoter might be activated indirectly by another myocardin-stimulated transcription factor. Careful analysis of myocardin binding to the endogenous 130-kDa MLCK promoter is required to distinguish between these possibilities.

How myocardin distinguishes smooth muscle-restricted genes from growth factor-responsive SRF-dependent genes remains elusive. Data obtained from analysis of the telokin and *c-fos* promoters suggest that additional *cis* elements and *trans* factors contribute to myocardin's promoter-specific effects (96). For example, binding of Elk-1 adjacent to the CArG box, within the *c-fos* serum response element, may in part prevent myocardin from activating this promoter (Fig. 4). Although Elk-1 and myocardin have been shown to compete for binding to SRF, the binding of Elk-1 to the *c-fos* promoter is not sufficient to explain why myocardin does not activate this promoter. In fact, Elk-1 also has been shown to bind to the telokin and SM22 α promoters and to partially block myocardin's interaction with SRF on these promoters, leading to downregulation of their activity (83,98). In addition, a chimeric telokin promoter containing the Elk-1 binding site and CArG box from the *c-fos* promoter was still strongly activated by

myocardin, suggesting that the presence of these elements alone is not sufficient to prevent myocardin from activating the promoter (96). Identification of additional factors that bind to the telokin promoter and cooperate with myocardin is required to help unravel the mechanisms of promoter selectivity by myocardin.

ACTIVATION OF *MYLK1* GENE BY THYROTROPH EMBRYONIC FACTOR

In addition to SRF-associated proteins, several other *trans* factors have been identified that regulate *mylk1* transcription, including thyrotroph embryonic factor (TEF) (97), Foxq1 (32), and Hox proteins (16) (Figs. 4 and 5). These proteins bind to the AT-rich region adjacent to the CArG box in the telokin promoter. TEF is a member of the PAR (proline- and acidic-amino acid-rich) subfamily of bZIP transcription factors. Overexpression of TEF β upregulated endogenous telokin and 130-kDa MLCK expression in A10 smooth muscle cells without affecting expression of other smooth muscle-restricted proteins, such as smooth muscle α -actin or calponin (97). Although overexpression of a dominant negative TEF repressed telokin and 130-kDa MLCK expression, no studies have examined the expression of these proteins following knockdown or knockout of endogenous TEF. The physiological role of TEF in regulating expression of the *mylk1* gene in different smooth muscle tissues thus remains to be determined.

REGULATION OF *MYLK1* GENE BY HOMEBOX PROTEINS

In addition to being regulated by the homeodomain containing protein Barx, which can increase SRF DNA binding activity (28), the telokin promoter is directly regulated by the products of clustered homeobox genes (*Hox*) (16). Homeobox genes encode a family of highly conserved transcription factors that share a conserved 60-amino acid homeodomain, which binds to AT-rich consensus sequences in DNA (67). Homeobox proteins are master regulatory proteins that control basic developmental processes. They are differentially expressed along the anteroposterior (AP) axis and are believed to play an important role in organogenesis and pattern formation during both vertebrate and invertebrate embryogenesis (51). Several studies also have indicated an important role of homeodomain-containing proteins in regulating differentiation, proliferation, and migration in different cell types (52). Homeodomain protein expression often persists in adult tissues, where these proteins are believed to play a role in maintaining cell type specification (54). The spatial pattern of homeobox protein expression makes them ideal candidates for specifying distinct smooth muscle cell phenotypes in different tissues. A number of Hox proteins have been identified in smooth muscle: Hoxa2, a4, a5, and b7 were identified in an adult rat vascular smooth muscle cDNA library (24), and Hoxa10 and b8 in an adult mouse bladder cDNA library (16). In addition, HOXA5, A11, B1, B7, and C9 have been detected in human adult and fetal aortic smooth muscle tissues (54), and HOXA10 in human adult myometrium and endometrium (74). Of the identified Hox proteins, only Hoxa10, b8, and b7 have been shown to have direct effects on the *mylk1* gene and other smooth muscle-specific genes. Hoxb7 overexpression promoted differentiation of C3H10T1/2 cells into myofibroblasts and induced expression of SM22 α and smooth muscle α -actin (5). However, a comparison of the increased expression of HOXB7 RNA in human atherosclerotic plaques to normal adult smooth muscle tissue suggests that HOXB7 plays a role in either smooth muscle cell proliferation or dedifferentiation under pathological conditions. Within the *mylk1* gene, Hoxa10 has been shown to bind directly to multiple sites in the telokin promoter and to specifically regulate telokin promoter activity (16) (Fig. 4A). Hoxa10 is expressed in adult uterine, colonic, and bladder smooth muscle tissues and cells. Several studies have indicated that Hoxa10-1 is important for the process of uterine epithelium cell differentiation and the development of subcellular structures required for implantation (73). In addition, the expression of Hoxa10-1 in the myometrium is regulated during the menstrual cycle, with high levels of estrogen increasing Hoxa10 expression (46). Previously we have shown that telokin

