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Sp1 and Sp3 transcription factors regulate the basal expression of human microsomal epoxide hydrolase (EPHX1) through interaction with the E1b far upstream promoter

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ABSTRACT

Microsomal epoxide hydrolase (mEH, EPHX1) is a critical biotransformation enzyme, catalyzing the metabolism of many xenobiotics. Human mEH is transcribed using alternative promoters. The upstream E1 promoter is active in liver while the far upstream E1b promoter drives the expression of mEH in all tissues, including liver. Although several liver-specific transcription factors have been identified in the regulation of E1 transcription, little is known regarding the mechanisms of E1b transcriptional regulation. Genome-wide mapping of DNase I hypersensitive sites revealed an open chromatin region between nucleotide −300 upstream and +400 downstream of E1b. This area coincides with a previously described promoter region responsible for maintaining high basal promoter activity. *In silico* analysis of this location revealed several Sp1/Sp3 binding sites. Site-directed mutagenesis of these motifs suppressed the transactivation activity of the E1b proximal promoter, indicating their importance as contributors to E1b promoter regulation. Further, E1b promoter activities were increased significantly following Sp1 and Sp3 overexpression, while Mithramycin A, a selective Sp1 inhibitor, reduced the promoter activities. EMSA studies demonstrated that Sp1 bound to two putative Sp1/Sp3 binding sites. ChIP analysis confirmed that both endogenous Sp1 and Sp3 were bound to the proximal promoter region of E1b. Knockdown of Sp1 expression using siRNA did not alter the endogenous E1b transcriptional level, while knockdown of Sp3 greatly decreased E1b expression in different human cell lines. Taken together, these results support the concept that Sp1 and Sp3 are functionally involved as transcriptional integrators regulating the basal expression of the derived mEH E1b variant transcript.

1. Introduction

Human microsomal epoxide hydrolase (mEH, EPHX1) contributes an important biotransformation function, catalyzing the hydrolysis of electrophilic epoxides generated from oxidative metabolism contributed by cytochrome P450 (CYP) enzymes (Fretland and Omiecinski, 2000). Epoxide moieties are potentially highly electrophilic and may react covalently with cellular DNA and other macromolecules resulting in mutagenesis and carcinogenesis outcomes. In contrast, dihydrodiols, the products of hydrolysis of epoxides, tend to exhibit less reactivity and greater water solubility and often more readily eliminated. In this sense, mEH serves a key detoxifying function. However, in the metabolism of polycyclic aromatic hydrocarbons (PAH), mEH plays an opposite role (Lu and Miwa, 1980). PAHs are ubiquitous pollutants and a number are known human carcinogens. The metabolism of PAHs involves the action of specific enzymes in a multistep process. CYP enzymes first convert PAHs to epoxides that are further hydrolyzed by mEH to form PAH dihydrodiols. These dihydrodiols may be further oxidized to yield diol epoxides, which are typically more reactive than the original epoxides. The necessity of mEH in the bioactivation of PAH procarcinogens was confirmed in mEH-null mice, which are highly resistant to PAH-induced carcinogenesis compared with wild type mice (Miyata et al., 1999). The balance between detoxification and bioactivation by mEH is important for protecting against many chemically-initiated diseases, such as cancer. Any aberrant change affecting protein levels and subsequent enzymatic activities of mEH may therefore represent a risk factor for various diseases (Omiecinski et al., 2000).

Human mEH is encoded by a single gene on chromosome 1. Driven by alternative promoters, it is transcribed from two distinct locations approximately 15 kb apart (Liang et al., 2005). Two resulting transcripts are termed as E1 and E1b. The more proximal E1 promoter is selectively active in liver, while the far upstream E1b promoter drives mEH expression in all tissues, including liver (Liang et al., 2005; Yang et al., 2009). Previous studies have shown that several liver-enriched transcription factors, in particular the C/EBPα, HNF3 and GATA transcription factors,

Abbreviations: mEH, microsomal epoxide hydrolase; EPHX1, microsomal epoxide hydrolase gene; CYP, cytochrome P450; PAH, polycyclic aromatic hydrocarbons; Mit A, Mithramycin A; CGI, CpG island; TSS, transcriptional start site; DBTSS, DataBase of Transcription Start Sites; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

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are involved in regulating E1 transcription (Zhu et al., 2004a,b; Liang et al., 2005). However, with regard to E1b, relatively little is known regarding its regulatory mechanisms. A recent study from our laboratory demonstrated that the presence of genetically polymorphic transposable elements within the promoter region of E1b functions to decrease luciferase reporter-based transcription activity (Yang et al., 2009). These data appear to explain some of the interindividual variability noted in mEH expression. However, they do not explain why human mEH levels vary across tissues within the same individual. In addition, how exactly these elements affect mEH promoter activity is unknown.

In this study, we sought to identify the transcription factors that are mechanistically involved in maintaining the basal expression of the human mEH alternative transcript variant, E1b. The discovery of a CpG island in the proximal promoter region of E1b indicated a potential link between E1b transcriptional regulation and GC-rich DNA motif-binding transcription factors, such as Sp1/Sp3. The results generated demonstrate that Sp1 and Sp3 are bound to the E1b proximal promoter region and functionally regulate its transcriptional activity.

2. Materials and methods

2.1. Materials

Mithramycin A was from Enzo Life Sciences (Farmingdale, NY); the protease inhibitor mixtures were from Calbiochem (Billerica, MA). FuGENE 6 Transfection Reagent and the dual luciferase reporter assay system were from Promega (Madison, WI). All cell culture media and supplies were from Invitrogen (Carlsbad, CA). The TRIzol Reagent was from Invitrogen and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Small interfering RNA (siRNA) targeting Sp1 mRNA (siRNA ID: s13319), Sp3 mRNA (siRNA ID: s13326), or Silencer® Select Negative Control No. 1 siRNA and the Lipofectamine RNAiMAX reagent were all purchased from Invitrogen. The mouse monoclonal anti-mEH antibody, rabbit polyclonal anti-Sp1 (H-255) and anti-Sp3 (D-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Another rabbit polyclonal anti-Sp1 antibody was from Millipore (Billerica, MA). The rabbit polyclonal anti-GAPDH was from Sigma and the normal rabbit IgG was from Cell Signaling Technology (Danvers, MA).

