Dual promoters control the cell-specific expression of the human cell death-inducing DFF45-like effector B gene

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CIDE-B [cell death-inducing DFF45 (DNA fragmentation factor 45)-like effector B] is a member of the CIDE family of apoptosis-inducing factors. The highly restricted pattern of expression of *CIDE-B* in the liver and spleen suggests that a mechanism exists for the tissue- and cell-specific regulation of transcription of this gene. We have analysed the promoters of the human *CIDE-B* gene, particularly the mechanism of cell-specific transcription. Expression of *CIDE-B* is driven by two promoters which are responsible for the synthesis of two types of transcript, and Sp1 and Sp3 are key regulators of basal transcription from both the upstream and the internal promoter, as indicated by EMSAs (electrophoretic mobility-shift assays) and site-directed mutagenesis. Bisulphite sequencing analysis demonstrated that the upstream promoter was hypermethylated in cells that did not express the long transcript of *CIDE-B*, but was hypomethylated in cells that expressed this transcript. Furthermore, methylation of this

region *in vitro* reduced the promoter activity to ~5% of the control. Thus methylation at CpG sites in the upstream promoter region appeared to be important for cell-specific synthesis of the long transcript. By contrast, HNF4 α (hepatocyte nuclear factor-4 α) bound to the internal promoter and enhanced its activity. Moreover, the short transcript of *CIDE-B* gene was expressed in cells which do not normally express this transcript upon introduction of exogenous HNF4 α , demonstrating the involvement of HNF4 α in the cell-specific synthesis of the short transcript. Thus our analysis revealed a novel mechanism for the cell-specific transcription of the human *CIDE-B* gene, which involves epigenetic and genetic control at separate respective promoters.

Key words: cell death-inducing DFF45 (DNA fragmentation factor 45)-like effector (CIDE), cell-specific expression, DNA methylation, promoter, hepatocyte nuclear factor-4 (HNF4), Sp1.

INTRODUCTION

Apoptosis or programmed cell death is an evolutionarily conserved phenomenon that removes redundant cells produced during animal development [1]. Changes in this normal process can result in the disruption of the delicate balance between cell proliferation and cell death, and can lead to a variety of diseases, including cancers, neurodegenerative disorders, autoimmune diseases and viral infections [2,3]. A novel family of cell death-inducing DFF45 (DNA fragmentation factor 45)-like effectors (CIDEs) has recently been identified, and it includes CIDE-A, CIDE-B, and CIDE-C (originally named CIDE-3) in humans, and fat-specific gene 27 (*FSP27*, a homologue of the human *CIDE-C* gene) in mice [4–6]. CIDEs share a conserved amino acid sequence similar to the CIDE-N domains in DFF40/CAD (caspase-activated nuclease) and its inhibitor [DFF45/ICAD (inhibitor of CAD)], which are two subunits of DFF complex [6–8]. Cleavage of DFF45/ICAD by caspase 3 releases DFF40/CAD from the complex and triggers DNA fragmentation and nuclear condensation [9,10]. The structure of the N-terminal domain of CIDE-B suggests that this domain might serve as a weak-interaction interface or regulatory domain [11]. Overexpression of *CIDE-B* results in cell death associated with the fragmentation of DNA [5]. Such CIDE-B-induced apoptosis can be inhibited by the NS2 (non-structural protein 2) of HCV (hepatitis C virus)

via interaction with the C-terminal domain of CIDE-B [12]. This domain is conserved in CIDEs, and is responsible for the mitochondrial localization and dimerization of CIDE-B and CIDE-B-induced cell death [13].

According to previous reports, the expression of *CIDEs* is strongly tissue specific. Two cDNA variants have been reported that encode human CIDE-B [5,11]. The major short transcript of *CIDE-B* was detected in adult and fetal liver, whereas the long transcript was detected at lower levels in fetal liver, spleen, peripheral blood lymphocytes and bone marrow [5]. Other *CIDE* genes also have multiple transcripts with different levels of tissue-specific expression. For example, the major large transcript of human *CIDE-C* was detected in the small intestine, heart, colon and stomach, whereas a small transcript was detected at a lower level in placenta [6]. Thus the transcription of *CIDE* genes appears to be regulated in a strictly tissue- and cell-specific manner. However, complete analysis of the regulation of transcription of human *CIDE* genes has not yet been performed. Studies of the adipocyte-specific gene *Fsp27*, a member of the mouse CIDE family, showed that the expression of *Fsp27* was regulated by C/EBP (CCAAT/enhancer-binding protein) and other C/EBP-like transcription factors [4], and that the expression of the gene was strongly induced in PPAR α ^{−/−} (peroxisome-proliferator-activated receptor α ^{−/−}) mouse livers with PPAR γ 1 overexpression [14]. It is of interest that both C/EBP and PPAR γ are critical transcription

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; CAD, caspase-activated nuclease; DFF, DNA fragmentation factor; CIDE, cell death-inducing DFF45-like effector; EMSA, electrophoretic mobility-shift assay; *FSP27*, fat-specific gene 27; (DN)-HNF, (dominant negative) hepatocyte nuclear factor; LTB4R, leukotriene B₄ receptor; MeCP, methyl-CpG-binding protein; ORF, open reading frame; Pi, internal promoter; PPAR, peroxisome-proliferator-activated receptor; Pu, upstream promoter; RE, response element; RT, reverse transcription.