expression is also regulated during the menstrual cycle, with high levels of expression being observed during the estrogen-dominant follicular phase and low levels of expression during the progesterone-dominant luteal phase. Telokin expression is also elevated in the uterus during pregnancy (71). A link between *Hoxa10-1* and telokin expression in uterine smooth muscle was established by studies in which small interfering RNA-mediated knockdown of *Hoxa10-1*, in uterine smooth muscle cells, resulted in decreased telokin expression (16). Previous studies also demonstrated that the telokin promoter is directly regulated by estrogen bound to the estrogen receptor (71). Together, these studies suggest that both *Hoxa10* and estrogen receptor may synergize with each other to mediate hormone-dependent changes in telokin expression in the uterus (Fig. 4). Because telokin has been shown to play a role in decreasing the calcium sensitivity of contraction through its activation of myosin light chain phosphatase (10,38), the elevated levels of telokin in the myometrium during pregnancy may contribute to the maintenance of uterine quiescence.

In contrast to *Hoxa10*, *Hoxb8* inhibits the activity of the telokin promoter as well as the promoters of a number of other smooth muscle-specific genes (16). The expression of both *Hoxa10* and *Hoxb8* mRNA in some of the same smooth muscle cells suggests that the regulation of telokin gene activity is determined by the relative ratio of *Hoxa10* and *Hoxb8* or that the activity of these proteins is modified posttranscriptionally. In support of this latter possibility, a microRNA has been identified that specifically represses *Hoxb8* expression at the posttranscriptional level (88). The physiological significance of *Hoxb8* in regulating the expression of smooth muscle-specific genes currently remains unclear. It will be interesting to determine whether increases in the level of *Hoxb8* protein expression occur in pathological conditions associated with downregulation of smooth muscle-specific gene expression, as reported previously for *HOXB7* (5). Finally, the restricted expression of Hox proteins along the AP axis suggests that other Hox proteins may be playing similar roles in regulating telokin expression in more anterior or posterior tissues. This spatial pattern of expression of the Hox proteins makes these proteins excellent candidates for helping to specify the unique identity of smooth muscle cells in different smooth muscle tissues.

INHIBITORY FACTORS CONTRIBUTE TO CELL-SPECIFIC TRANSCRIPTION OF TELOKIN

The tissue specific pattern of *mylk1* expression is likely to be the result of the combined actions of both positive- and negative-acting transcription factors. For example, although telokin is strongly activated by myocardin and myocardin is highly expressed in vascular smooth muscle cells, there is relatively little expression of telokin in these cells (18,29,96). This suggests that an inhibitory factor must be attenuating the activity of the telokin promoter in vascular smooth muscle cells. One possible candidate for this inhibitory factor is GATA-6 (89,90). A number of other proteins also have been reported to inhibit the activity of the telokin promoter, including *Hoxb8*, *Foxq1*, and *Elk-1* (16,32,76,98) (Fig. 4B). GATA-6 and *Elk-1* both appear to act by disrupting the interaction of myocardin and SRF, and the inhibitory activity of both proteins has been proposed to be dependent on the location of their cognate *cis*-acting elements in the telokin promoter adjacent to the CArG box (89,98). Interestingly, although GATA-6 also inhibits the 130-kDa MLCK promoter, and expression of the 130-kDa MLCK also can be induced by myocardin, overexpression of *Elk-1* had no effect on the levels of the 130-kDa MLCK in A10 smooth muscle cells (98). These data further highlight the differences in the regulation of the 130-kDa MLCK and telokin promoters by myocardin. The mechanisms by which *Foxq1* and *Hoxb8* inhibit telokin promoter activity have not yet been elucidated. However, the close proximity of the consensus binding sites for *Foxq1* and *Hoxb8* to the CArG box, the ability of a number of homeodomain proteins to interact with SRF (6,25,28,64,91), and a recent report describing the interaction of *Foxo4* (44) and myocardin make it tempting to speculate that these proteins also may disrupt key SRF activation complexes (Fig. 4B).

