Selective modulation of the SM22 α promoter by the binding of BTEB3 (basal transcription element-binding protein 3) to TGGG repeats

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We have previously identified a C2H2 zinc-finger transcription factor [BTEB3 (basal transcription element-binding protein 3)/KLF13 (Krüppel-like factor 13)] that activates the minimal promoter for the smooth muscle-specific SM22 α gene in other types of cell. We show that recombinant BTEB3 binds to three TGGG motifs in the minimal SM22 α promoter. By mutation analysis, only one of these boxes is required for BTEB3-dependent promoter activation in P19 cells and BTEB3 activates or inhibits reporter gene expression depending on the TGGG box to which it binds. Transient transfection experiments show that BTEB3 also activates reporter gene expression from the SM22 α promoter in

VSMCs (vascular smooth muscle cells). Similar studies showed that BTEB3 did not activate expression from the promoter regions of the smooth muscle myosin heavy chain or smooth muscle α -actin promoters, which contain similar sequences, implying that promoter activation by BTEB3 is selective. The expression of BTEB3 is readily detectable in VSMCs *in vitro* and is modulated in response to injury *in vivo*.

Key words: differentiation, gene expression, Krüppel-like factor (KLF), TGGG box, transcription factor.

INTRODUCTION

SM22 α is a small actin-binding protein, the expression of which is restricted to smooth muscle cells in normal adult rodents. Although the function of SM22 α in smooth muscle cells remains to be established, expression of the gene has proved to be a useful marker of smooth muscle tissue [1,2]. Furthermore, the SM22 α promoter has been analysed to determine sequences and transcription factors that are required for the expression of smooth muscle-specific genes [3,4]. For example, the rat and mouse SM22 α promoters contain two conserved CArG boxes [CC(A/T-rich)₆GG boxes; binding sites for SRF (serum response factor)], in addition to conserved GC-rich boxes that are likely to be binding sites for the KLFs (Krüppel-like factors) [5–7]. Transgenic analysis has shown that a 280-bp fragment of the mouse SM22 α promoter containing two CArG boxes and GC-rich boxes is sufficient to drive expression of a *lacZ* reporter gene in VSMCs (vascular smooth muscle cells) but not in visceral smooth muscle cells [3,4]. Similar studies have shown that mutation of either the CArG boxes [6,7] or a GC-rich box termed the TCE [TGF- β (transforming growth factor β) control element] results in loss of *lacZ* transgene expression [8]. *In vitro* analysis has indicated that a 118-bp fragment of the rat SM22 α promoter drives reporter gene expression in rat aortic VSMCs, but is inactive in a non-smooth muscle cell line (Rat-1 fibroblasts) [5].

These data implicate SRF and binding factors for GC-rich boxes in the control of SM22 α gene expression. Fully differentiated adult aortic VSMCs have been shown to express multiple forms of SRF capable of binding and activating the SM22 α promoter [9], but the factors that bind to the GC-rich sequences remain to be identified. Using a yeast one-hybrid screen, Adam et al. [8] showed that gut-enriched KLF (KLF4) bound to the SM22 α promoter but transfection analysis showed that gut-enriched KLF did not activate the promoter [8]. They also showed that a related

KLF, BTEB2 [BTE (basal transcription element)-binding protein 2; intestinal KLF/KLF5], bound and activated the SM22 α promoter. However, it has been reported that BTEB2 is not expressed in adult rat aorta and is therefore unlikely to regulate the basal expression of SM22 α in these cells [10].

We identified a novel KLF, BTEB3 (KLF13) that is widely expressed during development and in the adult animal and showed that BTEB3 is a potent activator of the SM22 α promoter in P19 embryonic carcinoma cells [11,12]. Here we have defined the BTEB3-binding sites on the rat SM22 α promoter and examined whether BTEB3 activated the SM22 α promoter in VSMCs. We have also examined the expression of BTEB3 in VSMCs *in vitro* and in response to vascular injury *in vivo*.

MATERIALS AND METHODS

Plasmids and cloning

The plasmids pCBTEB3 [11], pSM22 – 118/+ 65 and pSM22 – 197/+ 65 and pSM22 – 1500/+ 65 [5,13] have been described previously.