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The nucleotide sequence data reported will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers DQ017648 and DQ017647 for the upstream promoter and the internal promoter for the human *CIDE-B* gene respectively.

Table 1 Primers, sizes of PCR products and details of PCR protocols

F, forward; R, reverse; T°, annealing temperature.

Oligonucleotides (5' to 3')	Size (bp)	Protocol		
		T° (°C)	Time (s)	No. of cycles
PCR				
<i>CIDE-B</i> coding sequence F: GTGGACAGTGAGGACTTCTCCAG R: AGGCTGCCAAAGAGGTCTCG	200	60	30	30
Long transcript of <i>CIDE-B</i> F: GGCAGGAAAGCAGGGATTG R: TATTAGATACTGACCTGAGTAAGTCACTG	432	55	60	30
Short transcript of <i>CIDE-B</i> F: CTTGACTCATTCACCTTGTC R: AGGCTGCCAAAGAGGTCTCG	576	55	60	30
<i>HNF4α</i> F: ATGCACCCCATGCCACCACCCCC R: TGGTGATGGTCGGCTGGGGGATGGC	237	65	30	30
<i>β</i> -Actin F: TGACGGGGTCACCCACACTGTGCCATCTA R: CTAGAAGCATTTGCGGTGGACGATGGAGGG	661	60	60	25
Bisulphite sequencing F: AAATATCCCAAACACTAACCTCTA R: TTTATTTGAGTTTGAGATTTTGAT	622	50	60	35

factors in adipogenesis. These observations suggest that certain tissue-specific transcription factors might be involved in the activation of *CIDE* genes.

In addition to the genetic regulation of gene activation that involves transcription factors, epigenetic controls provide another important mechanism for the tissue- and cell-specific expression of genes. Major epigenetic mechanisms include DNA methylation and histone modification. In mammalian cells, DNA methylation occurs predominantly at cytosine residues in the dinucleotide sequence CpG, and such methylation regulates gene expression through several distinct mechanisms. It can act directly by blocking regulatory factors from binding to their target sequences, and it can repress gene expression via the actions of MeCPs (methyl-CpG-binding proteins) (reviewed in [15]). Moreover, the apoptotic pathway can be inactivated via DNA methylation [16], and several apoptosis-associated genes (*BNIP3* [17], *DAPK* [18], *p14^{ARF}* [19], *EDNRB* [20], *RASSF1A* [21], *CDH1* [22], *TMS1* [23], and *Apaf-1* [24]) whose expression is regulated directly or indirectly by methylation have been described. However, there are no reports, to our knowledge, of the epigenetic control of the expressions of *CIDE*s, and the mechanisms that regulate the specific expression of *CIDE* genes have not been conclusively defined.

We present here an analysis of the regulatory region of the human *CIDE-B* gene, and demonstrate that the cell-specific expression of two transcripts of *CIDE-B* is driven by upstream and internal promoters (Pu and Pi respectively) by epigenetic and genetic mechanisms respectively. Expression of the long transcript is regulated by DNA methylation of the Pu region, whereas that of the short transcript is activated by HNF4 α (hepatocyte nuclear factor α), a nuclear receptor that is required for the differentiation of mammalian hepatocyte and for the normal regulation of liver metabolism [25], via interaction with its RE (response element) in the Pi. Our results also show that Sp1 and Sp3 are key regulators that are required for basal activation of both promoters.

EXPERIMENTAL

Cell culture

All cell lines used in this study were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were maintained in our laboratory. BEL-7404 cells (human hepatocellular carcinoma), HEK-293T cells and HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) (Gibco BRL, Grand Island, NY, U.S.A.), Jurkat cells and SMMC-7721 (human hepatocellular carcinoma) cells were maintained in RPMI 1640 medium (Gibco BRL), and HepG2 cells were maintained in MEM (minimum essential medium) (Gibco BRL). All media were supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum (Gibco BRL). All the cells were cultured at 37°C in 5% CO₂.

RNA analysis

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA (5 μ g) was reverse transcribed using oligo(dT)₁₈ primer and SuperScript II First-Strand Synthesis System (Invitrogen) in a reaction volume of 20 μ l, according to the manufacturer's instruction. The primers used for the RT (reverse transcription)-PCR *CIDE-B*, *HNF4α* and β -actin and the PCR protocols are shown in Table 1.

CpG island predictions

The presence of a putative CpG island in proximity to the *CIDE-B* gene was analysed by the CpG Island Searcher program (<http://www.uscnorris.com/cpgislands/cpg.cgi>) [26] with the default setting (% GC > 55%, ObsCpG/ExpCpG > 0.65, length > 500 bp).

