Functional analysis of basic transcription element (BTE)-binding protein (BTEB) 3 and BTEB4, a novel Sp1-like protein, reveals a subfamily of transcriptional repressors for the BTE site of the *cytochrome P4501A1* gene promoter

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The Sp1-like family of transcription factors is emerging as an integral part of the cellular machinery involved in the control of gene expression. Members of this family of proteins contain three highly homologous C-terminal zinc-finger motifs that bind GC-rich sequences found in the promoters of a diverse number of genes, such as the basic transcription element (BTE) in the promoter of the carcinogen-metabolizing *cytochrome P4501A1* (*CYP1A1*) gene. In the present study, we report the molecular and functional characterization of BTE-binding protein (BTEB) 4, a novel ubiquitously expressed member of the Sp1-like proteins family. This protein represents a new homologue of BTEB1, originally described as a regulator of the BTE site in the *CYP1A1* gene promoter. Similarly to the recently described BTEB3, we demonstrate that the N-terminal region of BTEB4 directly

represses transcription and binds the co-repressor mSin3A. In addition, we show that the C-terminal zinc-finger domain of BTEB4 binds specifically the BTE site of the *CYP1A1* promoter, similar to BTEB1 and BTEB3. Also, we show that both BTEB3 and BTEB4 repress the *CYP1A1* gene promoter via the BTE site in HepG2 and BxPC3 cells. Thus the identification of this protein expands the repertoire of BTEB-like members of the Sp1-like protein family involved in transcriptional repression. Furthermore, our results demonstrate that the BTEB subfamily can repress the *CYP1A1* gene promoter via the BTE site.

Key words: carcinogen metabolism, GC-rich promoter, mSin3A, repression, zinc-finger protein.

INTRODUCTION

The Sp1-like family of transcription factors has been shown to regulate many cellular functions through the regulation of genes driven by GC-rich promoters [1–4]. The ability of Sp1-like proteins to bind these promoters is conferred by a C-terminal DNA-binding domain consisting of three highly conserved Cys₂His₂ zinc-finger motifs. The N-terminal domains of Sp1-like proteins, on the other hand, vary greatly between individual members and either activate or repress transcription. Thus Sp1-like proteins have the potential to bind similar promoters and to differentially regulate the expression of various target genes, which raises the question of how cells use this group of proteins to regulate gene expression in a tissue- and promoter-specific manner. Therefore functional studies on the activity of Sp1-like proteins in diverse tissue and promoter contexts are necessary to differentiate their unique cellular functions.

One well-characterized target site for Sp1-like proteins is the basic transcription element (BTE), which, in part, controls expression of the *cytochrome P4501A1* (*CYP1A1*) gene, an

enzyme that plays a well-documented role in the metabolism and activation of several carcinogens, such as dibenzo[*a,h*]pyrene [5–7]. The BTE site seems to play a modulatory role in *CYP1A1* expression and deletion of the BTE site not only leads to a decrease in constitutive expression, but also to a significant reduction of promoter activity in response to hydrocarbons, such as 2,3,6,7-tetrachlorodibenzo-*p*-dioxin (TCDD) or 3-methylcholanthrene (3-MC) [8]. A discrete number of Sp1-like proteins have been shown to regulate the *CYP1A1* promoter via the BTE site, including Sp1, BTE-binding protein (BTEB) 1 and gut-enriched Kruppel-like factor (GKLF) [9–13]. Sp1, for example, participates in activation of the promoter, whereas BTEB1 and GKLF have been shown to behave as transcriptional repressors [9,14,15]. Thus it is hoped that investigating transcription factors that bind the BTE site and potentially regulate *CYP1A1* expression will provide much needed insight into the regulation of carcinogen metabolism as well as reveal novel mechanisms for the regulation and function of the *CYP1A1* promoter.

We [11] and others [16–18] have recently reported the functional characterization of BTEB3, a new member of the BTEB

Abbreviations used: 3-MC, 3-methylcholanthrene; AhR, aryl-hydrocarbon receptor; Arnt, arylhydrocarbon nuclear translocator; BTE, basic transcription element; BTEB, BTE-binding protein; CHO, Chinese-hamster ovary; CYP1A1, cytochrome P4501A1, FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; GAL4 DBD, GAL4 DNA-binding domain; GKLF, gut-enriched Kruppel-like factor; GST, glutathione S-transferase; HA, haemagglutinin; RSV, Rous sarcoma virus; TCDD, 2,3,6,7-tetrachlorodibenzo-*p*-dioxin; TIEG(2), transforming-growth-factor- β -inducible early gene (2); XRE, xenobiotic-response element.