REGULATION OF MLCK AND TELOKIN EXPRESSION BY GATA FAMILY MEMBERS IN SMOOTH MUSCLE CELLS

GATA proteins are a family of transcription factors with two zinc fingers that directly bind DNA regulatory elements containing a consensus (A/T)GATA(A/G) motif. To date, six mammalian members of the GATA family have been identified that can be divided, on the basis of sequence and expression similarities, into two subgroups (63). The GATA-1/2/3 subfamily is preferentially expressed in the hematopoietic cells (86), and the GATA-4/5/6 subfamily is expressed within various mesoderm- and endoderm-derived tissues, including the heart, liver, lung, gonads, and small intestine (55). During early murine embryonic development, the expression patterns of GATA-6 and GATA-4 are very similar, with these factors being detected in the precardiac mesoderm, the embryonic heart tube, and the primitive gut. Notably, during development GATA-6 becomes the only member of the family expressed in vascular smooth muscle cells and has been linked to the differentiated phenotype of these cells (56). GATA-6 expression in adult vascular smooth muscle cells is rapidly down-regulated upon mitogen stimulation or vascular injury. Adeno-virus-mediated GATA-6 gene transfer to the vessel wall after balloon injury partially inhibited lesion formation and reversed the downregulation of smMHC, smooth muscle α -actin, calponin, vinculin, and metavinculin expression that is normally associated with injury-induced vascular smooth muscle cell phenotypic modulation (50). In addition to the critical role of GATA-6 in the maintenance of the differentiated phenotype in vascular smooth muscle cells, a recent report has revealed that GATA-6 may act as the predominant GATA factor in the maintenance of endodermal gene expression by TGF- β signaling in gastrulating embryos (1).

Recent studies have shown that the transcripts of the *mylk1* gene are differentially regulated by GATA-6 (89,90). Over-expression of GATA-6, by adenoviral transduction, significantly decreased endogenous telokin and 130-kDa MLCK expression in A10 vascular smooth muscle cells. In contrast, expression of the 220-kDa MLCK, SM22 α , and calponin were markedly increased (89,90). GATA-6 has been shown to bind directly to the telokin and 130-kDa MLCK promoters at consensus binding sites that are closely opposed to the CArG boxes within these promoters (Figs. 4 and 5). In addition to being recruited to promoters through binding to consensus GATA sites, GATA-6 also can be recruited to promoters through its binding to the MADS domain of SRF (60). Because both GATA-6/4 and myocardin interact with same domain of SRF, it is likely that a single SRF molecule is able to interact with either GATA-6/4 or myocardin but not with both molecules at the same time (Fig. 4). Whether GATA-6/4 or myocardin are bound to SRF appears to depend on the specific promoter context. In the case of telokin and 130-kDa MLCK promoters, there is a consensus GATA binding site adjacent to a CArG box, which would greatly facilitate the ability of GATA factors to compete with myocardin for interacting with SRF by increasing the local effective concentration of GATA-6. In contrast, GATA-6 binding to a more distal site in the smMHC promoter can activate the promoter synergistically with myocardin (78). In support of this model, the activation of both the 130-kDa MLCK and telokin promoters by myocardin can be blocked by GATA-6, and overexpression of GATA-6 also attenuates the ability of myocardin to induce expression of the endogenous 130-kDa MLCK and telokin genes (89,90). This mechanism to explain the GATA-6-mediated repression of the 130-kDa MLCK should be interpreted with caution, because myocardin has not yet been shown to directly activate this promoter or to bind to the promoter in vivo.

Through its interaction with SRF, GATA-6 also has been shown to form complexes with other proteins to regulate expression of a number of smooth muscle-specific genes. For example, an SRF-GATA-6-Nkx3.2 complex cooperatively transactivated α -integrin, SM22 α , and caldesmon promoters. This activation also required direct binding of each of these factors to their respective consensus sequences within the promoters (58). It has been reported that

cysteine-rich protein 1/2 (CRP1/2) may act as scaffold protein that can stabilize SRF and GATA-6 into a transcriptosome that confers strong activation of smooth muscle-specific promoters (7). However, there have been no reports demonstrating that any of these complexes regulate the activity of the *mylk1* gene. Unpublished studies from our laboratory have failed to show any differences in the regulation of the telokin promoter by a GATA-6-SRF-CRP2 complex compared with GATA-6 alone. It should be noted, however, that in our hands, adenovirus-mediated over-expression of SRF, GATA-6, and CRP2 together in 10T1/2 cells was not sufficient to induce expression of any of the smooth muscle-specific genes tested.

A number of other transcription cofactors also have been reported to be involved in GATA-induced smooth muscle-specific gene expression. For instance, the transcriptional coactivator p300, together with GATA-6 and NFATc1, form a large complex during the transcription of the smMHC gene. This complex has been shown to play a role in the maintenance of the differentiated phenotype of vascular smooth muscle cells (77,78). In contrast, the basic helix-loop-helix (bHLH) transcription factor CHF1/Hey2 can suppress the smMHC promoter activity by interacting with GATA-6 through its bHLH domain (69), and LMCD1/Dyxin has been identified to repress GATA-6 function by inhibiting its ability to bind to DNA (66). It will be important to determine whether these cofactors also are involved in GATA-6-mediated regulation of 130-kDa MLCK and telokin expression.