For the expression of BTEB3 in *Escherichia coli*, the full-length BTEB3 open reading frame was amplified by PCR using the primers 5'-GAAGATCTTCGACGCCGCCCTATGTGGAC-3' and 5'-GGAATTCCTCAGGGTGAGCTGGCCGGGC-3'. The PCR product was digested with *Bgl*II and *Eco*RI and ligated into pTrcHisA (Invitrogen). The resultant plasmid pTrcHis-BTEB3 was selected and sequenced.

Primers containing the mutant GC-rich boxes were used to amplify –118 to +65 bp of the SM22 α promoter by PCR (see Figure 5a, below). The amplified DNA fragments were cloned into pGEM-T-Easy, and positive colonies were selected and sequenced. The mutant pSM22 – 118/+ 65 promoter fragments were ligated into pCAT-Basic for transfection.

Abbreviations used: BTE, basal transcription element; BTEB, BTE-binding protein; rBTEB, recombinant BTEB; CAT, chloramphenicol acetyltransferase; EMSA, electromobility shift assay; KLF, Krüppel-like factor; SM α -actin, smooth muscle α -actin; SRF, serum response factor; TGF- β , transforming growth factor β ; TCE, TGF- β control element; VSMC, vascular smooth muscle cell; YY1, yin yang 1; RT, reverse transcriptase; SM-MHC, smooth muscle MHC.

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Transfection of P19 cells and CAT (chloramphenicol acetyltransferase) assay

P19 embryonic carcinoma cells were maintained and transfected as described in [11] with a total of 2 µg of plasmid DNA as detailed in the text. VSMCs were derived, grown and transfected as described previously [13–15]. Cells were transfected with 1.6 µg of DNA consisting of 1.2 µg of reporter construct and 0.4 µg of pCDNA3 or pCBTEB3.

The cells were harvested 48 h after transfection, lysed and CAT assays were performed as described previously [9]. CAT assays were normalized to the total protein content of the lysates, which was determined by Bradford assay, as described previously by Shimizu et al. [16]. All transfections were performed in at least three independent experiments with triplicate samples and the data are presented as means ± S.E.M.

Northern-blot analysis, RT (reverse transcriptase)-PCR and *in situ* hybridization

Total RNA was extracted from the intact rat aorta, freshly dispersed VSMCs and cultured rat VSMCs (after 10 passages) using Phaselock columns (Flowgen) according to the manufacturer's instructions. The RNA was separated using a formaldehyde/agarose gel and transferred to a nitrocellulose membrane before hybridizing overnight to a ³²P-labelled BTEB3 probe [11] or a SM22α probe [5]. After washing as described previously [14], the blots were exposed to a phosphor screen and bound probe was visualized using ImageQuant software (Molecular Dynamics). RT-PCR on RNA extracted from VSMCs and P19 cells was performed as described previously [11].

In situ hybridization with ³⁵S-labelled antisense probes (BTEB3 544–880 bp relative to the transcription start site and SM22α probe [5]) was carried out on injured and uninjured rat carotid arteries as described previously [17].

rBTEB3 (recombinant BTEB3) expression

For expression of the rBTEB3 protein, 2 ml of LB media supplemented with ampicillin (50 µg/ml) was inoculated with *E. coli* cells containing pTrcHis-BTEB3 and grown overnight at 37 °C with agitation. Then 1 ml of this suspension was diluted into 50 ml of LB containing ampicillin and grown at 37 °C with shaking until *D*₆₀₀ reached 0.5, when 50 µl of isopropyl β-D-thiogalactoside (100 mg/ml) was added to induce protein expression. After 4 h, the bacteria were centrifuged at 100 g for 15 min. The supernatant was removed and the pellet stored at –20 °C until use.

Bacteria were lysed using 5 ml BugBuster solution (Novagen) per g of wet cell paste for 20 min at room temperature, and rBTEB3 was purified using a HiTrap affinity column (Amersham Biosciences) according to the manufacturer's instructions. Fractions containing rBTEB3 were dialysed against water using a 3500-Da molecular-mass cut-off (0.5–3 ml) Slide-A-Lyser dialysis cassette (Pierce).

Preparation of nuclear extracts and EMSA (electromobility shift assay)

Double-stranded oligodeoxynucleotide probes were labelled with [γ -³²P]ATP using 1 µl of T4 polynucleotide kinase in 1 × T4 polynucleotide kinase buffer at 37 °C for 1 h and purified through a Sephadex G-25 column.