Plasmids

To clone the promoter region of the human *CIDE-B* gene, a 1534 bp genomic fragment (from -4177 to -2644 relative to the

translation start site), containing the 5' upstream region and exon 1 of the *CIDE-B* gene, was amplified by PCR, using high-fidelity LA Taq polymerase (Takara, Dalian, China) and human genomic DNA from BEL-7404 cells. A 2642 bp genomic fragment (from -2642 to -1) between a CpG island and the translation start site of the *CIDE-B* gene was amplified in a similar manner. Various deletions of the *CIDE-B* promoters were created using these two fragments as templates for PCR amplifications. Also, XhoI and HindIII restriction sites were added to the 5' end of the sense and antisense primers respectively. The amplified fragments were ligated into the XhoI-HindIII site of the pGL3-Basic vector (Promega, Madison, WI, U.S.A.) and the sequences of the products were confirmed by direct sequencing.

Site-directed mutagenesis of Pu-Sp1 (-3452/-3457) in p(-3628/-3345), Pi-Sp1 (-193/-188) in p(-204/-1), and two HNF4 α REs (RE1, -142/-130; RE2, -174/-162) in p(-204/-1) was introduced by PCR, and the mutagenesis confirmed by direct sequencing. Detailed procedures and conditions and various primer sequences are available upon request from the authors.

Expression vectors for human HNF4 α and DN-HNF4 (dominant-negative HNF4 α) [27] were kindly provided by Dr Todd Leff (Department of Pathology, Wayne State University School of Medicine, Detroit, MI, U.S.A.).

Methylation *in vitro*

The *CIDE-B* Pu-reporter construct and the pGL3-Basic vector were methylated by incubation with SssI methylase (New England BioLabs, Beverly, MA, U.S.A.) for 3 h at 37°C in the presence of 160 μ M S-adenosylmethionine. The methylation status was verified by digestion with a methylation-sensitive enzyme, HapII, or an insensitive enzyme, MspI.

Transfections and luciferase assays

For transient transfections, BEL-7404 and HEK-293T cells were plated into 24-well plates the day before transfection at a density of 4×10^4 and 2×10^5 cells/well respectively. Cells were transfected with 0.4 μ g of various luciferase constructs using LipofectamineTM reagent (Gibco BRL) according to the manufacturer's instructions. The pRL-TK plasmid (Promega; 40 ng per sample) containing the *Renilla* luciferase gene driven by the herpes simplex virus thymidine kinase promoter was co-transfected with the constructs, and the luciferase activity was normalized. In addition, 0.15 μ g of HNF4 α or DN-HNF4 α expression vector, or the empty vector, was included in co-transfection experiments. The preparation of cell lysates and measurements of luciferase activity were performed by using the Dual Reporter assay system (Promega) and Lumat LB 9507 luminometer (EG and G. Berthold, Berlin, Germany) according to the manufacturers' instructions. All transfections were performed three times in triplicate.

Sodium bisulphite genomic sequencing

Sodium bisulphite modification of genomic DNA was performed as described previously [28]. Briefly, 2 μ g of genomic DNA, which had been digested with EcoRI and EcoRV, was denatured in 0.3 M NaOH for 15 min at 37°C and then treated with 3 M sodium bisulphite (Sigma, St. Louis, MD, U.S.A.) and 10 mM hydroquinone (Sigma) for 16 h at 50°C. The DNA was then desalted using Wizard DNA Clean-Up System (Promega), desulphonated in 0.3 M NaOH and precipitated. The treated DNA was amplified by PCR in a 50 μ l reaction volume with the bisulphite-specific primers 5'-TTTATTTGAGTTTGGAGATT-TTGAT-3' (sense) and 5'-AAATATCCCAAACACTAACCTC-

Figure 1 Cell-specific expression and genomic structure of the human *CIDE-B* gene

(A) Analysis of the expression of *CIDE-B* in six human cell lines. Total RNA was extracted, reverse transcribed and amplified with primers specific for *CIDE-B* CDS (Table 1). The amplified fragment of the *CIDE-B* CDS was 200 bp. A 661 bp fragment of the β -actin gene was used as a positive control for RNA integrity. (B) Analysis of the expression of two transcripts of *CIDE-B*. The cDNA used in (A) was amplified with primers for the long (L) and the short (S) transcript of the *CIDE-B* gene. The primers were designed on the basis of the previously reported sequences of 2289 bp (long version, accession no. NM_014430) and 1511 bp (short version, accession no. AF218586) cDNAs (Table 1). The fragment amplified from the long transcript was 432 bp, and that from the short transcript was 576 bp. (C) Structure of the human *CIDE-B* locus. Boxed arrows represent published cDNAs that encode known proteins. The open, grey and black boxes represent the untranslated regions, ORFs and predicted CpG island respectively. Note that, within the CpG island, exon 1 of *CIDE-B* is located close to the exon 1 of *LTB4R* and overlaps with the ORF of *LTB4R2*. Accession numbers are as follows: *CIDE-B* short cDNA, AF218586; *CIDE-B* long cDNA, NM_014430; *LTB4R*, D89079; *LTB4R2*, NM_019839.

TA-3' (antisense). The purified PCR products were cloned into the pMD 18-T vector (Takara), and five clones were sequenced for each sample.