SUMMARY OF MECHANISMS REGULATING TISSUE-SPECIFIC EXPRESSION OF MYLK GENE

Figure 4 summarizes our current model describing the tissue-specific expression of telokin. In this model, high levels of telokin transcription in smooth muscle cells is mediated by a combination of cell- and tissue-restricted factors, acting together with more general transcription factors (Fig. 4A). Among different smooth muscle tissues, unique but overlapping sets of transcription factors are used to direct high levels of telokin expression. For example, in uterine smooth muscle, the estrogen receptor and Hoxa10-1 act together with myocardin to drive high levels of telokin expression. In contrast, in intestinal smooth muscle cells, the estrogen receptor is not required for telokin expression, and an as yet unidentified factor is required in its place (Fig. 4A). In contrast, negative-acting factors such as Foxq1 and Hoxb8, present in nonmuscle cell types, help to prevent telokin transcription in these cells (Fig. 4B). Unlike telokin, the 130-kDa MLCK, although expressed at highest levels in smooth muscle cells, is also expressed in most other cell types. Transcription of the 130-kDa MLCK is thus likely to be regulated by both smooth muscle-specific mechanisms, as well as by more general mechanisms. For example, expression of high levels of the 130-kDa MLCK in smooth muscle cells may result from the activity of myocardin. In nonmuscle cells, which express no detectable myocardin, the activity of more general factors such as Sp1 and cAMP response element binding protein (CREB) may be sufficient to direct the low levels of transcription of the 130-kDa MLCK in these cells (Fig. 5A). Analysis of the expression of the *mylk* gene in animal models harboring cell- and tissue-specific knockouts of factors shown to regulate the *mylk* gene is required to further unravel the mechanisms regulating its tissue-specific expression.

SUMMARY AND PERSPECTIVES

A number of studies have provided important insights into the transcriptional regulation of the *mylk1* gene, but this is at best a partial view of the regulatory mechanisms. To date, most studies of the promoters within the *mylk1* gene have primarily focused on the smooth muscle-specific regulation of telokin promoter, and many unanswered questions remain regarding the regulation of this complex gene. For example, how is the chromatin structure of the *mylk1* locus regulated? Do the MLCK promoters regulate the chromatin structure of the entire *mylk1* locus, including the telokin promoter? Are there additional promoters within the

mylk1 gene? What fragments of the 130-kDa MLCK promoter are required to mimic endogenous 130-kDa MLCK expression in vivo? Where are the promoters of the 220-kDa MLCK isoforms, and how are they regulated? The current studies also have been limited to defining the transcriptional mechanisms responsible for regulation of *mylk1* gene expression under normal physiological conditions. An important challenge of future studies will be to elucidate how these regulatory mechanisms are disrupted under pathological conditions. It also will be important to determine how the subsequent changes in expression of the *mylk1* gene products contribute to the pathology. By manipulating the regulatory elements that control expression of the *mylk1* gene under normal physiological conditions, researchers should be able to design in vivo mouse models with decreased expression of individual *mylk1* gene products. Analysis of the physiological and pathological properties of these mice can then be used to define the specific roles of individual *mylk1* gene products in vivo.