2.2. Plasmids

The E1b promoter luciferase reporter construct (E1b −320/+46-pGL3) containing 320 bp of the 5′-flanking region upstream of E1b was constructed as described previously (Liang et al., 2005). Site-directed mutagenesis of the putative Sp1/Sp3 sites in the E1b proximal promoter region was carried out with the use of the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) with the following primers: mut Sp#1 (5′-GCGGAGACTGaaaCGGGGCTGCTGA-3′ and 5′-TCAGCAGCCCCGtttCAGTCTCCGC-3′), mut Sp#2 (5′-AGGCCG GGCTTacatGGAGACTGCGC-3′ and 5′-GCGCAGTCTCCatgtAAGCCCGG CCT-3′), mut Sp#3 (5′-AGGCCGGGGAACaaaCCGCTCGGAGGC-3′ and 5′-GCCTCCGAGCGGtttGTTCCCCGGCCT-3′), mut Sp#4 (5′-GGAGCCTT AttCAttCCTAGAGACT-3′ and 5′-AGTCTCTAGGaaTGaaTAAGGCTCC-3′), mut Sp#5 (5′-GGCCGCGGACCaaaCTTTAAGTAGCCCG-3′ and 5′-CGGG CTACTTAAAGtttGGTCCGCGGCC-3′), and mut Sp#6 (5′-TCTGGCCG CGGaaaCGCGGACCGCCC-3′ and 5′-GGGCGGTCCGCGtttCCGCGGCCAG A-3′). The mutated nucleotides are in lowercase. The basal E1b promoter construct (E1b −320/+46-pGL3) was used as template for amplification. Expression plasmids were generated by inserting full length cDNA of Sp1 (NM_138473.2), Sp3 (NM_003111.4) and ZBTB10 (NM_001105539.1) into the p3XFLAG-CMV10 expression vector (Sigma). Primers for amplifying these genes were as follows: Sp1 (5′-GATCGAATTCAAGCGACCAAGATCACTCCATG-3′ and 5′-GAT CTCTAGAATCAGAAGCCATTGCCACTGAT-3′), Sp3 (5′-GATCGAATTC AACCGCTCCCGAAAAGCCCGTG-3′ and 5′-GATCGGATCCTTACTCCATT GTCTCATTTCCAG-3′), and ZBTB10 (5′-ATCGTCGTTCAGTGAAATGA ACCGC-3′ and 5′-GATCGGATCCTTAATCATCTAGAGACATACAAACTT CTCC-3′). All constructs were confirmed by DNA sequencing.

2.3. Cell culture, transient transfection and luciferase reporter assays

Human bronchial epithelial BEAS-2B cells and human hepatoma HepG2-derived C3A cells were purchased from American Type Culture Collection (Manassas, Virginia). Both cell lines were cultured in 5% CO_2 incubator at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM Non-Essential Amino Acids, 1.0 mM Sodium Pyruvate, 10 mM HEPES, 0.15% sodium bicarbonate, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were cultured in 24-well or 6-well plates and 60 mm or 100 mm Petri dishes and harvested according to the requirements of the experiments.

BEAS-2B and C3A cells were seeded a day before transfection in 24-well plates at a density of 5×10^4 cells per well. For assessing E1b promoter activities in response to ectopic expression of Sp1, Sp3 and other transcription factors, cells were co-transfected with E1b −320/+46-pGL3 reporter plasmid and the corresponding expression plasmid of the given transcription factor using a FuGENE 6 transfection protocol according to the manufacturer's instructions. The pRL-CMV plasmid containing *Renilla* Luciferase cDNA was also co-transfected as an internal control for transfection efficiency. Cells were harvested 24 h post transfection and luciferase activity was measured and analyzed in a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) using the Dual Luciferase Reporter Assay System (Promega) as described previously (Auerbach et al., 2005). For Mithramycin A treatment, the cells were transfected with E1b −320/+46-pGL3 and pRL-CMV reporter plasmids for 6 h and were incubated for 24 h in culture medium containing the indicated concentration of Mithramycin A or vehicle (0.1% DMSO). Luciferase activity was measured in the same manner as described above. All transfections were performed in triplicate and the results were expressed as means ± standard deviations (SD) of triplicates. The experiments were repeated three times and the most representative results were shown.

2.4. Sp1 and Sp3 siRNA knockdown studies

To reduce endogenous Sp1 or Sp3 and assess the effect on E1b promoter activity, BEAS-2B and C3A cells were transfected with the respective siRNAs at 25nM using the Lipofectamine RNAiMAX reagent and assessed with a Reverse Transfection Protocol according to the manufacturer's instructions. Briefly, the transfection complexes of the Lipofectamine RNAiMAX reagent and the given siRNA were prepared in 24-well plates before medium and cells at a density of 5×10^4 cells per well were added to each well. Following transfections, cells were allowed to recover for 24 h and sequentially transfected with E1b −320/+46-pGL3 and pRL-CMV reporter plasmids using FuGENE 6 as described above. Luciferase activities were measured and analyzed after 24 h as mentioned previously.

To assess endogenous E1b transcription and mEH protein level in response to the knockdown of Sp1 or Sp3, BEAS-2B and C3A cells were transfected with these siRNAs at 25 nM using the Lipofectamine RNAiMAX reagent with a Forward Transfection Protocol according to the manufacturer's instructions. Briefly, cells were seeded a day before transfection in 6-well plates at a density of 3×10^5 cells per well or in 60 mm Petri dishes at a density of 7×10^5 cells per dish. The transfection complexes of the Lipofectamine RNAiMAX reagent and the given siRNA were added to each well containing cells. After 48 h, siRNA-transfected cells in 6-well plates were harvested for RT-PCR analysis and cells in 60 mm Petri dishes were collected for western blotting.

2.5. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA from siRNA-transfected BEAS-2B and C3A cells in 6-well plates was extracted with TRIzol Reagent according to the manufacturer's instructions. Total RNA (2 μg) was converted to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). cDNAs were analyzed with CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, MD). The final concentration of primers in each reaction was 0.2 μM. The PCR conditions consist of an initial denaturation for 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was run in duplicate and the results were normalized to the level of GAPDH mRNA. The primers used for quantitative real-time PCR were as follows: E1b, 5′-GAGCCTGCGA GCCGAGAC-3′ (forward)/5′-CGTGGATCTCCTCATCTGACGTTT-3′ (reverse); Sp1, 5′-ATTGAGTCACCCAATGAGAACAG-3′ (forward)/5′- CAGC CACAACATACTGCCC-3′ (reverse); Sp3, 5′-CACTGGTCAGTTGCCAAATC-3′ (forward)/5′-GAGCTGCCACTCTTCAGGAT-3′ (reverse); and GAPDH, 5′-CCCATCACCATCTTCCAGGAG-3′ (forward)/5′-GTTGTCATGGATGACC TTGGC-3′ (reverse).

2.6. Western blotting

BEAS-2B and C3A cells were plated in 60 mm Petri dishes and transfected with siRNA as described above. Cells were washed with PBS, trypsinized and centrifuged at 1000 ×*g* for 3 min. For preparation of whole cell lysates, cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 1 × protease inhibitor cocktail (Cat # 539131, Calbiochem). The cell lysates were centrifuged at 16,000 ×*g* for 10 min at 4 °C and the supernatants were collected as whole-cell lysate. Protein concentrations were determined by Pierce 660 nm Protein Assay (Thermo Scientific, Waltham, MA). The extracted proteins (30 μg) were separated on a 10% denaturing polyacrylamide gel (Bio-Rad) and transferred to a PVDF membrane (Bio-Rad). After blocking in 5% skim milk for 30 min, the blots were incubated sequentially with primary antibodies at the dilution of 1:1000 and horseradish peroxidase-conjugated secondary antibodies at the dilution of 1:5000. The membranes were washed three times with 1 × TBS/0.1% Tween 20, treated with Pierce ECL Western Blotting Substrate (Thermo Scientific), and exposed to ImageTek-H X-ray films (American X-Ray & Medical Supply, Oilville, VA). The antibodies used for immunoblotting were as follows: anti-mEH (sc-135984, Santa Cruz Biotechnology), anti-Sp1 (17-601, Millipore), anti-Sp3 (sc-644, Santa Cruz Biotechnology) and anti-GAPDH (G9545, Sigma).