EMSA (electrophoretic mobility shift assays) and protein analysis

HEK-293T cells were transiently transfected with 10 μ g of HNF4 α expression vector or empty vector pcDNA3, as described above. Nuclear extracts were prepared from BEL-7404 and HEK-293T cells as described by Gazzoli and Kolodner [29]. Nuclear extracts containing 10 μ g of protein were pre-incubated in 20 μ l of binding buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 μ g/ml poly(dI-dC) · poly(dI-dC) and 4 % glycerol) with or without unlabelled competitor (a 200-fold molar excess). For supershift assays, 1 μ g of antibody or rabbit IgG (Sigma) was added to the pre-incubation mixture. After 30 min pre-incubation on ice, the DNA probe (20000 cpm) labelled with [α -³²P]dCTP was added, and the samples were incubated at room temperature for 30 min. Reaction mixtures were separated on 4 % polyacrylamide (Sp1/Sp3 assay) or 6 % polyacrylamide (HNF4 α assay) gels. The gel was dried and subjected to PhosphorImage analysis using a Storm 860 system and ImageQuant TL software (Amersham Biosciences, Sunnyvale, CA, U.S.A.). Antibodies used in supershift assays were rabbit polyclonal antibodies against Sp1 (sc-59X), Sp3 (sc-644) and HNF4 α (sc-8987X) from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The sequences of oligonucleotides used in EMSAs were as follows (binding sites of transcription factors are underlined): Pi-Sp1, 5'-AGGACCC-CATCTGGCCCCCTCCCTCATCCCTCCC-3' and 5'-AAGGGG-AGGGATGAGGGAGGGGCCAGATGGGGTC-3'; and HNF4 α

Figure 2 Identification of the Pu and regulation of the Pu activity by Sp1/Sp3

(A) Mapping of the minimal Pu region of the *CIDE-B* gene. The CpG island (black box) and exon 1 of *CIDE-B* (open box) are indicated. Numbers above the constructs indicate positions relative to the translation start site. The activity of each promoter construct is shown as the luciferase activity relative to that of the pGL3-Basic vector (a promoterless vector). The activities in BEL-7404 cells (black bars) and HEK-293T cells (grey bars) are shown as the means $\pm$ S.D. for three independent experiments, each of which was performed with triplicate samples. (B) Mutation or deletion of the Sp1-binding site represses the activity of the Pu. Horizontal arrows on the upper left indicate the minimal promoters of the *CIDE-B* and *LTB4R* genes. The Sp1-binding site in the overlapping region is indicated by a black ellipse. Numbers above constructs indicate positions relative to the translation start site. Mutations are shown in bold next to the construct. Each promoter activity is shown as the luciferase activity relative to that of the pGL3-Basic vector. The activities in BEL-7404 cells (black bars) and HEK-293T cells (grey bars) are shown as the means $\pm$ S.D. for three independent experiments, each of which was performed with triplicate samples.

RE1, 5'-CCAGGCAGGGGGGCCAGAGTCCAGGCTTGACT-CATTC-3' and 5'-GGGAATGAGTCAAGCCTGGACTCTGG-CCCCCTGC-3'. Mutant probes were created by changing the Sp1-binding sequence from CCTCCC to CTTTTC to create Sp1m and by changing HNF4 α RE1 from GGGCCAGAGTCCA to GGGCTTTTTTCTT to create HNF4 α RE1m.

For Western blotting analysis, total cell samples were harvested by applying 2 $\times$ SDS Laemmli sample buffer directly on to cell monolayers after three washes with PBS. Electrophoresis, transfer of proteins on to nitrocellulose membranes and blocking of membranes were performed as described previously [30]. Antibodies specifically against HNF4 α (anti-HNF4 α , sc-8987X) and tubulin (Ab-4; NeoMarkers, Fremont, CA, U.S.A.) were incubated, at dilutions of 1:10 000 and 1:500 respectively, with the membranes for 1 h at room temperature. Secondary antibody [goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz)] was used at a dilution of 1:2000, and incubated with membranes for 1 h at room temperature. Proteins revealed by Western blotting were visualized by chemiluminescence.

RESULTS

Cell-specific control of the generation of two transcripts of *CIDE-B*, and the structure of the *CIDE-B* locus

To confirm that the transcription of the *CIDE-B* gene is regulated in a tissue- and cell-specific manner, we investigated the expression of *CIDE-B* in various cell lines using the primers for *CIDE-B* CDS (coding sequence), the long and the short transcript of *CIDE-B* respectively (Table 1). RT-PCR analysis revealed that BEL-7404 and HepG2 cells expressed *CIDE-B* mRNA, whereas SMMC-7721, HEK-293T, HeLa and Jurkat cells did not (Figure 1A). Furthermore, both the long and the short transcript were detected in BEL-7404 cells, but the long version was not detected in HepG2 cells (Figure 1B).