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subfamily of Sp1-like proteins, which can either activate the simian virus 40 ('SV40') [10,18], RANTES (regulated upon activation, normal T-cell expressed and secreted) [16] and γ -globin promoters [17] or repress promoters containing the CYP1A1 BTE site [11]. Sequence comparisons showed that BTEB3 is highly related to BTEB1 and both proteins bind the BTE sites with an affinity similar to Sp1 [11,12]. In addition, we found that BTEB3 represses BTE-dependent transcription via mechanisms involving competition for DNA binding with the activator Sp1 and recruitment of the mSin3A-histone deacetylase co-repressor complex [11]. In the present study, we have pursued the identification of novel BTE-binding proteins with the aim of understanding better the mechanisms involved in CYP1A1 promoter regulation. As a result of these studies, we report the molecular and biochemical characterization of BTEB4, a novel Sp1-like transcriptional repressor that is significantly related to BTEB1 and BTEB3. Biochemical studies reveal that BTEB4 behaves as a BTE-binding transcriptional repressor, and reporter assays demonstrate that, in contrast with Sp1, both BTEB3 and BTEB4 repress the CYP1A1 promoter via the BTE site, as reported previously for BTEB1 [10]. In conclusion, the identification of this protein expands the repertoire of BTEB-related Sp1-like transcription factors and suggests that this group of proteins may have evolved, at least in part, to balance the activating function of Sp1 in the induction of the CYP1A1 promoter.

MATERIALS AND METHODS

Plasmid construction

Using standard molecular biology protocols, the full-length *BTEB4* gene was cloned in-frame with the His/Xpress epitope of the pcDNA3.1/His vector and the Asp-Tyr-Lys-Asp-Asp-Asp-Lys (FLAG) epitope of the pCMVtag2 vector (Invitrogen, Carlsbad, CA, U.S.A.). N-terminal (amino acids 1–128) and C-terminal (amino acids 214–252) deletions of *BTEB4* were cloned in-frame with the GAL4 DNA-binding domain (GAL4 DBD) of the pM vector (ClonTech, Palo Alto, CA, U.S.A.) as indicated. The zinc-finger (amino acids 118–252) and N-terminal domains of *BTEB4* were cloned into the pGEX vector (Amersham Biosciences, Piscataway, NJ, U.S.A.) for expression as a glutathione S-transferase (GST) fusion protein. For reporter assays, we used the previously described 5 $\times$ GAL4, pBTE₆, pBTE₀ and Rous sarcoma virus (RSV)–*Renilla* reporters [11]. Wild-type and BTE-deleted mutant rat CYP1A1 promoters were obtained from Dr Yoshiaki Fujii-Kuriyama (Department of Chemistry, Tohoku University, Sendai, Japan) and subcloned into the pGL3 basic firefly luciferase vector (Promega, Madison, WI, U.S.A.). The BTEB3 pcDNA3.1/His and FLAG-tagged constructs have been described previously [11]. Sp1 was cloned in-frame with the pcDNA3.1/His construct. All constructs were verified by sequencing.

Cell culture and reporter assays

Chinese-hamster ovary (CHO) cells were cultured as described previously [11,19]. HepG2 and BxPC3 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and were cultured in α -minimal essential medium (MEM) and RPMI 1640 respectively, supplemented with 5% (v/v) foetal bovine serum, 5% (v/v) normal calf serum, 100 units/ml streptomycin and 100 units/ml penicillin (Life Technologies, Rockville, MD, U.S.A.). GAL4 and pBTE reporter assays were performed as described previously [11,20,21]. Briefly, CHO cells were plated in 24-well culture dishes and co-transfected with various *BTEB4* expression vectors along with GAL4 or pBTE reporter plasmids

using LIPOFECTAMINE™ (Life Technologies) according to manufacturer's instructions. Basal transcriptional activity was determined using the reporter constructs co-transfected with the GAL4 or the pcDNA3.1/HisC parental vectors. The RSV–*Renilla* reporter was added to each transfection as an indicator of transfection efficiency. Reporter activity was measured using the Dual Luciferase Kit (Promega). For GAL4 assays, relative luciferase activity was determined as a ratio of the GAL4 reporter activity to RSV–*Renilla* activity. For BTE assays, the relative luciferase activity was expressed as a ratio between the RSV–*Renilla*-normalized activities of pBTE₆ to pBTE₀ reporters. HepG2 and BxPC3 cells were plated in 24-well culture dishes and transfected with full-length *BTEB*, *BTEB3* or *Sp1*, the CYP1A1 promoter luciferase plasmids and RSV–*Renilla* using FuGene (Roche, Indianapolis, IN, U.S.A.), according to manufacturer's suggestions. Control experiments were performed using the parental pcDNA3.1/His vector. Transfected cells were treated with various concentrations of TCDD (Wellington Laboratories, Guelph, ON, Canada) or 3-MC (Sigma–Aldrich, St. Louis, MO, U.S.A.) solubilized in DMSO to induce the CYP1A1 promoter. Control cells were treated with an equal volume of DMSO alone. Luciferase activity was measured as above and relative luciferase activity was determined as the ratio of CYP1A1 reporter activity normalized to RSV–*Renilla* values. All studies were performed in triplicate in at least three independent experiments with similar results. Error bars indicate S.D. Statistical significance was determined using squared *t* test analysis.