2.7. Electrophoretic mobility shift assays (EMSA)

BEAS-2B cells in 100 mm Petri dishes at 80% confluence were transfected with the Sp1 expression plasmid using FuGENE 6 as described above. After 24 h, the nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. Double-stranded probes containing putative Sp1/Sp3 sites were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). For EMSA, the DNA-binding reactions, containing 2 μg of nuclear extracts, 20 fmol of labeled probes, 0.01 mg/ml sonicated salmon sperm DNA (D7656, Sigma), 2 μl of 5× binding buffer [20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5), 0.25 mg/ml poly(dI-dC)] in a final volume of 10 μl, were incubated with or without unlabeled competitor for 20 min at room temperature. For supershift assays, 2 μg of anti-Sp1 antibody (17-601, Millipore) or normal rabbit IgG (2729 s, Cell Signaling Technology) was added to the binding reaction mixture without the labeled probe and incubated at 4 °C for 30 min before addition of the labeled. The DNA–protein complexes were resolved by electrophoresis through a nondenaturing 4% polyacrylamide gel in 0.5× TBE buffer. Subsequently, the gels were dried and exposed to X-ray film with intensifying screens at −70 °C. The sense sequence of probes were as follows: Sp site#1, 5′-GCGGAGACTGCGCCGGGGCTGCTGA-3′; Sp site#2, 5′-AGGCCGGGCTTG GGCGGAGACTGCGC-3′; Sp site#3, 5′-AGGCCGGGGAACGCCCCGCTCGGA GGC-3′; Sp site#4, 5′-GGAGCCTTAGGCAGGCCTAGAGACT-3′; Sp site#5, 5′-GGCCGCGGACCGCCCTTTAAGTAGCCCG-3′; Sp site#6, 5′-TCTGGCCG CGGGGCCGCGGACCGCCC-3′; Sp1 consensus oligos, 5′-ATTCGATCGGGG CGGGGCGAGC-3′; and mutant Sp1 consensus oligos, 5′-ATTCGATC GGttCGGGGCGAGC-3′. The mutated nucleotides in the mutant Sp1 consensus oligos are in lowercase.

2.8. Chromatin immunoprecipitation (ChIP) assay

BEAS-2B and C3A cells, grown to 80–90% confluence in 100 mm Petri dishes, were harvested by trypsinization and fixed in 1% formaldehyde at room temperature for 10 min with slow agitation. The fixation was stopped by addition of glycine to a concentration of 0.125 M. After a 5 min incubation at 25 °C, cells were pelleted by centrifugation at 1000 ×*g* for 5 min and then washed twice with ice-cold phosphate-buffered saline. Cells were lysed for 10 min on ice in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–HCl, pH 8) with 1X protease inhibitor cocktail (539131, Calbiochem). Cells were then sonicated with a Bioruptor sonicator (Diagenode, Liège, Belgium) for 5 cycles of 30 s ON and 30 s OFF at HIGH setting in a refrigerated water bath. Sheared cross-linked chromatin was centrifuged at 12,000 ×*g* for 10 min at 4 °C and diluted 10-fold in ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH8, and 167 mM NaCl) with 1X protease inhibitor cocktail. The diluted chromatin was pre-cleared overnight at 4 °C with 35 μl protein A/G Plus-agarose beads (sc-2003, Santa Cruz Biotechnology) which were pre-blocked with sonicated salmon sperm DNA (201190, Stratagene, Santa Clara, CA) and BSA (2930, EM Science, Billerica, MA). Pre-cleaned chromatin was then incubated overnight at 4 °C with 4 μg of anti-Sp1 antibody (17-601, Millipore), anti-Sp1 antibody (sc-14027, Santa Cruz Biotechnology), anti-Sp3 antibody (sc-644, Santa Cruz Biotechnology) or normal rabbit IgG (2729 s, Cell Signaling Technology). To collect the antibody–chromatin complex, 75 μl protein A/G Plus-agarose beads pre-blocked as above were added, incubated for 3 h with rotation at 4 °C and pelleted by centrifugation at 5000 ×*g* for 1 min. The pelleted complexes were then washed sequentially with Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8, 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8, 500 mM NaCl), and LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris–HCl, pH 8), followed by two washes with TE buffer. Precipitated protein–DNA complexes were eluted twice with 100 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at room temperature. To reverse crosslinks, the eluates were incubated at 65 °C for 4 h in the presence of 8 μl of 5 M NaCl and 1 μl of 10 mg/ml RNase A. Proteins were digested with 2 μl of 10 mg/ml proteinase K for 2 h at 45 °C in the presence of 4 μl of 0.5 M EDTA, pH 8, and 8 μl of 1 M Tris–HCl, pH 8. DNA was purified with ChIP DNA Clean & Concentrator kit (D5205, Zymo Research, Irvine, CA). Immunoprecipitated DNA was amplified and the PCR amplicons were analyzed on 1.5% agarose gels. PCR amplification with appropriate primers was performed to analyze immunoprecipitated DNA. The PCR amplicons were subjected to 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The immunoprecipitated DNA was also subjected to the analysis of quantitative RT-PCR. The primers used for detecting E1b proximal promoter were: forward (5′- ACCG CCCTTTAAGTAGCCCGTTT-3′) and reverse (5′- TTACGGTCTCGGCTCG CA-3′).

2.9. Statistical analyses

Data are expressed as means ± standard deviations (SD). The statistical significance of the differences between samples was determined using one-way analysis of variance (ANOVA) in combination with Dunnett's test or one-tailed Student's *t*-test, dependent on the design of the experiments. Differences were considered significant for samples with *p*-values <0.05.

3. Results

3.1. Identification of the critical promoter region for the basal expression of E1b

To investigate the basal transcriptional regulation of the E1b variant, we analyzed the promoter region of the EPHX1 gene using the UCSC Genome Browser. Genome-wide mapping of DNase I hypersensitive sites revealed an open chromatin region in several cell types containing the alternative first exon, E1b (Fig. 1A). This region, which spans from −300 bp upstream to +400 bp downstream of E1b, coincides with a previously described promoter region that contributes to high basal promoter activity (Liang et al., 2005). The localization of an active promoter adjacent to the alternative first exon, within the context of an open nucleosomal structure that is conserved across multiple cell types and likely highly accessible to transcription factors, supports the concept that this architecture functions to drive basal expression of the gene. As indicated in Genome Browser display of the E1b flanking region (Fig. 1A), this area also maps to a putative CpG island (CGI). This CpG-rich region is characterized with a GC content of 66.3% and an Obs/Exp ratio of CpG dinucleotide of 0.937 (Fig. 1B).