The sequences corresponding to the two reported cDNAs that encode *CIDE-B* [5,11] were mapped to chromosome 14q11. As shown in Figure 1(C), the short transcript [5] consists of five exons (exons 3A, 4, 5, 6 and 7), whereas the long version [11] includes two additional 5' untranslated exons (exon 1 and exon 2) and a

variant exon 3 (exon 3B). Both transcripts have the same ORF (open reading frame). This region also includes two other genes located on the reverse strand of *CIDE-B* [31]. These genes encode the leukotriene B₄ receptor (LTB₄R; GenBank® accession no. D89079) and LTB₄R2 (GenBank® accession no. NM_019839). The reported transcription initiation site of the *LTB₄R* gene [32] is located only 113 bp upstream from the first exon of *CIDE-B*.

The CpG Island Searcher program revealed a CpG island (from –3965 to –2674) spanning the 5′ upstream area and exon 1 of the *CIDE-B* gene. To simplify the nomenclature, we attributed the position number +1 to the translation start site of the *CIDE-B* gene.

Identification of the Pu of *CIDE-B*

We first constructed a luciferase reporter plasmid [p(–4177/–2644)] that contained a 1534 bp genomic DNA fragment (from –4177 to –2644), spanning the 5′ upstream region and exon 1 of *CIDE-B*. This plasmid was used for transient transfection of BEL-7404 and HEK-293T cells. BEL-7404 cells expressed *CIDE-B*, whereas HEK-293T cells did not (Figure 1). The cloned fragment exhibited significant promoter activity in both cell lines, indicating that we had isolated an active Pu sequence (Figure 2A). Next, we created a series of deletion constructs to identify the minimal promoter required for activation. As shown in Figure 2(A), deletion in the 3′ region from –3345 to –3478 completely abolished the activity of the reporter gene, and allowed us to locate the minimal Pu within the 284 bp region between positions –3628 and –3345.

The minimal Pu overlapped, on the reverse strand, the *LTB₄R* promoter. The overlapping region contains an Sp1-binding site that has been shown to bind both Sp1 and Sp3, with activation of the basal transcription of *LTB₄R* [32]. To investigate the role of this element in the activity of the Pu, deletion and site-directed mutagenesis of the Sp1 site were performed. In both BEL-7404 and HEK-293T cells, the luciferase activities of the mutated constructs were reduced to ~30% or even less of the wild-type activity (Figure 2B), indicating that Sp1 and Sp3 are major regulators of the expression of both *CIDE-B* and *LTB₄R*.

Methylation of CpG sites in the Pu

The Pu is in a CpG island and partially overlaps the *LTB₄R* promoter, whose activity is influenced by DNA methylation [32]. We then examined the methylation status of 32 CpG sites in the minimal Pu region by sodium bisulphite sequencing. As shown in Figure 3(B), the overall percentage of methylated CpG sites was only 10% in BEL-7404 cells, and thus this region was almost unmethylated. By contrast, in the cells in which the long transcript of *CIDE-B* was silenced (HepG2, SMMC-7721, HEK-293T, HeLa and Jurkat cells), there was a high degree of methylation at each CpG site examined. These results led us to the conclusion that methylation inhibits the synthesis of the long transcript of *CIDE-B*. Thus the effect of methylation on the Pu activity was investigated. We modified promoter constructs with SssI methylase and examined the activity of the methylated promoter. When we introduced these constructs into BEL-7404 cells, the activity of the methylated construct was only ~5% of that of the unmethylated construct (Figure 3C). We obtained similar results after transfection of HEK-293T cells with these constructs. These observations support the conclusion that methylation of the CpG sites inhibited the activity of the Pu.

Identification of the Pi of *CIDE-B*

The 5′ untranslated regions of the two transcripts of *CIDE-B* are different, although the translation start site is identical (Figure 1C).

Figure 3 Methylation of CpG sites in the Pu

(A) Representation of the *CIDE-B* 5′ region. Numbers indicate positions relative to the translation start site. The minimal Pu is indicated by the grey box. The position of each CpG dinucleotide is indicated by a short vertical line. (B) Pu bisulphite sequencing. DNA from BEL-7404, HepG2, SMMC-7721, HEK-293T, HeLa and Jurkat cells was treated with bisulphite, amplified by PCR, subcloned and sequenced. For each cell line, the methylation status of 32 CpGs in the promoter region is shown for five clones. White circles indicate unmethylated CpGs and black circles represent methylated CpGs. Circles enclosed by the boxes represent the CpG in the Sp1-binding site. (C) Effect of *in vitro* methylation on the activity of the Pu. pGL3-Basic and p(–3628/–3345) were methylated *in vitro* by SssI methylase. Methylated and unmethylated pGL3-Basic and p(–3628/–3345) were introduced into BEL-7404 and HEK-293T cells and assayed for activity. Results are shown as percentages, with luciferase activity due to unmethylated p(–3628/–3345) designated 100%. Results are the means ± S.D. for three independent experiments, each of which was performed with triplicate samples.

Moreover, the transcripts have different tissue- [5] and cell-specific (Figure 1B) expression patterns. These observations suggest that the expression of the short transcript might be regulated by another cryptic promoter.