Nuclear extracts were prepared as described previously [9]. For EMSA, a 20-µl reaction comprising ³²P-labelled DNA, binding buffer [50 mM Tris/HCl (pH 8.0), 100 mM KCl, 12.5 mM MgCl₂, 1 mM EDTA, 1 µg of poly(dI-dC) and 1 mM dithiothreitol in

Figure 1 EMSA analysis of the BTE sequence with rBTEB3

Bacteria containing either the empty pTrcHis vector or the pTrcHis-BTEB3 vector were harvested 4 h after isopropyl β-D-thiogalactoside induction and lysed. The lysates were mixed with ³²P-labelled BTE oligonucleotide in the presence (+) or absence (–) of an unlabelled competitor (CC) and separated through a 6% acrylamide Tris/borate/EDTA gel (see the Materials and methods section).

20% (v/v) glycerol] and either 5 µl of nuclear extract or 5 µl of rBTEB3 were mixed and incubated for 1 h at room temperature. For competition reactions, a 50–400-fold excess of unlabelled DNA probes was incorporated into the reaction mix. Samples were electrophoresed in 6% (w/v) acrylamide non-denaturing gels. The gels were pre-run at 100 mA for 1–2 h before loading the samples. On completion the gel was dried and visualized using ImageQuant software (Molecular Dynamics).

RESULTS

BTEB3 binds the minimal SM22α promoter at a conserved TGGG site

To show that induction of rBTEB3 production from pTrc-His BTEB3 produced a protein that binds GC-rich sequences, EMSA analysis was carried out using a ³²P-labelled BTE probe, to which BTEB3 made by *in vitro* translation has previously been shown to bind [11]. Incubation of rBTEB3 with the BTE probe resulted in the formation of a complex that was not formed when the probe was incubated with lysate purified from cells containing empty vector (pTrcHis; Figure 1). Complex formation was effectively competed by a 100-fold excess of the unlabelled competitor sequence.

To determine whether rBTEB3 binds to the SM22α promoter, the protein was incubated with a ³²P-labelled fragment of the SM22α promoter containing the minimal promoter (bases –118 to +65). A complex was formed that was effectively competed by an excess of unlabelled SM22α minimal promoter, indicating that BTEB3 binds to the –118 to +65 bp region of the rat SM22α promoter (Figure 2b).

The –118 to +65 bp region of the minimal rat SM22α promoter contains three repeats of a TGGG box (Figure 2a), similar to sequences present in the SMα-actin (smooth muscle α-actin) and SM-MHC (smooth muscle MHC) promoters. To determine whether rBTEB3 binds to the TGGG boxes, oligonucleotides containing the GC-rich boxes at –107 and –85 bp (107/85, 5'-GTGGGAAGCCAAGCAGACTTCCATGGGC-3'), or the GC-rich boxes at –85 and –66 bp (85/66, 5'-CCATGGGCAGG-AGGGGCGCCAGTGGGCAGG-3'), or a mutant of the –85 and

Figure 2 EMSA analysis of the minimal region of the SM22 α promoter using rBTEB3

(a) Sequence of the rat SM22 α promoter from -59 to -114 showing the TGGG sequences, boxes 107, 85 and 66 (bold). (b) rBTEB3 was mixed with unlabelled competitor (CC; SM22 α promoter, bases -118 to +65) as indicated and incubated with 32 P-labelled SM22 α promoter probe (bases -118 to +65) at RT for 1 h before electrophoresis [6% acrylamide TBE (Tris/borate/EDTA) gel]. (c) rBTEB3 was mixed with unlabelled competitors as indicated (box 107/85, 85/66 or m85/66; see Materials and methods section) and incubated with 32 P-labelled SM22 α promoter probe (bases -118 to +65) at RT for 1 h before electrophoresis (6% acrylamide TBE gel). (d) rBTEB3 was mixed with unlabelled competitor (box 85/66) as indicated and incubated with 32 P-labelled box 85/66 probe at RT for 1 h before electrophoresis (6% acrylamide TBE gel). (e) rBTEB3 was mixed with the unlabelled competitors as indicated and incubated with 32 P-labelled box 85/66 probe at RT for 1 h before electrophoresis (6% acrylamide TBE gel).