3.2. Analysis of the E1b proximal promoter region for the transcription factor Sp1/Sp3 binding sites

The proximal promoter region of E1b, specifically the nucleotide sequence region between position −320 bp and +46 bp relative to the transcriptional start site (TSS), was retrieved using the UCSC Genome Browser (Fig. 2A). The TSS for E1b variant was determined by the 5′ end of Exon E1b as reported previously (Liang et al., 2005) and defined as +1 (Fig. 2A). We noted a discrepancy on the start position of E1b as reported by Liang et al. (termed as TSS1) with GenBank entry NM_001136018 (termed as TSS2). TSS1 is ~30 bp downstream of TSS2. To resolve this inconsistency, an analysis was conducted using the DataBase of Transcription Start Sites (DBTSS) (Yamashita et al., 2012). Both TSS1 and TSS2 were detected by TSS-seq, but the usage of the former was greater than the latter. Therefore, TSS1 appears to represent the major start site for the E1b transcript.

The discovery of a CpG island in the region of the TSS indicated possible regulation of E1b promoter by transcription factors that bind to GC boxes. A search for transcription factor binding sites in this proximal promoter region using MatInspector (http://www.genomatix.de) and TESS (http://www.cbil.upenn.edu) indicated that the −320 bp/+46 bp region lacked canonical CCAAT and TATA boxes, but contained 6 potential Sp1/Sp3 binding sites (Fig. 2A).

To evaluate the contribution of these Sp1/Sp3 binding sites to the basal promoter activity of E1b proximal promoter, we introduced point mutations and measured their effects on transcriptional activity in BEAS-2B and C3A cells with the luciferase assay (Fig. 2A). As shown in Fig. 2B, all of site mutations resulted in a strong negative effect on E1b promoter activity; especially mutations at sites #2, #3 and #6, which reduced the promoter activity to less than 35% of the wild-type activity. Therefore, the results indicated that these putative Sp1/Sp3 sites in the E1b proximal promoter contribute to the basal transcription of E1b although not all of the Sp1/Sp3-binding sites are functionally equivalent.

3.3. Sp1/Sp3 is involved in activation of the E1b proximal promoter

The presence of canonical Sp1/Sp3 binding sites in the proximal promoter region of E1b variant suggested that Sp1/Sp3 was involved in the regulation of E1b promoter activity. Therefore, we co-transfected the E1b −320/+46 luciferase construct and Sp1 or Sp3 expression plasmid into BEAS-2B and C3A cells. Overexpression of Sp1 and Sp3 significantly increased reporter activity in BEAS-2B cells (Fig. 3A). Interestingly, Sp1

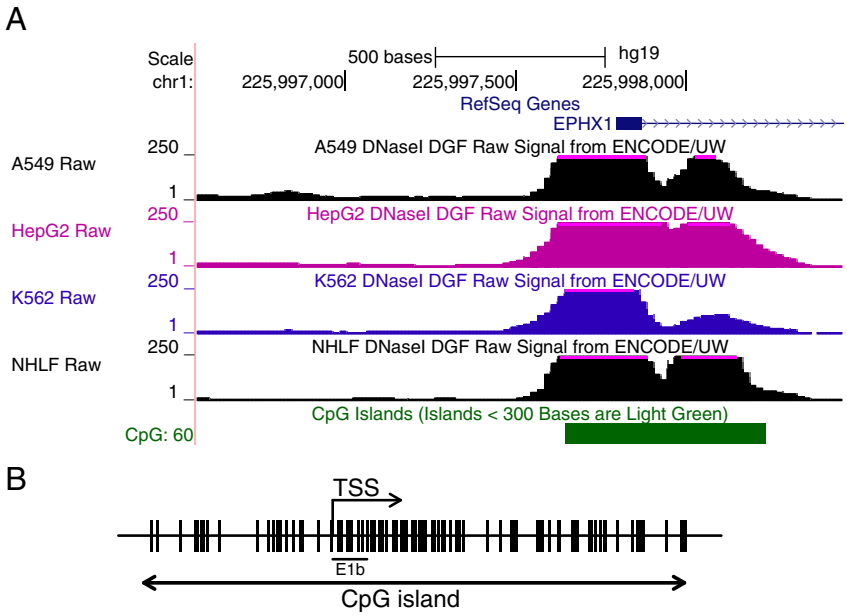

Fig. 1. Location of E1b proximal promoter. A) Genome browser display of the hg19 human assembly showing the alternative E1b promoter region of human mEH. Data tracks shown are DNaseI Digital Genomic Footprinting from ENCODE/University of Washington for A549, HepG2, K562 and NHLF cells and CpG islands. B) A schematic structure of the putative E1b promoter CpG island. CpG sites within the CpG island are shown as short vertical lines.

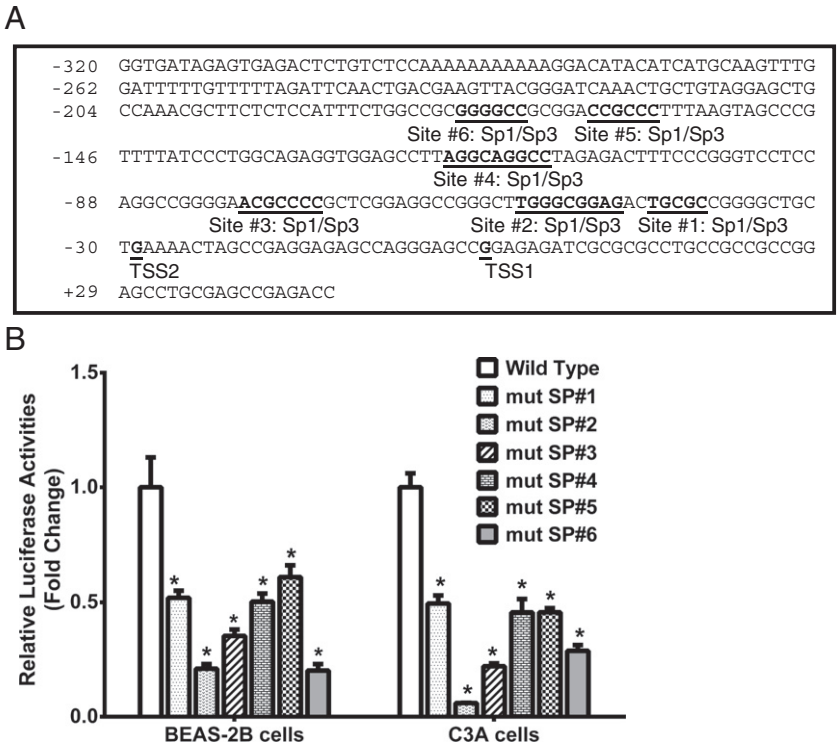

Fig. 2. Identification and mutational analysis of Sp1/Sp3 binding sites within the E1b proximal promoter region. A) Nucleotide sequence of the E1b −300 bp proximal promoter. The predicted binding sites of Sp1 are underlined. The sites indicated with 'TSS1' and 'TSS2' denote the transcription start sites (TSS) of the E1b transcript. TSS1 represents the major TSS of E1b. B) Mutation analysis of Sp1/Sp3 binding sites in the E1b proximal promoter. Individual Sp1/Sp3 sites were mutated and their effects on the E1b −300 promoter activity were measured by luciferase assays in human bronchial epithelial BEAS-2B cells and human hepatoma HepG2-derived C3A cells. Statistically significant differences on the luciferase activity compared to the wild type (WT) are indicated by an "*" ($p < 0.05$).