To test this hypothesis, we constructed a luciferase reporter plasmid [p(–2642/–1)] that contained the 2.6 kb region (positions –2642 to –1) between the CpG island and the translation start site, as well as several constructs that contained fragments of this region with stepwise deletions from the 5′ and 3′ side. These plasmids were used for transient transfection of

Figure 4 Mapping the minimal Pi region of the *CIDE-B* gene

(A) Serial deletion analysis of the Pi. Horizontal arrows indicate the long and the short transcript of the *CIDE-B* gene. The Pu is indicated by a black box. Numbers above constructs indicate the positions relative to the translation start site. Each promoter activity is shown as the luciferase activity relative to that of the pGL3-Basic vector. The activities in BEL-7404 cells (black bars) and HEK-293T cells (grey bars) are shown as the means $\pm$ S.D. for three independent experiments, each of which was performed with triplicate samples. (B) Nucleotide sequence of the minimal Pi region. The Sp1-binding site and two HNF4 α response elements (RE1 and RE2) are boxed. The consensus sequences recognized by Ap1 and CREB (cAMP-response-element-binding protein) are underlined.

BEL-7404 and HEK-293T cells. The luciferase activity of the 2.6 kb promoter construct [p(-2642/-1)] was approx. 10-fold higher than that of a promoterless control construct (pGL3-Basic) in BEL-7404 cells, whereas the activity was approx. 3-fold higher than that of the control vector in HEK-293T cells, indicating that the 2.6 kb region contains an internal promoter with significant cell-specific activity (Figure 4A). The highest promoter activity was observed in BEL-7404 cells that had been transfected with the construct p(-204/-1), which contained the region between -204 and -1. This activity was completely abolished by further deletion of the region from -204 to -149. These results suggest that the cell-specific activity of the Pi promoter is determined by the region between -204 and -1, and that the region from -204 to -149 is critical for basal promoter activity. The nucleotide sequence of the Pi region is shown in Figure 4(B). There is only one CpG dinucleotide, located at positions -8/-9. We searched for transcription factor binding sites using the MatInspector program [33] and found consensus sequences for the binding of Sp1/Sp3, HNF4 α , Ap1 and CREB (cAMP-response-element-binding protein).

Sp1 and Sp3 bind to the Pi and regulate its activity

Luciferase assays demonstrated that the region between -204 and -149 represented a *cis* element that is important in the

basal transcription of the short transcript of *CIDE-B*. This region includes a consensus sequence for binding of Sp1 (Figure 4B). We first performed EMSAs to determine whether Sp1 and Sp3 bind to this region. Several DNA-protein complexes were observed in nuclear extracts of both BEL-7404 and HEK-293T cells (Figure 5A, lanes 2 and 10). These complexes represented sequence-specific interactions of proteins with this region, since the addition of a 200-fold molar excess of unlabelled probe eliminated the detectable complexes (Figure 5A, lanes 3 and 11), whereas an excess of an oligonucleotide with a mutated putative Sp1-binding site (5'-CTTTTC-3' at -193 to -188; mutation sites are indicated in bold) did not compete away the binding (Figure 5A, lanes 4 and 12). The slowest migrating DNA-protein complex was supershifted by the Sp1-specific antibody (Figure 5A, lanes 6 and 14), whereas the two rapidly migrating bands were supershifted by the Sp3-specific antibody (Figure 5A, lanes 7 and 15). No supershift was observed, however, when we used control IgG instead of specific antibodies (Figure 5A, lanes 5 and 13). Our results showed that Sp1 and Sp3 bound to the Sp1 site at -193/-188 in the Pi. We next investigated the effect of Sp1 binding on the Pi activity by site-directed mutagenesis (Figure 5B). The luciferase activity of the mutated construct was decreased to $\sim$ 14% and $\sim$ 25% of the wild-type in BEL-7404 and HEK-293T cells respectively. These results indicated that Sp1 and Sp3 bound to the Pi and regulated basal promoter activity.

Figure 5 Sp1 and Sp3 bind to and regulate the Pi

(A) We performed EMSAs using nuclear extracts prepared from BEL-7404 and HEK-293T cells with a 32 P-labelled oligonucleotide that encompassed the Sp1-binding site. The DNA-protein complexes were detected in both lines of cells (lanes 2 and 10). They were eliminated by an excess of unlabelled wild-type oligonucleotide (Sp1 wt × 200; lanes 3 and 11), but not by an excess of a mutated oligonucleotide (Sp1m × 200; lanes 4 and 12). The complexes were supershifted in the presence of Sp1-specific antibody (Sp1 Ab; lanes 6 and 14), Sp3-specific antibody (Sp3 Ab; lanes 7 and 15), and both antibodies (lanes 8 and 16), but not in the presence of the control IgG (lanes 5 and 13). (B) The effect of mutation of the Sp1-binding site on the activity of the Pi. Luciferase activities due to the wild-type construct [p(−204/−1)] and the mutated construct [p(−204/−1)Sp1m] in BEL-7404 cells (black bars) and HEK-293T cells (grey bars) are shown as the means ± S.D. for three independent experiments, each of which was performed with triplicate samples. Mutations are shown in bold above the histogram.