GST pull-down, immunoprecipitation and Western blot

GST pull-down assays and anti-(FLAG) antibody immunoprecipitations were performed as described previously [11,19]. Briefly, the GST fusion protein expressing the *BTEB4* N-terminus used for these experiments was generated in isopropyl β -D-thiogalactoside ('IPTG')-induced BL21 cells and purified using glutathione-S-Sepharose chromatography, according to manufacturer's instructions (Promega). CHO cell extracts were prepared using a lysis buffer described previously [11,19] and incubated with either the GST–N-terminal fusion protein or GST alone. Complexes were isolated and Western-blot analysis was performed using polyclonal anti-mSin3A antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) as described previously [19]. For immunoprecipitation, CHO cells were transfected with FLAG-tagged *BTEB4* (FLAG–*BTEB4*), lysed, and incubated with agarose-conjugated anti-(FLAG) antibody (Sigma–Aldrich). The resulting complexes were analysed by Western-blot analysis as for GST pull-down assays.

Chromatin immunoprecipitation

HepG2 cells were transfected with *BTEB4* and *BTEB3* full-length FLAG-tagged plasmids using the BTX Electro square porator T820 (Genetronic Biomedical, San Diego, CA, U.S.A.) according to manufacturer's instructions. At 24 h post-transfection, cells were cross-linked with formaldehyde for 20 min at 25 °C, harvested in SDS-lysis buffer (Upstate Biotechnology, Lake Placid, NY, U.S.A.), and sheared to fragment DNA to approx. 500 bp. Samples were then immunoprecipitated using an agarose-conjugated anti-(FLAG) antibody (Sigma–Aldrich) or agarose beads alone at 4 °C overnight. Following immunoprecipitation, samples were washed and eluted using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) according to manufacturer's instructions. Cross-links were removed at 65 °C for 4 h and immunoprecipitated DNA was purified using phenol/chloroform extraction (500 μ l) and ethanol precipitation.

A 249 bp region of the CYP1A1 promoter containing the BTE site was detected in immunoprecipitated samples by PCR using 5'-TCCGCCACCTTTCTCTCCAATC-3' (forward) and 5'-AAGTCCCCCAGCAACTCACCTGA-3' (reverse) primers. Prior to PCR, the forward primer was end-labelled using T4 kinase and [γ - 32 P]ATP. PCR products were separated by non-denaturing PAGE and visualized using the Storm 860 Imager system (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Gel-shift assays were performed as described previously [11,22]. Briefly, a double-stranded DNA probe containing the BTE element (5'-AGCTTGAGAAGGAGGCGTGGCCAACGCA-TG-3') was end-labelled with [γ - 32 P]ATP and incubated with equal amounts of GST or a GST-BTEB4-zinc-finger fusion protein, followed by non-denaturing gel electrophoresis. The mutant BTE probe has the underlined bases mutated to TT. Where indicated, an excess of unlabelled wild-type BTE probe, unlabelled activator protein 1 (AP1) probe, unlabelled mutant

Northern blots of RNA from various human tissues (ClonTech) were probed as described previously [22] using a BTEB4 cDNA probe. The blots were stripped and rehybridized using a probe for β -actin. The subcellular localization of BTEB4 was determined essentially as described previously [22,23]. Briefly, XpressTM-tagged BTEB4 was transfected into HepG2 cells plated on poly-L-lysine-coated coverslips. The localization of XpressTM-tagged BTEB4 after treatment in the absence or presence of TCDD was performed using an anti-(XpressTM) antibody (Invitrogen) and an FITC-conjugated secondary antibody (Molecular Probes, Portland, OR, U.S.A.). To localize the nuclei, cells were

counterstained with Hoechst. Cells were observed with a Zeiss LSM-510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

BTEB4 is a novel widely expressed Sp1-like protein

Evidence for the existence of a novel Sp1-like zinc-finger-encoding cDNA was gathered during the process of comparing our transforming-growth-factor- β -inducible early gene 2 (TIEG2) sequence published previously [22] against the I.M.A.G.E. Washington University expressed sequence tag ('EST') database using the BLAST software. We subsequently cloned the full-length cDNA, and sequence comparisons revealed that it was highly homologous to both BTEB1 and BTEB3. Thus we called the novel Sp1-like gene *BTEB4*. The full-length BTEB4 cDNA encoded a protein containing three C-terminal zinc-finger domains, with approx. 80% similarity to both BTEB3 and BTEB1 (Figures 1A and 1C). In addition, the N-terminus of BTEB4 contained motifs that were similar to the R1 transcriptional repressor domains found in both BTEB1 and BTEB3 [11,19]. The total predicted size of BTEB4 was slightly smaller than that of BTEB3, but larger than BTEB1. The region located between the R1 domain and the zinc fingers was the most variable in sequence among the three proteins. However, the total amino acid composition was similar, with a predominance of proline and alanine residues. Similar to the other BTEB proteins, BTEB4 also contained a putative nuclear localization site immediately upstream of the zinc-finger domain [11]. Northern-blot analysis in Figure 1(B) shows that, like many other members of the Sp1-like family, BTEB4 was expressed to a varying degree in a number of human tissues, although it was enriched in liver, where the levels of the other BTEBs and the TIEG transcripts are very low [16,22,24,25]. Thus BTEB4 represents the third member of this subfamily of transcription factors to be identified.