NOTE ADDED IN PROOF

Since completion of this review, two additional studies have been published that describe the transcriptional regulation of the *mylk1* gene. Graham et al. describe the regulation of the human 220-kDa MLCK E1 promoter and its activation by TNF α (Graham WV, Wang F, Clayburgh DR, Cheng JX, Yoon B, Wang Y, Lin A, and Turner JR. TNF induced long myosin light chain kinase transcription is regulated by differentiation-dependent signaling events: Characterization of the human long myosin light chain kinase promoter. *J Biol Chem* 2006). Han et al. demonstrated that the rat 130-kDa MLCK promoter is also SRF dependent (Han YJ, Hu WY, Chernaya O, Antic N, Gu L, Gupta M, Piano M, and de Lanerolle P. Increased Myosin Light Chain Kinase Expression in Hypertension: Regulation by SRF via an Insertion Mutation in the Promoter. *Mol Biol Cell* 17:4039–4050, 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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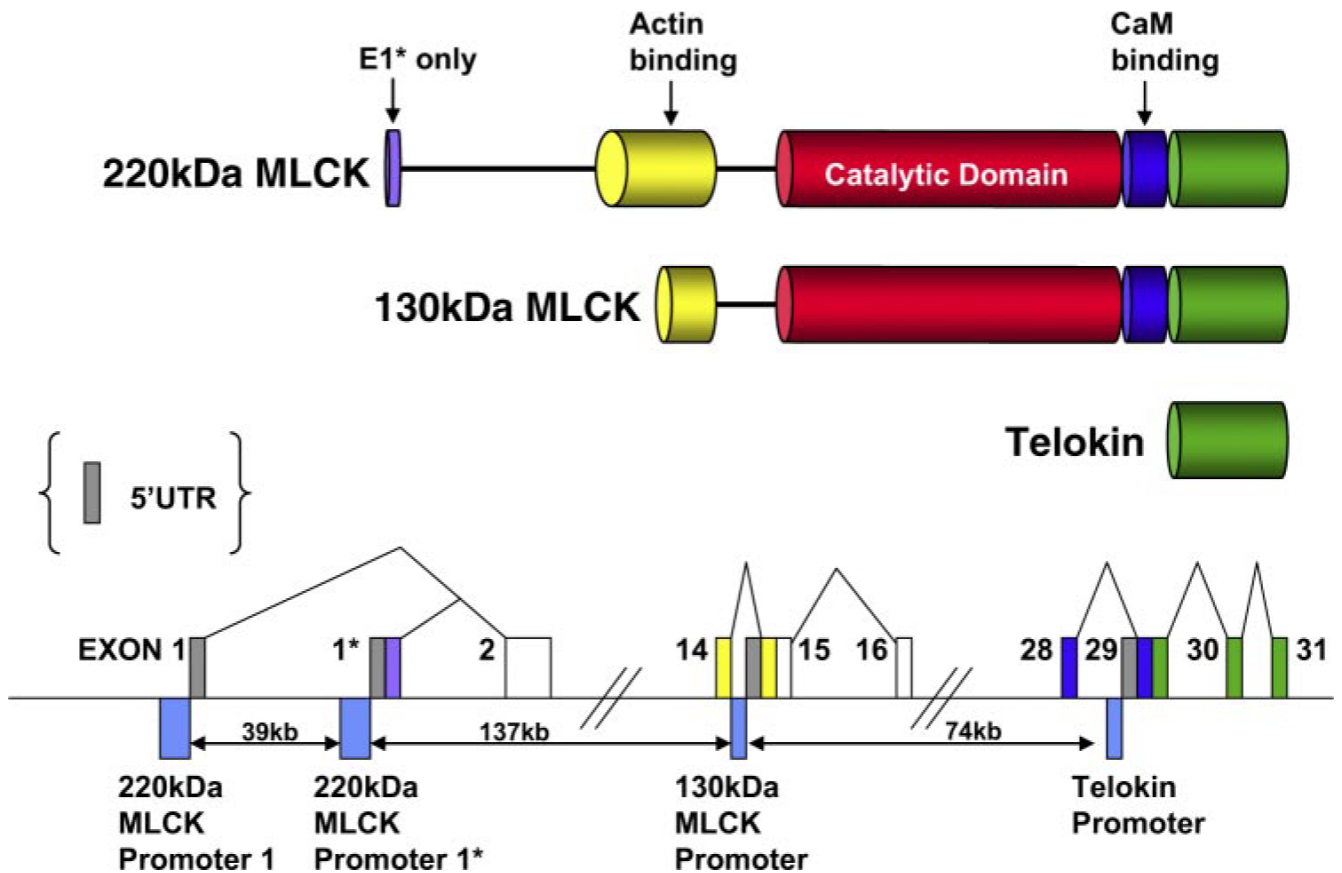


Fig. 1.

Structure of the mouse *mylk1* gene. *Top* schematic is a representation of the domain structure of protein products of the *mylk1* gene. As illustrated, the amino acid sequence of telokin is identical to the COOH terminus of the myosin light chain kinase (MLCK) molecules. Likewise, the amino acid sequence of the 130-kDa MLCK is identical to the common COOH-terminal portion of the 220-kDa MLCK. *Bottom* schematic is a representation of a portion of the mouse *mylk1* gene (not to scale; see Supplemental Data for sequence information).² Exons are indicated by boxes and are color coded to match the domains in the *top* schematic. The dark gray boxes represent the unique 5'-untranslated region (UTR) segments of each transcript. Promoter regions are indicated by the blue boxes below the line. Genomic sequence predicts that two 5' promoters direct the expression of the two isoforms of the 220-kDa MLCK that differ from each other only in the sequence encoded by exon 1* of the 220-kDa MLCK E1*. The protein produced from usage of exon 1* is predicted to be 9 amino acids longer than the protein resulting from usage of exon 1. Two internal promoters direct the expression of transcripts encoding the 130-kDa MLCK and telokin. (For specific sequences, see Supplemental Data).