-66 boxes (m85/66, 5'-CCATACGCAGGAGGGGCGCCAG-TACGCAGG-3') were used as competitors for the binding of rBTEB3 to the -118/+65 fragment of the SM22 α promoter. Both of the wild-type oligonucleotides (107/85 and 85/66) competed with the minimal SM22 α promoter, whereas the mutant oligonucleotide (m85/66) did not, suggesting that rBTEB3 binds to the SM22 α minimal promoter region through one or more of the TGGG sequences (Figure 2c). Binding of rBTEB3 to the TGGG sequence in 85/66 was confirmed by EMSA analysis and was inhibited by addition of unlabelled competitor DNA (Figure 2d). Formation of this complex could also be inhibited by the addition of 107/85 oligonucleotide but not by m85/66 (Figure 2e).

Binding studies were performed to confirm that BTEB3 produced in mammalian cells bound to the 85/86 oligonucleotide. EMSA showed that nuclear extracts from P19 cells did not contain a protein that bound to the 85/66 oligonucleotide, but that P19 cells transfected with pCBTEB3 formed a complex with this oligonucleotide (Figure 3a). This observation was consistent with the lack of BTEB3 mRNA detectable by RT-PCR in these cells (Figure 3b) and indicated that BTEB3 produced by mammalian cells bound to the oligonucleotide.

Figure 3 BTEB3 RNA and protein in P19 cells and VSMCs

(a) Nuclear extracts were prepared from VSMCs, P19 cells or P19 cells 48 h after transfection with pCBTEB3. Each extract (5 μ l) was incubated with 32 P-labelled box 85/66 oligonucleotide for 1 h in the presence (+) or absence (-) of an excess of unlabelled competitor box 85/66 oligonucleotide (CC) and analysed on a 6% acrylamide gel. (b) Expression of BTEB3 in VSMCs and P19 cells was determined by RT-PCR as described previously [11]. RT-PCR for BTEB1 was performed on the same cDNA samples in a parallel reaction to test for the quality of the cDNA from each cell type.

To determine the sequence of the binding site present in the SM22 α promoter further competition experiments were performed using oligonucleotides that only contained one of the repeated sequences. Nuclear extracts prepared from P19 cells that had been transfected with pCBTEB3 were incubated with the 85/66 probe in the presence or absence of an excess of wild-type or mutated box 85 oligonucleotides (sites 85 WT and 85 m1-m6 respectively, Figure 4). In these experiments the single-site 85 oligonucleotides were weak competitors of binding compared with the double-site 85/66 oligonucleotide as competition for binding required a 200–400-fold excess of competitor with the single-site oligonucleotides to inhibit binding, whereas the 85/66 oligonucleotide completely inhibited binding with a 50-fold excess of competitor. Furthermore, whereas the site 85 WT oligonucleotide inhibited the formation of the complex formed with 85/66 at high concentrations a small amount of a complex with a lower mobility was formed. The composition of this complex is not clear but this observation was consistent using different concentrations of oligonucleotide and over multiple repeats of the experiment. These data support the suggestion that BTEB3 binds preferentially to multiple binding sites. Competition experiments using the mutated box 85 oligonucleotides to delineate the monomer binding site showed that mutant competitors m1, m2 and m5 did not compete with the wild-type sequence for binding, whereas competitors m3, m4 and m6 inhibited binding to 85/66 (Figure 4b). These data indicate that the sequence TGGGNNNNAG is required for binding of BTEB3 to box 85.

BTEB3 activates the SM22 α promoter through a TGGG site

To examine the activation of the SM22 α promoter by BTEB3, P19 embryonic carcinoma cells were chosen because they do

Figure 4 Determination of the BTEB3-binding site

(a) Wild-type and mutant oligonucleotides used to determine the binding site for BTEB3. (b) Nuclear extracts were prepared from P19 cells 48 h after transfection with pCBTEB3; 5 μ l of each extract was incubated with labelled 85/66 oligonucleotide for 1 h in the presence of a 400-fold or 50-fold excess of the indicated oligonucleotide. Samples were analysed on a 6% acrylamide gel. The arrow indicates the position of the BTEB3-DNA complex.