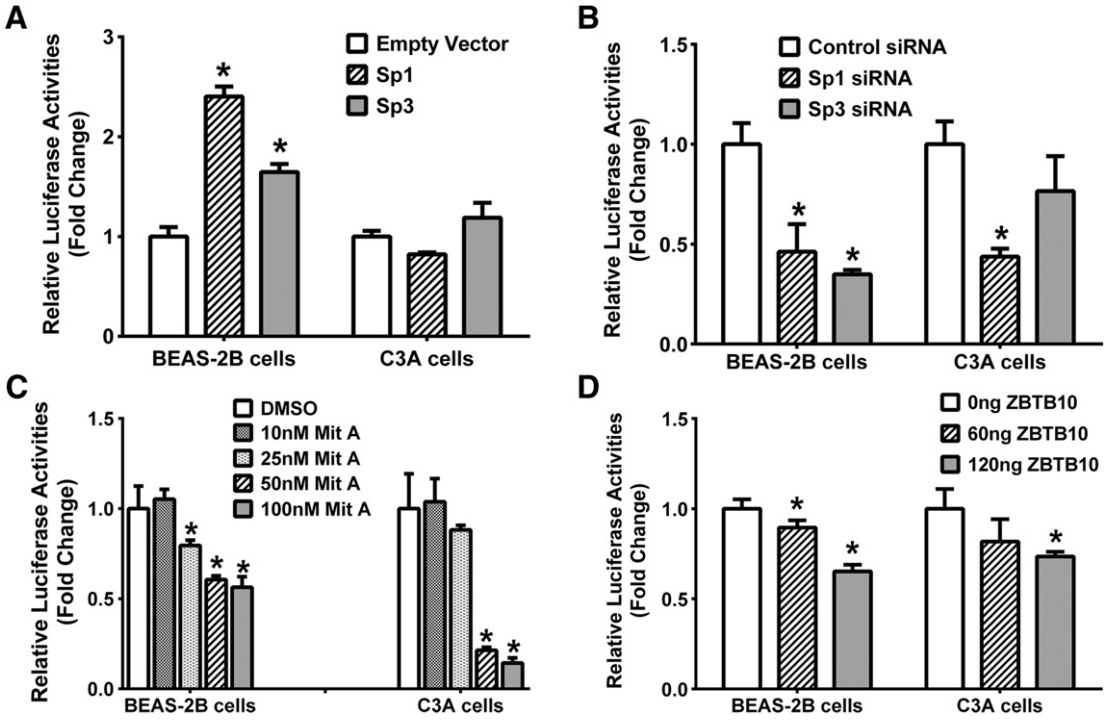

Fig. 3. Sp1 and Sp3 regulated E1b proximal promoter activities. A) Overexpression of Sp1 and Sp3 activated E1b proximal promoter activity. BEAS-2B and C3A cells were co-transfected with Sp1 or Sp3 expressing plasmid and E1b −300 promoter-luciferase reporter. After 24 h, luciferase activity was measured. B) Knockdown of Sp1/Sp3 by siRNAs reduced E1b promoter activity. Cells were transfected with 25 nM Sp1 or Sp3 siRNA and E1b −320/+46-pGL3 as described under "Materials and methods". C) The Sp1/Sp3 inhibitor Mithramycin A attenuated E1b −300 promoter activity. Luciferase assay was performed after cells transfected with E1b −300 promoter-luciferase reporter were treated with Mithramycin A for 24 h. D) Overexpression of Sp protein repressor ZBTB10 decreased E1b −300 promoter activity. BEAS-2B and C3A cells were co-transfected with ZBTB10 expressing plasmid and E1b −300 promoter-luciferase reporter. After 24 h, luciferase activity was measured. Statistically significant differences between treatment groups compared to the empty vector control, the control siRNA, or the vehicle-treated control are indicated by an "*" ($p < 0.05$).

and Sp3 contributed only a small effect on reporter activity in C3A cells. This result may be due to the presence of endogenous Sp1 or Sp3 proteins, as well as other Sp family proteins that may virtually saturate the reporter read out. We next evaluated the effect of siRNA knockdown of Sp1 and Sp3 on E1b promoter activity. In BEAS-2B cells, knockdown of Sp1 and Sp3 significantly attenuated E1b proximal promoter activity, while in C3A cells only knockdown of Sp1 had a significant effect on E1B promoter activity (Fig. 3B). Overall, these results suggest that both Sp1 and Sp3 can regulate the basal activity of the E1b proximal promoter.

To further confirm the involvement of Sp1/Sp3 for the basal activity of E1b proximal promoter, we performed a series of transient transfection experiments using the E1b −320/+46 promoter-luciferase reporter construct and tested the converse hypothesis that interference in the interaction of Sp1 or Sp3 with the E1b promoter would reduce its promoter activity. First, cells were treated with Mithramycin A which binds to GC rich DNA sequences and prevents Sp1 binding to its target DNA motif (Snyder et al., 1991). Treatment with Mithramycin A significantly blocked E1b promoter activity in a dose-dependent manner in both BEAS-2B and C3A cells (Fig. 3C). The cells were then co-transfected with the E1b −300 luciferase construct and increasing amounts of a ZBTB10 expressing plasmid. ZBTB10 is a suppressor of Sp-dependent transactivation, and competes with Sp1 binding sites to decrease the expression of Sp1 and Sp3 (Tillotson, 1999; Mertens-Talcott et al., 2007). Overexpression of ZBTB10 resulted in a dose-dependent reduction in reporter activity (Fig. 3D). These data indicate that inhibition of Sp1/Sp3 protein binding to the E1b proximal promoter inhibits basal promoter activity.