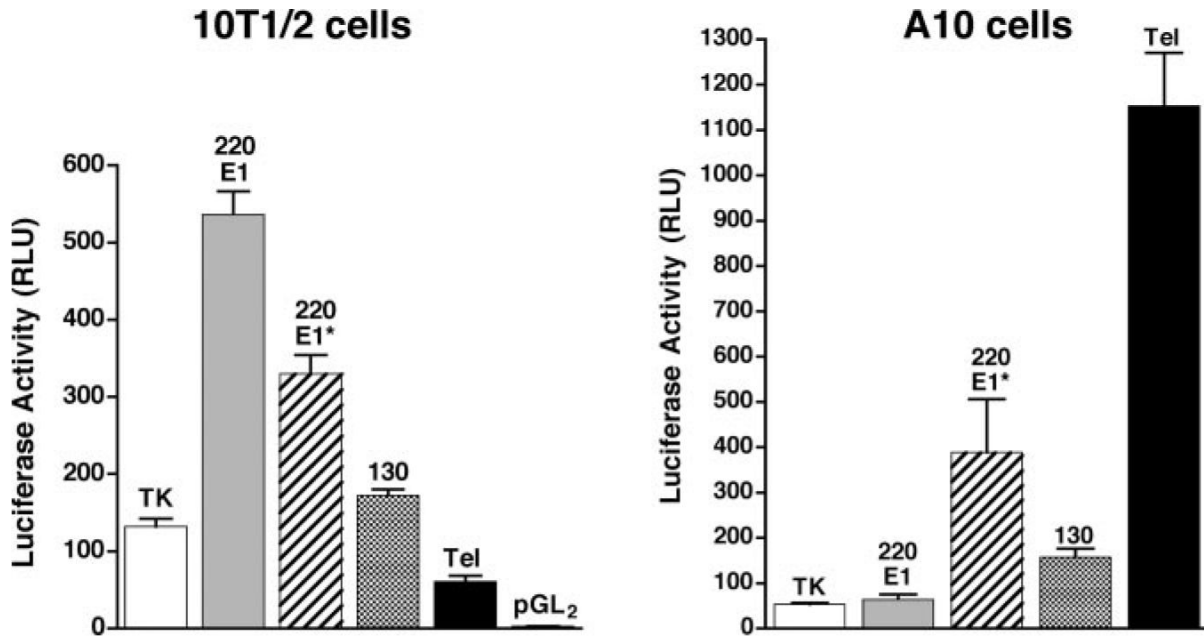


Fig. 2.

Transcriptional activity of the 4 predicted promoters within the *mylk1* gene. Luciferase reporter genes were generated containing genomic fragments (700 and 750 bp, respectively) immediately 5' of exons E1 (220 E1) or E1* (220E1*) or comprising nucleotides -389 to +115 of the 130-kDa MLCK (130) promoter, -190 to +180 of the telokin (Tel) promoter, or -113 to +20 of the thymidine kinase (TK) promoter. These reporter plasmids or the empty luciferase vector pGL₂B were transfected into 10T1/2 embryonic fibroblasts of A10 smooth muscle cells, together with an internal control renilla luciferase plasmid. At 24 h after transfection, lysates were prepared and analyzed for luciferase activity. Results presented are relative light units (RLU) of luciferase activity normalized to the renilla luciferase internal control. Values are means \pm SE of 6 samples.