Figure 5 Effect of BTEB3 on different TGGG boxes in the SM22 α promoter

(a) Mutations in the pSM22 – 118/+65 promoter. Primers were designed to incorporate the mutant sites and the SM22 α fragments amplified by PCR and ligated into pCAT-Basic. The three GC-rich boxes are highlighted in bold and the mutated bases underlined. (b) pSM22 – 118/+65 or the mutant vectors (1.5 μ g of each) were transfected with 0.5 μ g of either pcDNA3 (white bars) or pCBTEB3 (black bars) into P19 cells. CAT activity was determined 48 h after transfection and the results were normalized to protein content of the cell lysates. (c) pSM22 – 197/+65 CAT or pSM22 – 197/+65 Δ TCE CAT (1.5 μ g of each) were transfected with 0.5 μ g of either pcDNA3 (white bars) or pCBTEB3 (black bars). CAT activity was determined and normalized as above. In (b) and (c) transfections were performed in triplicate and repeated three times; data are shown as means $\pm$ S.E.M. of fold activation.

not express detectable BTEB3 mRNA or protein and therefore represent a null background (Figure 3).

To determine which of the three GC-rich boxes in the SM22 α promoter fragment were required for activation by BTEB3,

mutant forms of the SM22 α promoter were made in which each TGGG site in pSM22 – 118/+65 was inactivated by mutation to TACG, separately and in all possible combinations. The sequences of the mutant promoters are shown in Figure 5(a).

The wild-type and mutant SM22 α promoter CAT constructs were transfected into P19 cells in the presence of either pcDNA3 or pCBTEB3. BTEB3 activated the wild-type construct (pSM22 – 118/+65) 11-fold, whereas pSM22m107/85/66 containing three mutant sites was not activated, indicating that one or more of the TGGG sites are required for activation of the promoter by BTEB3 (Figure 5b). Mutation of the three TGGG sites separately indicated that pSM22m85 was not activated by BTEB3, whereas pSM22m107 and pSM22m66 were both activated by BTEB3 by 2.5- and 10-fold respectively. The double-mutant promoters showed that pSM22m107/85 (with only box 66 functional) was activated approx. 7-fold by BTEB3 whereas the basal activity of pSM22m85/66 (with only box 107 functional) was inhibited by BTEB3. The basal activity of pSM22m107/66 (only box 85 functional) was activated 25-fold by BTEB3 (Figure 5b). Taken together these data indicate that the major activation of the SM22 α promoter by BTEB3 is through box 85 whereas BTEB3 acts as an inhibitor through box 107. Activation by BTEB3 through box 66 is only observed in the absence of a functional box 107. These data also show that BTEB3 acts as an inhibitor as well as an activator, depending on the site to which it binds.

BTEB3 does not activate the TCE in the SM22 α promoter

The sequence to which BTEB3 binds is very similar to the TCE sequence that has been shown to be required to drive the expression of SM22 α in transgenic mice [8]. To determine whether BTEB3 activated gene expression through the TCE in the rat SM22 α promoter, activation of SM22 α promoter fragments that contained the wild-type (pSM22 – 197/+65 CAT) TCE or a previously described mutant TCE [8] (pSM22 – 197/+65 Δ TCE CAT) was analysed. In these experiments BTEB3 activated expression from pSM22 – 197/+65 CAT by 3-fold and pSM22 – 197/+65 Δ TCE CAT by 5-fold (Figure 5c), indicating that the TCE was not involved in the activation of the SM22 α promoter by BTEB3. These data are consistent with the observation that BTEB3 activates expression via some but not all sequences containing TGGG boxes. Furthermore, BTEB3-dependent activation of pSM22 – 197/+65 CAT by BTEB3 was not enhanced by the addition of TGF- β (results not shown), unlike the activation of the SM22 α promoter by BTEB2 [8].

BTEB3 activates the SM22 α promoter in VSMCs

The transfection studies described demonstrate that exogenous BTEB3 controls SM22 α promoter activity in P19 cells, in which BTEB3 is not normally expressed. Experiments were performed to determine whether VSMC nuclear extracts contained a protein that bound to the same 85/66 sequence as rBTEB3. Incubation of nuclear extracts from VSMCs with 85/66 produced a complex detected by EMSA (Figure 6a). No complex was formed when the same extract was incubated with labelled m85/66 oligonucleotide, indicating that the protein present in VSMCs and rBTEB3 binds to similar sequences. To determine whether the complex formed between VSMC nuclear extracts and the 85/66 oligonucleotide had a similar mobility to that formed between BTEB3 and the box 85/66 oligonucleotide, EMSA analysis was performed using nuclear extracts from control untransfected P19 cells, that is P19 cells that had been transfected with pCBTEB3 and VSMCs. The nuclear extracts from the P19 cells transfected with pCBTEB3 formed a complex with the 85/66 oligonucleotide of similar mobility to that formed between the