BTEB4 N-terminus is a potent repressor domain that interacts with mSin3A

To characterize the transcriptional regulatory activity of BTEB4, we used the well-described GAL4-based reporter system [11,20,21]. Mutants containing N-terminal or C-terminal regions of BTEB4 (Figure 2A) were independently cloned in-frame with GAL4 DBD and co-transfected into CHO cells together with a GAL4 reporter plasmid, containing a thymidine kinase basal promoter, and five tandem GAL4 binding sites. Basal levels of GAL4 activity were determined by co-transfection with the parental GAL4 DBD vector, which exhibits some transactivation of the GAL4 reporter plasmid. Figure 2(B) shows that the BTEB4 N-terminus repressed reporter activity approx. 50-fold compared with GAL4 DBD alone [8.4 ± 0.20 (GAL4 DBD) versus 0.17 ± 0.005 (BTEB4 N-terminus)]. The C-terminus, on the other hand, displayed only 1.5-fold repression activity (5.7 ± 0.5). A control Western-blot analysis showed that both BTEB4 GAL4 constructs were expressed at similar or higher levels than GAL4 DBD (Figure 2B, inset). In addition, control gel-shift assays using a GAL4 probe showed that each construct bound DNA in a similar manner (results not shown). We conclude that, compared with the potent repression activity of the N-terminus, the C-terminus of BTEB4 exhibits negligible repression. Thus this study demonstrates that the N-terminus of BTEB4 behaves as a potent transcriptional repressor.

The sequence analysis shown in Figure 1(C) shows that BTEB4 contains a 26 amino acid domain (boxed sequence) that is highly

Figure 2 Transcriptional regulatory activity of BTEB4

(A) N-terminus (N-term) and C-terminus (C-term) of BTEB4 were fused to the GAL4 DBD and used in a GAL4 assay as described in the Materials and methods section. (B) Relative luciferase activity using the GAL4 reporter construct with GAL4 DBD or the deletion constructs of BTEB4. Results from a representative experiment are plotted. Note that the N-terminal region (N-term) of BTEB4 displays approx. 50-fold transcriptional repressor activity, whereas the C-terminal region (C-term) displays only 1.5-fold transcriptional regulatory activity over the GAL4 DBD control. * $P = 0.0001$ and ** $P = 0.001$ compared with the GAL4 DBD control. The inset shows a Western-blot analysis of the expression of the GAL4 constructs. Note that all GAL4 constructs were expressed at levels similar to or higher than GAL4 DBD. (C) Pull-down assays were performed using CHO cell lysates and GST alone or a GST fusion protein expressing the BTEB4 N-terminus (N-term), followed by Western-blot analysis using antibodies against mSin3A. Note that mSin3A is detected in complexes pulled-down with the BTEB4 N-terminus, but not GST alone. (D) CHO cells were transfected with FLAG-BTEB4 or the parental vector (control) and subjected to immunoprecipitation using the anti-(FLAG) antibody. The immunocomplexes were analysed by Western-blot analysis using antibodies against mSin3A. Note that mSin3A is present in the immunocomplexes from cells transfected with FLAG-BTEB4, but not in the control.

similar to repression domains of BTEB1 and BTEB3, proteins shown recently to repress transcription by recruiting mSin3A via the paired amphipathic helix 2 ('PAH2') domain [11,19]. Thus we decided to test whether BTEB4 does indeed interact with mSin3A by performing pull-down experiments from CHO cell lysates using the N-terminus of BTEB4 cloned as a GST fusion protein. Western-blot analysis of the pull-down complexes demonstrated that the N-terminus of BTEB4, which exhibits potent transcriptional repression activity, specifically interacted with mSin3A *in vitro* (Figure 2C). As a control, GST alone did not pull-down mSin3A. Subsequently, we transfected full-length FLAG-BTEB4 into CHO cells and performed anti-(FLAG) antibody immunoprecipitations. The presence of mSin3A in the immunocomplex was detected by Western-blot analysis in cells transfected with FLAG-BTEB4, but not in cells transfected with the parental vector (control) (Figure 2D). Together, these results