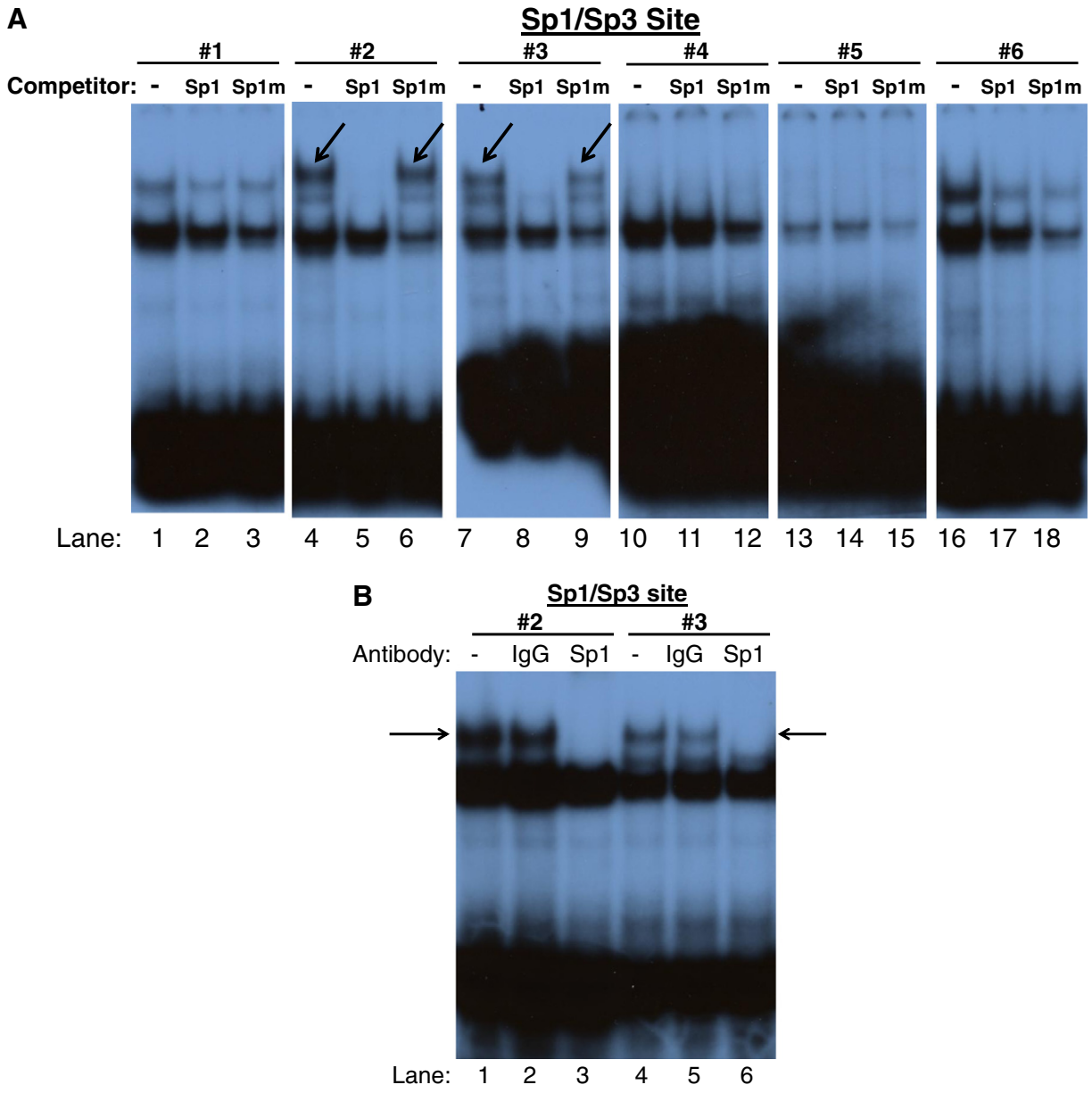

Fig. 4. EMSA and supershift analyses show Sp1 binding to putative Sp1/Sp3 binding sites on E1b −300 promoter. A) An EMSA was performed using ^{32}P-labeled oligonucleotide probes containing the putative E1b −300 Sp1/Sp3 binding sites. The labeled probes were incubated with nuclear extracts from BEAS-2B cells overexpressing Sp1. A fifty fold excess of unlabeled competitor oligonucleotide was used for competition assays. Competitors used were the consensus Sp1 oligonucleotide (Sp1, Lane 2, 5, 8, 11, 14, and 17) and a mutant Sp1 oligonucleotide (Sp1m, Lane 3, 6, 9, 12, 15, and 18). The arrows show specific binding. B) Supershift analysis was performed using a polyclonal antibody against Sp1 (see "Material and methods" for detailed experimental conditions) (Lane 3 and 6). Supershift with a rabbit normal IgG was used as a negative control (Lane 2 and 5). The arrows show specific binding which was interrupted by addition of anti-Sp1 antibody.

3.4. Sp1 and Sp3 bind and interact with the E1b proximal promoter region

To extend the promoter transactivation analysis and determine whether Sp1 or Sp3 binds to the E1b proximal promoter region via the putative binding sites for Sp1 and Sp3, EMSA was performed with oligonucleotide probes containing these sites. When these labeled probes were incubated with nuclear extracts from BEAS-2B cells transfected with Sp1 expression plasmid, several DNA–protein complexes were produced (Fig. 4A). The specificity of binding was confirmed by loss of labeled complexes in the presence of 50-fold excess of unlabeled Sp1 consensus oligonucleotide but not by the unlabeled mutated Sp1 consensus oligonucleotide. Sp1/Sp3 binding sites #2 and #3 displayed a substantial competition effect, which suggested that Sp1 preferentially binds to these two sites compared to the other 4 sites (Fig. 4A). Furthermore, addition of anti-Sp1 antibody, but not normal rabbit IgG, disrupted the DNA–protein complexes formed with Site#2 and #3, indicating that Sp1 is involved in the formation of these DNA–protein complexes (Fig. 4B). These results suggested that Sp1 protein specifically binds to the E1b proximal promoter *in vitro*. In these studies, we did not test whether Sp3 interacts with these putative binding sites in E1b proximal promoter. Because Sp1 and Sp3 share more than 90% sequence homology in the DNA-binding domain and bind to the same cognate DNA-element, it is likely that Sp3 can also bind to sites #2 and #3.

Next, we examined whether Sp1 or Sp3 binds to the E1b proximal promoter using the chromatin immunoprecipitation (ChIP) assay. Chromatin from BEAS-2B and C3A cells was sonicated and immunoprecipitated using antibodies against Sp1 and Sp3. The precipitated DNA was subjected to PCR (Fig. 5A) and qPCR (Fig. 5B) analyses using primers for the −320/+46 bp region of E1b proximal promoter. E1b promoter-specific primers amplified this promoter region from DNA that was immunoprecipitated by either Sp1 or Sp3 antibody in BEAS-2B and C3A cells. In the same experiment, no signal was observed when chromatin was immunoprecipitated with control rabbit IgG. These results clearly showed that Sp1 and Sp3 directly interact with the E1b upstream promoter region.

3.5. Knockdown of Sp1 and Sp3 regulated expression of E1b variant

Results from luciferase reporter-based promoter characterization as well as EMSA and ChIP analyses indicated an important role for Sp1 and Sp3 in transactivation of the E1b proximal promoter. We tested whether Sp1 and Sp3 siRNA knockdown would reduce the endogenous expression of the E1b transcript. Transfection with Sp1 and Sp3 siRNAs in BEAS-2B and C3A cells resulted in significant knockdown of Sp1 and Sp3 mRNA and protein levels (Figs. 6A and B). Surprisingly, down-regulation of Sp1 did not significantly affect the mRNA level of E1b in either cell line (Fig. 6A). In contrast, siRNA knockdown of Sp3 resulted in a 65–75% reduction in E1b transcript levels, as well as mEH protein levels in BEAS-2B and C3A cells (Fig. 6B). As a control for these experiments, we measured Sp1 expression in Sp3 siRNA-transfected BEAS-2B and C3A cells and found that knocking down Sp3 had no effect on Sp1 expression (data not shown). This result indicates that Sp3 siRNA does not cross react with Sp1 and that the reduction of E1b expression detected in the study is the direct consequence of specific Sp3 knockdown. These data support the conclusion that Sp1/Sp3 interactions influence basal endogenous expression of E1b variant.