Transactivation of the Pi by HNF4α

Analysis of the sequence of the Pi revealed two potential REs of HNF4α (Figure 4B), a nuclear receptor that is expressed at high level in liver. RT-PCR analysis showed high expression levels of *HNF4α* mRNA in HepG2 and BEL-7404 cells and very weak expression in SMMC-7721 cells, whereas there was no expression of *HNF4α* mRNA in HEK-293T, HeLa and Jurkat cells (Figure 6A). Given the significant activity of the Pi in liver cells, and the similar expression patterns of *CIDE-B* short transcript and *HNF4α*, we postulated that HNF4α might be involved in the synthesis of the short transcript of *CIDE-B*.

To test our hypothesis, the BEL-7404 and HEK-293T cells were transfected with the Pi reporter construct [p(−204/−1)] together with expression vectors for HNF4α or DN-HNF4α (Figure 6B). In BEL-7404 cells, the expression of exogenous HNF4α resulted in a 1.4-fold increase in luciferase activity, whereas expression of DN-HNF4α decreased the promoter activity to below the

control (empty vector) level, suggesting that HNF4α can activate the Pi and that DN-HNF4α can repress the promoter activity by forming defective heterodimers with endogenous HNF4α [27]. More significant activation of luciferase activity by HNF4α was observed in HEK-293T cells (4-fold increase) (Figure 6B), confirming the importance of HNF4α in the activation of the Pi.

We then mutated the two putative HNF4α REs to examine their effects on the activity of the Pi promoter. As shown in Figure 6(B), mutation of RE1 reduced the luciferase activity to ~16% of that of the wild-type and, in addition, completely abolished the HNF4α-dependent induction of the activity of the Pi. Although mutation of RE2 also reduced the luciferase activity, co-transfection of the HNF4α expression vector doubled the luciferase activity. We obtained similar results after transfection of HEK-293T cells with these same constructs. These observations indicate that RE1 does, indeed, confer responsiveness on HNF4α on the Pi.

To investigate whether HNF4α activates endogenous Pi, we introduced exogenous HNF4α into HEK-293T cells and examined the expression of *CIDE-B* by RT-PCR. We detected the short transcript of *CIDE-B* in the HNF4α-transfected cells, but not in untransfected cells and control cells (Figure 6C, upper panel), which suggested the activation of the endogenous Pi by HNF4α. No expression of the long transcript of *CIDE-B* was detected after introduction of exogenous HNF4α, indicating that HNF4α does not influence the activity of the Pu. The expression of exogenous HNF4α was confirmed by Western blotting as shown in Figure 6(C, lower panel).

We next investigated the binding of HNF4α to RE1 by EMSA, using a probe that encompassed this region (Figure 6D). We performed the assay using the nuclear extract of HNF4α-transfected HEK-293T cells. We observed a shifted band in the analysis of HNF4α-transfected cells, but not in that of control cells (Figure 6D, lanes 2 and 1). This binding was almost abolished in the presence of an excess of unlabelled oligonucleotide (a 200-fold molar excess) (Figure 6D, lane 3). However, an excess of mutant competitor did not eliminate the binding (Figure 6D, lane 4). Inclusion of a HNF4α-specific antibody in the reaction mixture resulted in a supershift (Figure 6D, lane 6), but control IgG did not have such an effect (Figure 6D, lane 5). These results, together with those of our transactivation studies, suggested that HNF4α binds specifically to RE1 and activates the synthesis of the short transcript of *CIDE-B* gene.

DISCUSSION

In the present study, we characterized two mechanisms that control the *CIDE-B* gene cell-specific expression. We identified two promoters of the *CIDE-B* gene using 5' flanking region-luciferase fusion plasmids. The region (Pu) between −3628 and −3345 is required for the basal expression of the long transcript of *CIDE-B*, whereas the region (Pi) between −204 and the translation start site is required for the expression of the short transcript. An Sp1 site (−3452/−3457) in the Pu promoter with Sp1- and Sp3-binding ability has been reported previously [32]. We found that another Sp1 site (−193/−188) in the Pi region can also bind Sp1 and Sp3, as demonstrated by EMSAs (Figure 5A). The activities of Pu and Pi were greatly reduced when we introduced mutations at the Pu-Sp1 and Pi-Sp1 sites. Our results showed clearly that Sp1 and Sp3 are important for the basal expression of both the long and the short transcript of the *CIDE-B* gene. Sp1 and Sp3 are ubiquitous transcription factors that bind to DNA with similar specificity and affinity. It has been suggested that the relative levels of Sp1 and Sp3 can vary during cellular