Figure 3 BTEB4 binds the BTE box in a sequence-specific manner

(A) Gel-shift assays with a probe containing either the wild-type BTE site (wt BTE; lanes 1–7) or a mutated BTE site (mut BTE; lanes 8 and 9) with 200 ng of GST protein (lane 2), 200 ng of GST–BTEB4 ZF (BTEB4 ZF; lanes 3–7 and 9) or probe alone (lanes 1 and 8). Where indicated, the following were added to the binding reactions: 500-fold molar excess unlabelled BTE probe (cold wt BTE; lane 4); 500-fold excess unlabelled non-competitor probe (cold AP1; lane 5); 250 ng of anti-(GST) antibody (αGST; lane 6); 250 ng of anti-HA antibody (αHA; lane 7). The specific complexes that form between BTEB4 ZF and the probe are indicated on the right. Note that, although the anti-(GST) antibody shifted the BTEB4–BTE complex (lane 6), the anti-HA antibody (concentration matched) did not (lane 7). Also note that the addition of an excess of unlabelled wild-type BTE probe competed for the binding (lane 4), whereas the addition of the unrelated AP1 probe did not (lane 5). In addition, BTEB4 ZF did not shift the mutant BTE probe (lane 9). (B) Full-length BTEB4 (BTEB4 FL) was generated by *in vitro* translation and used in a gel-shift assay along with the wild-type BTE probe (wt BTE; lanes 3–7) or a mutant BTE probe (mut BTE, lanes 8 and 9). Where indicated, the following were added to the binding reactions: 50-fold molar excess of unlabelled BTE probe (cold wt BTE; lane 4), 50-fold excess of unlabelled mutant BTE probe (cold mut BTE; lane 5), anti-His antibody (from Santa Cruz Biotechnology; αHis; lane 6) and IgG (lane 7). The specific complexes are indicated on the right. Note that anti-His antibody (lane 6), but not IgG (lane 7), supershifted the BTE–BTEB4 FL complex. Also note that an excess of unlabelled BTE probe competed for the binding (lane 4), whereas mutant BTE probe did not (lane 5). Also note that BTEB4 FL does not shift the mutant BTE probe (lane 9). Control *in vitro* translated proteins using no DNA template (lane 1) or the parental pcDNA vector (lane 2 and 8) show no significant BTE-binding activity. (C) HepG2 cells were transfected with FLAG–BTEB4 and FLAG–BTEB3 constructs and subjected to chromatin immunoprecipitation as described in the Materials and methods section. A 249 bp fragment of the CYP1A1 promoter containing the BTE site was amplified by PCR from anti-(FLAG) or mock-immunoprecipitated DNA samples. Note that the CYP1A1 promoter is amplified from anti-(FLAG) immunoprecipitated samples (αFLAG), but not from mock-immunoprecipitated samples (mock). As a control, the input (INP) shows the presence of the CYP1A1 promoter prior to immunoprecipitation.

indicate that BTEB4 can interact with mSin3A in mammalian cells through its N-terminus.

BTEB4 specifically binds the BTE site and represses a BTE-containing promoter

Next, we investigated whether, similarly to BTEB1 and BTEB3, BTEB4 also binds to the BTE site from the CYP1A1 promoter [10,11]. We performed gel-shift assays using the three zinc-finger motifs of BTEB4 cloned as a GST fusion protein (GST–BTEB4 ZF) and a DNA probe containing the BTE site. Figure 3(A) shows that BTEB4 ZF (Figure 3A, lane 3), but not GST alone

(Figure 3A, lane 2) bound and shifted the BTE probe. The interaction between GST–BTEB4 ZF and the BTE probe was specific as indicated by the fact that an excess of unlabelled BTE probe (Figure 3A, lane 4), but not AP1 (Figure 3A, lane 5), competed with the radiolabelled BTE probe. Furthermore, GST–BTEB4 ZF did not bind to a mutated BTE probe (Figure 3A, lane 9). We also tested the specificity of the complex using an anti-(GST) antibody, which resulted in a super shift of the GST–BTEB4 ZF–BTE complex (Figure 3A, lane 6). The non-specific anti-HA antibody of the same immunoglobulin class as the anti-(GST) antibody did not super-shift the GST–BTEB4 ZF–BTE complex (Figure 3A, lane 7). In addition, we performed

Figure 4 BTEB4 represses a heterologous BTE promoter

(A) Schematic representation of the pBTE heterologous reporter plasmids and BTEB4 expression plasmid. FL BTEB4, full-length BTEB4. (B) Increasing amounts of full-length BTEB4 were co-transfected into CHO cells together with pBTE₆ or pBTE₀ reporter plasmids as described in the Materials and methods section. Transcriptional activity specific to the BTE sites was determined by calculating the ratio of pBTE₆/pBTE₀ luciferase activities after normalization to RSV-*Renilla*. Basal transcription activity was measured in cells transfected with the parental pcDNA3.1/His vector. Note that BTEB4 repressed transcription of the BTE heterologous promoter in a dose-dependent manner. * $P \leq 0.0004$ compared with control. To control for expression of BTEB4, a Western blot was performed using the anti-His (α His) antibody, demonstrating that BTEB4 was overexpressed in increasing amounts. (C) The BTE reporter assay was performed as above, with the addition of an Sp1 expression construct as indicated. Note that Sp1 relieved the repression mediated by BTEB4 compared with BTEB4 alone (* $P \leq 0.005$).

the gel-shift assay using *in vitro* translated full-length BTEB4 protein. Figure 3(B) shows that, similar to the findings with the zinc-finger domain alone, full-length BTEB4 specifically bound the BTE site from the CYP1A1 promoter. These data suggest that BTEB4 can bind the BTE site *in vitro*, similar to other Sp1-like proteins, including Sp1 and BTEB3 [5,11]. To test whether

both BTEB4 and BTEB3 can bind the BTE site *in vivo*, we performed chromatin immunoprecipitation assays. For this purpose, HepG2 cells were transfected with FLAG-BTEB4 and FLAG-BTEB3 constructs, cross-linked with formaldehyde, and subjected to immunoprecipitation using the anti-(FLAG) antibody. DNA was then isolated and the CYP1A1 promoter in the region of the BTE site was amplified using PCR. Figure 3(C) shows that the CYP1A1 promoter was amplified in DNA samples obtained from BTEB4- and BTEB3-transfected cells when immunoprecipitated using the anti-(FLAG) antibody. No promoter was detected in mock-immunoprecipitated samples. In conclusion, the novel Sp1-like transcription factor, BTEB4, and BTEB3 can bind the endogenous CYP1A1 promoter in HepG2 cells *in vivo*.