4. Discussion

This investigation characterized the far upstream alternative E1b promoter region of EPHX1 and provided new insights into the regulation of human mEH gene expression. The E1b proximal promoter was localized within the first 300 bp upstream of the alternative E1b exon and contains at least two TSSs, CGI and several potential Sp1/Sp3 binding sites. Further characterization revealed that Sp1 and Sp3 do bind to the E1b promoter and that these interactions are important determinants of basal promoter activity. For example,

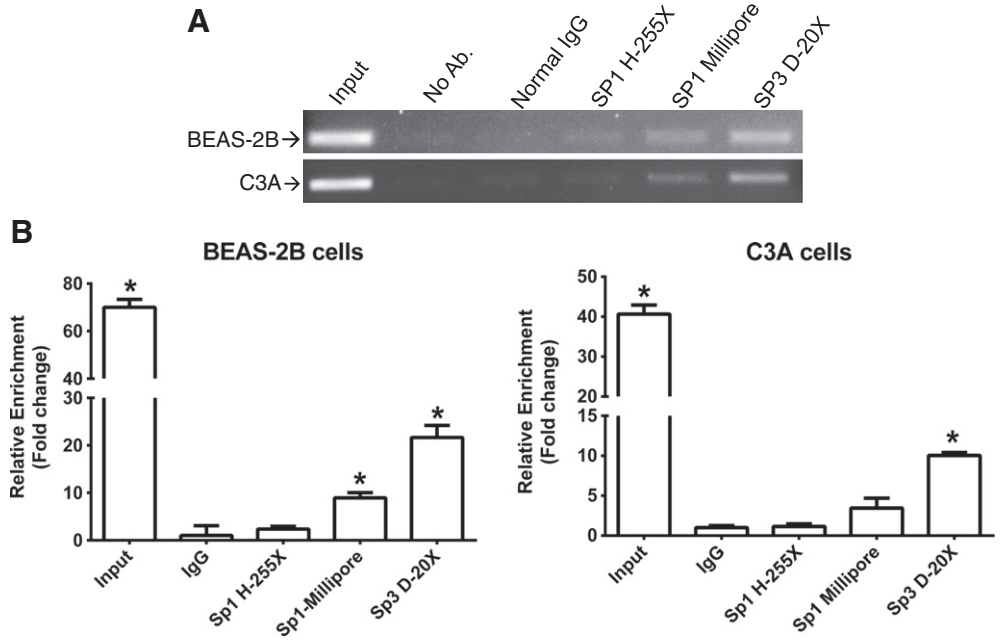

Fig. 5. ChIP assay for Sp1 and Sp3 binding to the E1b proximal promoter in BEAS-2B and C3A cells. ChIP assay was performed to confirm the binding of Sp1 and Sp3 to the E1b proximal promoter. The DNA–protein complexes were incubated with polyclonal antibodies against Sp1 or Sp3 and isolated by immunoprecipitation. The antibodies used for immunoprecipitation were as follows: SP1 H-255X (anti-Sp1 antibody from Santa Cruz Biotechnology), SP1 Millipore (anti-Sp1 antibody from Millipore), SP3 D-20X (anti-Sp3 antibody from Santa Cruz Biotechnology). For the negative controls, the DNA–protein complexes were incubated without antibodies or with normal IgG. The immunoprecipitated DNA fragments were analyzed by conventional PCR analysis (A) and qPCR analysis (B). Statistically significant differences on the binding to the E1b promoter region compared to the rabbit normal IgG are indicated by an "*" ($p < 0.05$).