Figure 6 BTEB3 in VSMCs *in vitro*

(a) A nuclear extract from VSMCs (5 μ l) was incubated with 32 P-labelled box 85/66 or box m85/66 probe for 1 h and analysed on a 6% acrylamide gel. (b) Rat aortic VSMCs were transfected with 1.2 μ g of reporter plasmid and 0.4 μ g of either pcDNA3 (white bars) or pCBTEB3 (black bars). CAT activity was determined 48 h after transfection and the results were normalized to protein content of the cell lysates. Transfections were performed in triplicate and repeated three times. Data shown are means $\pm$ S.E.M. of fold activation. (c) RNA (10 μ g) extracted from the intact rat aorta (lane 1), from freshly dispersed VSMCs (lane 2) and from VSMCs that had been cultured for 10 passages (lane 3) was electrophoresed in a 1% agarose formaldehyde gel and transferred to a nylon membrane. The blot was hybridized to a 32 P-labelled BTEB3 probe washed and exposed to a Phosphorimager screen. The blot was stripped and reprobed for SM22 α mRNA as described previously [17].

VSMC nuclear extracts and the oligonucleotide (Figure 3a), consistent with the presence of BTEB3 protein in VSMCs.

To determine whether BTEB3 activated the SM22 α promoter in VSMCs, rat aortic VSMCs were transfected with the pSM22 – 118/+65 CAT or the pSM22 – 1500/+65 CAT promoter together with either BTEB3 or the control vector pcDNA3. BTEB3 activated transcription from both pSM22 – 118/+65 (3-fold) and pSM22 – 1500/+65 (3.5-fold; Figure 6b).

The selective activation of TGGG-containing sequences by BTEB3 and the observation that smooth muscle-specific genes are activated in a distinct temporal sequence raised the question of whether BTEB3 activates other smooth muscle-specific genes. VSMCs were transfected with pSM α -actin-CAT (1 kb of the rat SM α -actin promoter cloned into pCAT-Basic) and pSM-MHC-CAT (2 kb of the rabbit SM-MHC promoter cloned into pCAT-Basic [18]) in the presence or absence of pCBTEB3, both of which contain TGGG repeat sequences. BTEB3 did not activate the SM-MHC promoter and inhibited the activity of the SM α -actin promoter, indicating that promoter activation by BTEB3 is promoter-dependent (Figure 6b).

BTEB3 is expressed in VSMCs *in vitro* and *in vivo*

We have previously shown that BTEB3 is expressed in RNA extracted from the intact aorta but these data do not show that this gene is expressed in the smooth muscle cells. To confirm the expression of BTEB3 mRNA in VSMCs *in vitro*, Northern

Figure 7 Expression of SM22 α and BTEB3 mRNA in response to balloon catheter injury of the carotid artery

In situ hybridization was performed on tissue sections from both uninjured (a and b) and injured rat carotids 4 (c, d), 7 (e, f) and 14 (g, h) days post injury, as described in the Materials and methods section, for BTEB3 (a, c, e, g) and SM22 α (b, d, f, h). The intima (I), media (M) and adventitia (A) are marked.

analysis of RNA extracted from VSMCs freshly isolated from rat aorta and from cells after 10 passages was performed (Figure 6a). These data showed that BTEB3 was expressed in VSMCs derived from the rat aorta. To confirm the expression of BTEB3 in VSMCs *in vivo* and to determine whether the expression of BTEB3 was regulated in proliferating VSMCs *in vivo*, the expression of BTEB3 was determined by *in situ* hybridization in response to balloon injury of the rat carotid artery. *In situ* hybridization with antisense probes for BTEB3 and SM22 α showed that both BTEB3 and SM22 α were expressed uniformly in the media of the uninjured, left carotid artery (Figures 7a and 7b). No hybridization was detected in sections that were treated with the corresponding sense probes (results not shown). The expression of SM22 α and BTEB3 was increased in the media and adventitia of the injured artery at 4 days post-injury (Figures 7c and 7d). At 7 and 14 days post-injury, expression of both genes was maintained in the media, but was markedly higher in the intima (Figures 7e–7h). BTEB3 expression was also readily detectable in the adventitia at 7 and 14 days post injury (Figure 7h). These data show that BTEB3 is expressed in VSMCs *in vitro* and *in vivo* and that the expression of BTEB3 is regulated in response to vascular injury.