We next tested whether BTEB4 regulates transcription from a BTE-containing promoter. CHO cells were co-transfected with full-length BTEB4 and the heterologous pBTE₆ or pBTE₀ luciferase reporter plasmids, an approach used previously [9, 11,22,23] to characterize the transcriptional activity of several Sp1-like proteins (Figure 4A). The reporter activity specifically corresponding to the BTE sites was calculated from the ratio of values obtained with the pBTE₆ and pBTE₀ reporters (pBTE₆/pBTE₀). Figure 4(B) shows that, as BTEB4 was expressed in increasing amounts, the heterologous BTE promoter was repressed in a dose-dependent fashion. The repression activity at the highest level of BTEB4 expression was approx. 10-fold [1.15 ± 0.21 (full-length BTEB4) versus 17.5 ± 2.82 (control)]. We have shown previously [11] that BTEB3 can compete with Sp1 for DNA binding and thus repress transcription of a heterologous BTE reporter plasmid. We then tested the ability of BTEB4 to repress transcription in the presence of exogenous Sp1 expression. Figure 4(C) shows that the expression of exogenous Sp1 relieved the repression mediated by BTEB4 in a dose-dependent manner. These data suggest that BTEB4 can regulate expression of a promoter containing the BTE site and expression of the activator Sp1 relieves the repression mediated by BTEB4.

BTEB3 and BTEB4 repress the CYP1A1 promoter via the BTE site

The ability of BTEB4 to repress the BTE-containing CYP1A1 promoter was then tested. For this purpose, we co-transfected HepG2 cells with full-length BTEB4 and either the wild-type or BTE-deleted mutant CYP1A1 promoter reporters (Figure 5A). Following transfection, the cells were induced with 10 nM TCDD prior to luciferase assay. Figure 5(B) shows that BTEB4 reduced TCDD-induced wild-type CYP1A1 promoter activity in a dose-dependent fashion. At maximal BTEB4 expression, the wild-type CYP1A1 promoter activity induced by 10 nM TCDD was reduced by approx. 50% [85 ± 8.6 (control) versus 40 ± 4.4 (BTEB4)]. As shown previously [5], the induction of the BTE-deleted mutant CYP1A1 promoter was reduced compared with the wild-type [85 ± 8.6 (wild type) versus 41 ± 5.9 (mutant)]. Interestingly, BTEB4 expression had no statistically significant effect on the BTE-deleted mutant promoter [40 ± 5.9 (control) versus 26 ± 1.6 (BTEB4)]. We also tested the ability of BTEB4 to inhibit the CYP1A1 promoter induction in the pancreatic BxPC3 cell line. Similarly, BTEB4 reduced TCDD-mediated induction of the wild-type CYP1A1 promoter in a dose-dependent fashion, but had no effect on the BTE-deleted mutant promoter (Figure 5C). In parallel experiments, we tested whether both BTEB3 and BTEB4 reduced the induction of the CYP1A1 promoter by another hydrocarbon 3-MC. Indeed, similar to TCDD induction, the activity of the 3-MC-induced wild-type CYP1A1 promoter was reduced by the expression of both BTEB3 and BTEB4

Figure 5 BTEB4 and BTEB3 reduce CYP1A1 promoter induction via the BTE site

(A) Schematic representation of the wild-type and BTE-deleted mutant CYP1A1 promoter-containing luciferase reporters. (B) HepG2 cells were co-transfected with the wild-type or BTE-deleted CYP1A1 promoter reporters and increasing amounts of BTEB4, followed by treatment with 10 nM TCDD. Note that BTEB4 reduced the induction of wild-type CYP1A1 in a dose-dependent manner compared with control ($*P \leq 0.02$). Also note that BTEB4 had no significant effect on the BTE-deleted promoter compared with control ($**P \geq 0.09$). (C) BxPC3 cells were co-transfected with the CYP1A1 reporter constructs together with increasing amounts of BTEB4, followed by TCDD (10 nM) treatment. Note that BTEB4 reduced the induction of the CYP1A1 wild-type promoter ($*P \leq 0.05$), but not the BTE-deleted mutant ($**P \geq 0.21$) compared with control. (D) HepG2 cells were transfected with wild-type and BTE-deleted CYP1A1 constructs and BTEB3 and BTEB4 expression vectors, followed by treatment with 1 μ M 3-MC. Note that both BTEB3 and BTEB4 reduced the induction of the CYP1A1 wild-type reporter ($*P \leq 0.02$) compared with control. Also note that the activity of the BTE-deleted promoter was unaffected by BTEB3 and BTEB4 expression ($**P \geq 0.16$).