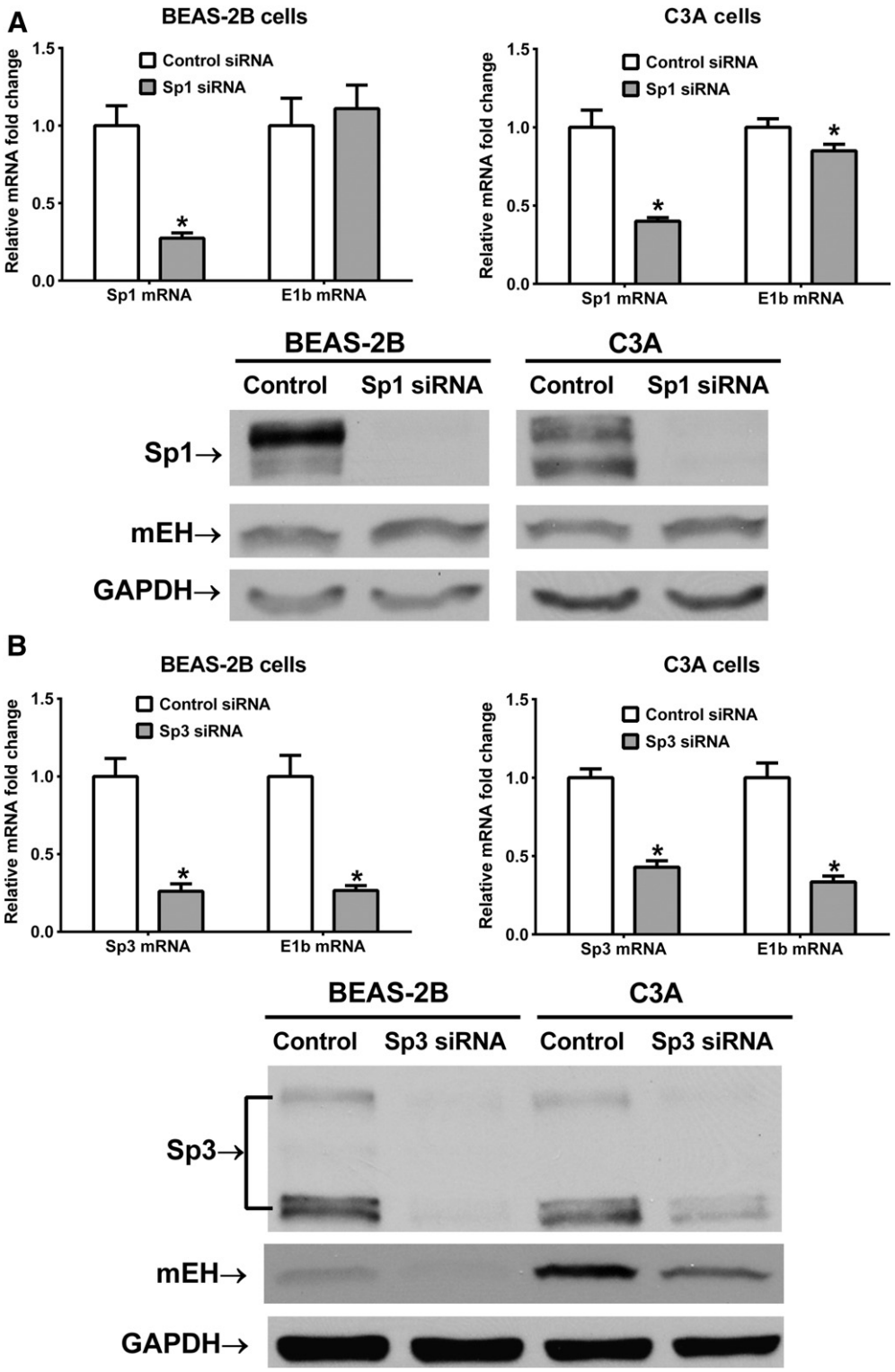

Fig. 6. Effect of Sp1 and Sp3 knockdown on E1b transcript and mEH protein. Cells were transfected with Sp1- (A) or Sp3- (B) specific siRNAs. After 48 h, total RNA was collected for assessment of transcript levels by real time quantitative PCR (upper panels) and total protein extracts were immunoblotted with anti-Sp1, Sp3 and mEH antibodies (lower panels). A) The mRNA and protein levels of Sp1 and E1b in Sp1-specific siRNA-transfected BEAS-2B and C3A cells. B) The mRNA and protein levels of Sp3 and E1b in Sp3-specific siRNA-transfected BEAS-2B and C3A cells. GAPDH was used as an internal control for real-time qPCR and a loading control for western blotting. Statistically significant differences compared to the negative control siRNA are indicated by an "*" ($p < 0.05$).

Sp1 and Sp3 overexpression and knockdown significantly impacted E1b promoter activity. Collectively, the data strongly support the contention that Sp1 and Sp3 are key regulators of E1b basal transcription.

Bioinformatics analysis of the E1b proximal promoter revealed a CGI overlapping with this region. CGIs are associated with promoter regions in 70% of human genes (Saxonov et al., 2006) and have been identified in all house-keeping genes and approximately 40% of

tissue-specific genes (Illingworth and Bird, 2009). CGI promoters typically lack TATA and other core promoter elements, but contain multiple Sp1 binding sites (Butler and Kadonaga, 2002). CGI promoters are also reported to contain multiple TSS over a stretch of about 50 to 100 nucleotides (Smale and Kadonaga, 2003; Juven-Gershon et al., 2008). The E1b promoter has no TATA box or other core promoter elements, but several potential Sp1/Sp3 binding sites within the upstream region of E1b and perhaps more within the rest of the CGI. Through literature review and DBTSS searches, we identified two TSSs within the E1b proximal promoter that are approximately 30 nucleotides apart. The DBTSS inquiry results also indicated that additional TSSs exist in this region and that these TSSs show tissue-dependent patterns. Although the mechanistic basis of the differential TSS usage within the E1b promoter is unknown, it may involve variations in the availability of transcription factors at different sites on the E1b promoter or/and changes in the patterns of epigenetic modifications, such as DNA methylation (Kawaji et al., 2006). In addition, how tissue-specific usage of TSSs contributes to the total transcription level of E1b needs further investigation. We speculate that various transcriptional factors and cofactors may utilize distinct TSSs to control the expression of E1b depending on the cell context.

Six potential Sp1/Sp3 binding sites were identified in the bioinformatics analysis of the E1b promoter DNA sequence. The mutation of any site significantly reduced the transactivation activity of E1b promoter, indicating these sites are important for basal promoter activity. EMSA established that Sp1 binds two out of the six sites. The remaining binding sites may interact with other related transcription factors that may also be important for the maintenance of the basal transcription of E1b. Possible candidates are transcription factors from Sp-like/KLF family, as they share more than 65% sequence homology on the DNA binding-domains composed of three adjacent Cys_2His_2-type zinc finger motifs (Kaczynski et al., 2003). The structural similarities among family members enable these factors to bind similar DNA sequences. In this respect, Sp1 is reported to compete for the same sites with Sp3, KLF4, KLF6, KLF9 and KLF13 (Kaczynski et al., 2003; Grande et al., 2012). In addition, there appear to exist potential binding sites for other transcription factors that overlap with putative Sp1/Sp3 binding sites. For example, site #6 overlaps with a predicted binding site for transcription factor AP-2α (TFAP2A), and the introduced mutation within site #6 is projected to disrupt that binding interaction. Therefore, the inference from the mutagenesis results is that AP-2α may also play an important role in regulating the basal expression of E1b.

Sp1 and Sp3 are ubiquitous transcription factors that bind to GC-rich motifs in the proximal promoter of a wide variety of genes, such as housekeeping and tissue-specific genes (Li and Davie, 2010). Sp1 acts as a transcriptional activator, whereas Sp3 can function either as a transcriptional activator or repressor (Suske, 1999). In the context of the E1b promoter, Sp1 and Sp3 both serve as activators as indicated by the data generated from overexpression of Sp1 and Sp3, mutagenesis analysis and Mithramycin A treatment coupled with luciferase reporter assays. Further, siRNA knockdown of Sp3 expression resulted in a reduction of the endogenous E1b transcription level, apparently confirming that Sp3 is an activator. However, the E1b transcription level remained unchanged when Sp1 was knocked down by Sp1 siRNA, suggesting perhaps that Sp1 does not regulate E1b expression. The inconsistency of the lack of effect of Sp1 on E1b expression might be explained by at least two considerations. First, the cancer cell lines used have high basal expression level of Sp1 and incomplete knockdown of Sp1 by siRNA may leave sufficient levels of Sp1 for normal function. Second, transcription factors from the Sp-like/KLF family may bind to the E1b proximal promoter and share overlapping function with Sp1. Knockdown of Sp1 by siRNA could result in occupancy by these related transcription factors on the sites that normally are bound by Sp1. In these respects, it seems plausible that E1b expression was not changed due to functional redundancy of the transcription factor family members.

As well, Sp1 is reported to regulate target gene transcription cooperatively through interaction with other transcription factors that bind to DNA motifs proximal to Sp1-binding sites (Safe and Abdelrahim, 2005). Although this study focused on Sp1/Sp3 sites, in our analysis of the E1b promoter sequence we also identified putative binding motifs for other cell type-specific transcription factors, including AP-2, E2F, NF*k*B and GATA-1. The interaction between Sp1/Sp3 and these other transcription factors may also be important determinants contributing to the regulation of E1b expression through differential cellular expression of the respective proteins. Thus, E1b expression is likely subject to combinatorial regulation in both ubiquitous and cell-type-specific manner. Further work is required to understand how the E1b promoter activity is altered with regard to cell background.

5. Conclusion

The link between E1b expression and Sp1/Sp3-mediated transcriptional regulation via the E1b proximal promoter is an intriguing development, given that little is known about the transcriptional regulation of the E1b promoter. The features identified in the context of the E1b proximal promoter indicate that its regulation is complex. The complexity is manifested in at least two respects: 1) transcription from multiple TSSs and 2) combinatorial regulation by Sp1/Sp3 and likely other transcription factors whose binding sites are in close proximity within the E1b proximal promoter. Therefore, the E1b proximal promoter provides a platform that can accommodate diverse transcription factors to control the expression of E1b in different cells. The characterization of the E1b promoter and the fundamental insights provided here with respect to E1b transcriptional regulation will serve as a valuable basis for further elucidation of normal and pathological conditions involving mEH expression.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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