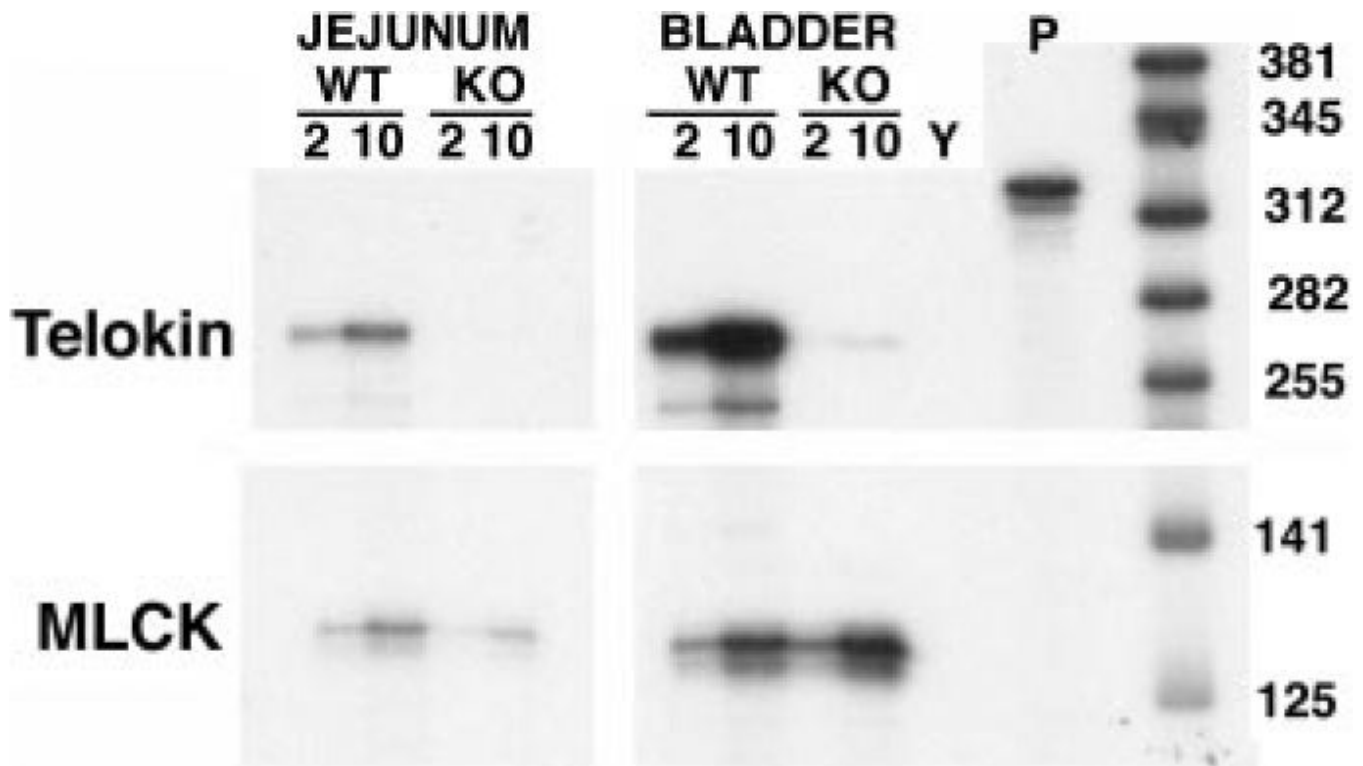


Fig. 3. Deletion of the AT-rich region and CArG box from the endogenous telokin promoter specifically abolished telokin expression. An RNase protection assay was performed on RNA isolated from jejunum and bladder of wild-type (WT) and telokin knockout (KO) mice. The probe used (P) protects an ~255-bp fragment of the telokin mRNA and a 125-bp fragment of the MLCK mRNA. The positions of RNA size markers are indicated at *right* of the blot in base pairs. Either 2 or 10 μ g of RNA were used in the assay, as indicated. Y, yeast RNA.

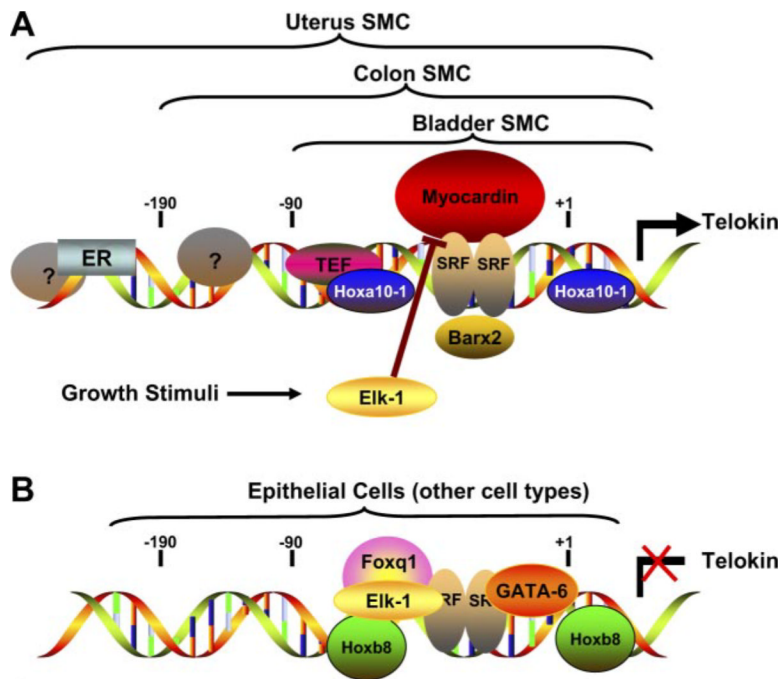


Fig. 4.