(Figure 5D). In addition, the expression of both proteins had no effect on the BTE-deleted CYP1A1 promoter. Thus repression by BTEB3 and BTEB4 can reduce, via the BTE site, the maximal levels of activity of the CYP1A1 promoter following xenobiotic induction in a variety of cells and under a variety of inducing agents.

To determine whether the BTEB4-dependent decrease in the induction of the CYP1A1 promoter is related to the dose of the inducer used, we performed additional reporter assays using a range of TCDD concentrations. Figure 6(A) shows that BTEB4 was able to inhibit CYP1A1 induction by approx. 50% across a range of TCDD concentrations in HepG2 cells (left panel). As a control, the BTE-deleted promoter, which exhibited less induction than the wild-type, was unaffected by BTEB4 expression (right panel). We also performed the reporter assay under basal conditions with no inducer present. Figure 6(B) shows that both BTEB3 and BTEB4 similarly inhibited the wild-type CYP1A1 promoter under basal conditions in HepG2 cells. In addition, the reporter activity of the BTE-deleted promoter was unaffected by expression of either gene, suggesting that the BTE site was vital

to this phenomenon. Subsequently, we performed subcellular localization studies to determine whether TCDD regulated the nuclear translocation of BTEB4, as has been shown previously [6] for the aryl-hydrocarbon receptor (AhR). Figure 6(C) shows that BTEB4 was localized to the nucleus under both basal and TCDD-inducing conditions. These results suggest that BTEB4 is expressed constitutively in the nucleus under these conditions.

We have shown in Figure 4 that Sp1 relieved BTEB4-mediated repression of a heterologous BTE reporter plasmid, similar to that shown previously for BTEB3 [11]. To test whether a similar mechanism occurred on the CYP1A1 promoter, we performed additional reporter assays in the presence of exogenous Sp1. Indeed, Figure 7 shows that Sp1 relieved both BTEB4- and BTEB3-mediated repression of the CYP1A1 promoter in a dose-dependent manner under basal conditions or 3-MC induction in HepG2 cells. Together, our data suggest that BTEB3 and BTEB4 inhibit transcription not only from a synthetic promoter containing a multimerized BTE site, but also within the context of a naturally occurring promoter, such as that of CYP1A1. Furthermore, the inhibition of the CYP1A1 promoter through the

Figure 6 BTEB3 and BTEB4 repress basal CYP1A1 promoter via the BTE site

(A) HepG2 cells were co-transfected with the wild type CYP1A1 promoter reporter and BTEB4, followed by treatment with increasing amounts of TCDD. Note that BTEB4 reduces the induction of wild type CYP1A1 under all TCDD concentrations tested ($P \leq 0.02$) compared with control. (B) HepG2 cells were co-transfected with the mutant CYP1A1 promoter reporter and BTEB4, followed by treatment with increasing amounts of TCDD. Note that BTEB4 has little or no effect on the BTE-deleted promoter under the various TCDD concentrations ($P \geq 0.12$) compared with control. (C) HepG2 cells were co-transfected with the CYP1A1 reporter constructs along with BTEB4 and BTEB3. No hydrocarbons were used to induce the CYP1A1 promoter. Note that BTEB3 and BTEB4 reduce basal activity of CYP1A1 wild-type promoter ($P \leq 0.02$), but not the BTE-deleted mutant ($P \geq 0.34$) compared with control. (D) HepG2 cells were transfected with a Xpress™ epitope-tagged vector carrying the full-length BTEB4 gene. Where indicated, cells were treated with 10 nM TCDD for 24 h. Detection of BTEB4 was performed using an anti-Xpress antibody as described in the Materials and methods section. Note that BTEB4 is localized to the nucleus and that TCDD does not change the subcellular localization of BTEB4. As a control for nuclear localization, cells were counter-stained with Hoechst.

BTE site occurs both under basal and hydrocarbon-induced conditions in a variety of cell types.

DISCUSSION

The induction of the CYP1A1 promoter by various hydrocarbons via the xenobiotic-response elements (XRE) has led to the identification and extensive characterization of the AhR and arylhydrocarbon nuclear translocator (Arnt) transcriptional activators [6]. In addition, deletion of the BTE site in the CYP1A1 promoter, a target of Sp1 and Sp1-like proteins, not only leads to a significant loss of constitutive expression, but also to a significant reduction in the inducibility of the promoter [8]. Thus both types of *cis*-regulatory elements appear to be necessary to achieve the highest levels of CYP1A1 necessary for proper xenobiotic metabolism. Unfortunately, in contrast with the extensive characterization of the XRE/AhR/Arnt pathway, little is known about the molecular machinery that can bind and regulate the BTE site. In the present study, we have characterized BTEB4, a novel BTE-binding transcriptional repressor. We show that BTEB4 is highly homologous both structurally and func-

tionally, to BTEB1 and the recently characterized BTEB3, which justifies the inclusion of these three genes in the same subfamily of Sp1-like proteins. Interestingly, the brain-enriched gene dopamine receptor-regulating factor ('DRRF') has recently been described and appears to be the murine orthologue of BTEB4 [26]. Since we found that BTEB4 expression in human tissues is also enriched in the brain, it is possible that it may also have an important role in dopamine receptor expression in addition to the regulation of the BTE-containing CYP1A1 promoter, although this hypothesis remains to be tested.