DISCUSSION

The data presented show that BTEB3 binds to three TGGG boxes in the minimal region of the SM22 α promoter. BTEB3 activates the minimal rat SM22 α promoter through box 85 and inhibits it through box 107. Box 85 is the only TGGG sequence conserved between the rat and mouse minimal promoters. These findings, together with the observation that BTEB3 is expressed in VSMCs, identify BTEB3 as a candidate regulator of SM22 α promoter activity in adult VSMCs. The readily detectable expression of BTEB3 in the VSMCs of adult rats and the inability of TGF- β to activate BTEB3 suggest that it is involved in the basal regulation of SM22 α gene expression. The closely related protein BTEB2 also activates the SM22 α promoter, but is not detectable in the normal adult aorta [10]. The activity of BTEB2 on the SM22 α promoter is modified by TGF- β [8], suggesting that BTEB2 is involved in the induced expression of SM22 α . Together these data imply a role for the BTEB subfamily of KLFs in regulating SM22 α expression.

Previous studies on BTEB3 have shown that it is able to both activate and inhibit gene expression. For example, we [11] and others [19] have shown that BTEB3 can activate a number of promoters, and the studies of Kaczynski et al. [20] have shown that BTEB3 can inhibit gene activity by binding to the transcription co-repressor mSin3A. Studies on BTEB1, another member of the BTEB subfamily of transcription factors, have suggested that BTEB1 acts as an activator of promoters with multimerized binding sites but as an inhibitor of promoters with single binding sites [21]. Transcription factors with dual actions as activators or inhibitors include other zinc-finger transcription factors; for example YY1 (yin yang 1) activates some promoters (e.g. c-Myc) [22] and inhibits others (e.g. SM α -actin and SM22 α) [13,22]. The ability of YY1 to act as an activator or inhibitor depends on the phase and orientation of the specific YY1-binding site [23,24]. Our data for BTEB3 indicate that on the SM22 α promoter its activity is also context-dependent, in that binding to either of two sites (boxes 85 and 66) stimulates transcription to different extents, whereas binding to box 107 is inhibitory.

BTEB3 is widely expressed, in contrast with SM22 α , which is restricted to cells of the smooth muscle lineage in adult animals [25]. Consequently, expression of BTEB3 alone is not sufficient for the activation of the endogenous SM22 α gene in cells that are not already competent to express SM22 α . The widespread expression of BTEB3 does not disqualify it as a regulator of a tissue-specific gene, but suggests that BTEB3 requires additional factors for this function. A requirement for accessory factors is observed for the tissue-specific activities of a number of widely expressed transcription factors. For example, SRF is a major regulator of tissue-specific gene expression in muscle cells, but is also an activator of immediate early genes in many cell types. SRF interacts with MyoD [26] and Nkx 2.5 [27] to activate skeletal muscle and cardiac muscle-specific genes respectively and also with myocardin [28]. Conversely, at the promoters of immediate early genes SRF interacts with the Ets family of transcription factors [29,30]. Other examples of transcription factors that are widely expressed, yet activate tissue-specific genes, include the C2H2 zinc-finger transcription factor Sp1, a protein closely related to BTEB3. This factor appears to be important in the control of expression of both cardiac and skeletal muscle-specific genes and in skeletal muscle Sp1 has been shown to form a complex with MyoD [31]. It is therefore likely that BTEB3 activates the SM22 α promoter in smooth muscle cells in combination with other transcription factors and accessory factors. However, the specific combination of factors that leads to tissue restriction of SM22 α gene expression remains to be determined.

Whereas BTEB3 was capable of activating the SM22 α promoter in VSMCs as well as P19 cells, it is of interest that it did not activate either a 2-kb fragment of the SM-MHC promoter or a 1-kb fragment of the SM α -actin promoter in VSMCs. Both of these promoters contain a number of TGGG sequences, and Sp/xKLF transcription factors have been implicated in activating them [32–34]. It is possible that the inability of BTEB3 to activate these promoters is due to a lack of intronic sequences that have been shown to be required for smooth muscle-specific activity of these promoters *in vivo*. However, the transcription factor programmes that regulate the activity of these smooth muscle-specific promoters are likely to be similar but distinct because each promoter is activated in a distinct temporal pattern [1]. We therefore speculate that BTEB3 is involved in the temporal regulation of SM22 α expression rather than its restriction to smooth muscle cells.

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