Telokin promoter regulation in smooth muscle and nonmuscle cells. A schematic model is shown of the transcription factors that bind to the telokin promoter in smooth muscle cells (SMC) (A) and in epithelial or nonmuscle cells (B). Analysis of promoter fragments in transgenic mice has suggested that the core of the telokin promoter, which includes a CARG box and adjacent AT-rich region, is required for expression in all smooth muscle tissues (38), and a -90 to $+180$ -bp fragment that includes this region is sufficient for expression in bladder smooth muscle cells (33). Additional unidentified elements between -90 and -190 bp are required for telokin expression in smooth muscle cells in the gastrointestinal tract (33). In most higher mammals, with the exception of rodents, the estrogen receptor (ER) and perhaps other proteins that bind to more distal sequences are required for expression in uterine smooth muscle cells (71). Transcription factors that have been found to bind to the core of the telokin promoter directly [Hoxa10-1, thyrotroph embryonic factor (TEF), and serum response factor (SRF) (16,30,97)] or indirectly through their association with SRF [Barx2 and Myocardin (28,96)] and activate the promoter are shown in A. Transcriptional inhibitors that repress the activity of the telokin promoter in epithelial cells or other nonmuscle cell types are shown in B. The relative contribution of each of the transcription factors to the transcriptional activity of the telokin promoter is likely to be different in smooth muscle cells from distinct tissues and in different nonmuscle cells. GATA-6 may inhibit telokin promoter activity in vascular smooth muscle cells by competing with myocardin for binding to SRF. Similarly, because Elk-1 also can compete with myocardin for binding to SRF, growth factor stimulation of smooth muscle cells may result in activation of Elk-1 and inhibition of telokin expression (98).

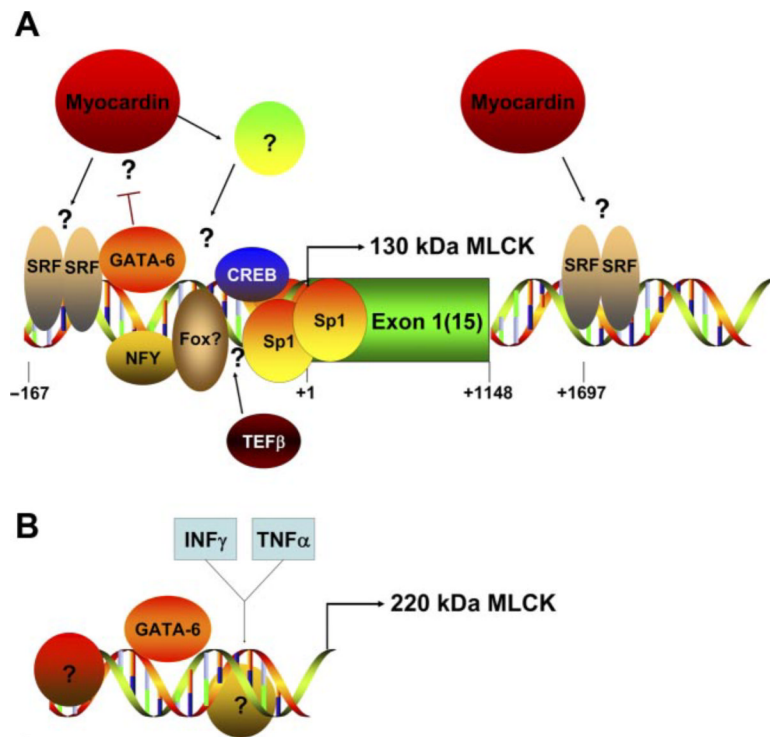


Fig. 5. Regulation of the MLCK promoters. In *A*, the schematic representation depicts the transcriptional regulation of the 130-kDa MLCK promoter (90). Two conserved CArG boxes, one in the proximal promoter and one in the first intron, have been shown to bind SRF in vivo (90). Whether myocardin directly or indirectly regulates the promoter remains to be determined. GATA-6 has been shown to bind to the promoter and inhibit its activity. TEF β has been shown to increase expression of the 130-kDa MLCK, although its binding site on the promoter has not been defined (97). The presence of the other factors (Fox, Sp1, CREB, NFY) on the promoter is based purely on their consensus binding sites within the promoter sequence and has not yet been verified experimentally. In *B*, the putative structure of the 220-kDa MLCK promoter(s) is indicated. To date, these promoters have not been characterized, except for the observations that GATA-6 and INF- γ /TNF- α stimulation can increase 220-kDa MLCK expression (47,82,90).