BTEB1 and BTEB3 have been described previously as activators of certain promoters, such as the simian virus 40 [10,18], RANTES [16] or γ -globin [17] promoters, and it is likely that BTEB4 may also act as an activator in various promoter contexts. However, our data strongly support a repressor role for both BTEB3 and BTEB4. First, using the GAL4 assay, we show that, similar to BTEB3, the BTEB4 N-terminus is a potent transcriptional repression domain. Secondly, GST pull-down and immunoprecipitation assays show that BTEB4 can interact with the co-repressor mSin3A *in vitro* and in intact cells, similar to BTEB3. Thirdly, reporter assays in HepG2 and BxPC3 cells

Figure 7 Sp1 relieves the repression mediated by BTEB4 and BTEB3

(A) HepG2 cells were transfected with the CYP1A1 wild-type reporter construct along with BTEB4, BTEB3 and Sp1 expression plasmids as indicated. No hydrocarbons were used to induce the promoter. Note that Sp1 relieves the repression mediated by both BTEB4 and BTEB3 ($*P \leq 0.03$) compared with BTEB4 or BTEB3 alone. (B) HepG2 cells were transfected with the wild-type CYP1A1 reporter construct and BTEB4, BTEB3 and Sp1 expression plasmids as indicated, followed by 1 μ M 3-MC treatment. Note that Sp1 decreases the ability of BTEB4 and BTEB3 to reduce the induction of the CYP1A1 promoter ($*P \leq 0.01$) compared with BTEB4 or BTEB3 alone.

demonstrate that, in contrast with Sp1, both BTEB3 and BTEB4 can repress the TCDD- and 3-MC-induced CYP1A1 promoter activity via the BTE site. We also show that, as opposed to AhR, BTEB4 is constitutively expressed in the nucleus and its subcellular localization is not regulated by TCDD. These findings are significant, because it suggests that the BTE site and the proteins that regulate it, for example Sp1 and BTEB, may have evolved to differentially activate and repress respectively, the CYP1A1 promoter and the XRE. We also find that BTEB3 and BTEB4 repress the basal activity, which suggests that the associated transcriptional complexes are regulating the CYP1A1 promoter under both basal and hydrocarbon-induced conditions. Thus we propose a model for BTEB-like proteins in which BTEB proteins actively bind the BTE site of the CYP1A1 promoter by competing with Sp1 and, subsequently, repress transcription by interacting with the co-repressor mSin3A. Indeed, our data show that exogenous expression of Sp1 reduces the ability of BTEB4

and BTEB3 to repress CYP1A1 promoter activity. In addition, this idea is also supported, at least in part, by recent reports from our laboratory demonstrating that BTEB3 can compete with Sp1 [11], and by the fact that the zinc-finger domains of all the BTEB proteins are nearly identical at the residues predicted to mediate DNA binding.

Functionally, it is tempting to speculate that the BTEB proteins, by repressing the expression from the CYP1A1 gene promoter, are involved in the regulation of carcinogen metabolism by CYP1A1 *in vivo*, effectively reducing the conversion of certain procarcinogens to active DNA-adduct-forming metabolites; a hypothesis currently being tested in our laboratory. Paradoxically, CYP1A1 also metabolizes other drugs and molecules that are relevant to cancer, such as chemotherapeutic agents. In this situation, one would predict that repression of the CYP1A1 promoter might reduce the efficacy of treatment with these drugs [27]. One would then wonder what mechanisms will prevail when cells are exposed to both types of xenobiotics. In light of the limitations of the current methodology to perform these types of studies, we predict that addressing this question will be arduous and will need to recruit the efforts of many laboratories. Interestingly, the promoters of genes encoding other cytochrome P450 enzymes also exhibit functionally important BTE-like elements [28–32]. Since BTEB4 is highly enriched in the liver, it is possible that it may regulate the expression of a number of liver-specific *cytochrome P450* genes. Thus the mechanism of CYP1A1 repression, like the one described in the present study, may have broad implications for cell and cancer biology.

In summary, the findings reported in this study, together with biochemical data published previously from our laboratory [11] and others [16–18], show that mammalian cells contain three BTEBs that potentially repress the CYP1A1 promoter. We have shown that two of these proteins, BTEB3 and BTEB4, bind to mSin3A *in vitro* and *in vivo*, revealing a potential role for mSin3A as a co-repressor for the BTEB subfamily of transcription factors. Functionally, this work reveals potentially important mechanisms underlying the regulation of CYP1A1-dependent chemical-induced carcinogenesis. Thus, when combined, these findings extend our understanding of the biochemical and functional properties of Sp1-like transcriptional repressors, such as BTEBs and TIEGs, and help to reduce the difference in the amount of knowledge that exists between these proteins and the better-studied Sp1-like transcriptional activators.

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