Figure 6 HNF4α transactivates the Pi

(A) RT-PCR analysis of expression of the *HNF4α* gene in six human cell lines. The amplified fragment of the *HNF4α* gene was 200 bp. A 661 bp fragment of the *β-actin* gene was used as a positive control for RNA integrity. (B) HNF4α increased the activity of the Pi *in vitro*. Promoter-reporter constructs were co-transfected with the HNF4α expression vector or the vector that expressed DN-HNF4α into BEL-7404 cells and HEK-293T cells. Each promoter activity is shown as the luciferase activity relative to that of the pGL3-Basic vector. The activities are shown as the means ± S.D. for three independent experiments, each of which was performed with triplicate samples. Mutations are shown in bold below the histogram. (C) HNF4α activated expression of the short transcript of *CIDE-B* *in vivo*. Upper panel, RT-PCR analysis of *CIDE-B* expression. The short transcript (S) was detected in HEK-293T cells transfected with the HNF4α expression vector, but not in control (pcDNA3-transfected) or untransfected cells. The long transcript (L) was not detected in cells that expressed HNF4α transgene. A fragment of the *β-actin* gene was used as a positive control for RNA integrity. Lower panel: Western blotting analysis of expression of exogenous HNF4α in transfected HEK-293T cells. A tubulin antibody was used as a protein-loading control. (D) Binding of HNF4α to RE1. We performed EMSAs using nuclear extracts of HEK-293T cells transfected with the HNF4α expression vector with a ³²P-labelled oligonucleotide that encompassed the HNF4α RE1. The DNA-protein complex was detected in the transfected cells (lane 2). It was competed by an excess of unlabelled wild-type oligonucleotide (RE1 wt × 200; lane 3), and not by an excess of mutated oligonucleotide (RE1m × 200; lane 4). The band of the complex was supershifted in the presence of HNF4α-specific antibody (HNF4α Ab; lane 6), but not in the presence of the control IgG (lane 5).

differentiation, thereby modulating the responses of target genes [34]. We detected significant basal activity of the Pu in HEK-293T cells which do not normally express *CIDE-B*. Thus it appears that Sp1 and Sp3 might not determine the cell-specific expression of the long transcript of *CIDE-B*. However, given the rather low activity of the Pi promoter in HEK-293T cells, we cannot exclude the possible effects of Sp1 and Sp3 on the cell-specific expression of the short transcript.

We found that the Pu-Sp1 site functions, in both directions, as an activator of the expression of the *CIDE-B* and *LTB4R* genes. A similar bidirectional Sp1-binding site has also been found in the ORF28 and ORF29 intergenic region of VZV (varicella-zoster virus) [35]. Furthermore, the tissue-specific expression pattern of the long transcript of *CIDE-B* is similar to that of the *LTB4R* gene, with both transcripts being detected in peripheral blood

lymphocytes/leucocytes, spleen and bone marrow, and not in many other tissues [5,36,37]. These findings are consistent with a previous report [38] that the majority of bidirectional genes are co-expressed and regulated by their shared regulatory elements.

Bisulphite sequencing analysis revealed that CpG sites in the Pu were hypomethylated in long transcript-expressing BEL-7404 cells, but they were hypermethylated in HepG2, SMMC-7721, HEK-293T, HeLa and Jurkat cells, which do not express the long transcript of *CIDE-B*. This result suggested a significant correlation between the expression of the long transcript and the methylation status of the Pu. Such a correlation is strongly supported by the observation that methylation of the Pu by SssI methylase significantly reduced the promoter activity. These results demonstrate clearly that the cell-specific expression of the long transcript is primarily dependent on the methylation of

the Pu region. There are two possible mechanisms by which CpG methylation can inhibit the binding of Sp1. The two methylated DNA-binding proteins MeCP1 [39] and MeCP2 [40] can compete with Sp1 for the same binding sites. Alternatively, the methylation of CpG sites can directly inhibit the binding of Sp1, as seen in a study of retinoblastoma gene expression [41]. It has been reported that Sp1 binds similarly to the methylated and the unmethylated Pu-Sp1 site [32], suggesting that MeCPs might play a role as repressors of the expression of *CIDE-B* long transcript and *LTB4R* gene.

There is only one CpG site within the 204 bp region of the Pi of the *CIDE-B* gene, which suggests that another regulatory mechanism, different from the epigenetic control of the Pu, might also be operative. This hypothesis is supported by results of reporter gene assays, which showed that the Pi promoter was strongly activated in BEL-7404 cells, but not in HEK-293T cells. This cell-specific activity of the Pi was consistent with the cellular expression pattern of the short transcript of *CIDE-B*. Moreover, we identified a functional HNF4 α RE between positions –142 and –130 in the Pi. This element bound HNF4 α in an EMSA, and efficiently mediated transactivation of the Pi by HNF4 α in BEL-7404 cells. HNF4 α is a nuclear hormone receptor that contributes to regulation of a large part of the liver transcriptome by binding directly to almost half of the actively transcribed genes [42]. Many nuclear hormone receptors interact with transcriptional co-activators with histone acetylase activity (reviewed in [43]). It has been shown that HNF4 α interacts directly with the co-activator complex TRAP/SMCC/Mediator, and its function on chromatin templates is TRAP/SMCC/Mediator dependent and stimulated by the histone acetylase co-activator p300 [44]. It is reasonable to postulate that the primary function of HNF4 α is to recruit co-activators to facilitate chromatin remodelling and the initiation of transcription [25]. This hypothesis is supported by our demonstration that expression of the short transcript of *CIDE-B* was re-activated in non-expressing HEK-293T cells upon introduction of a transgene for HNF4 α . Thus our results clearly indicate that HNF4 α is important for the cell-specific expression of the short transcript of *CIDE-B*. To our knowledge, this is the first demonstration that HNF4 α can regulate the expression of an apoptotic factor.

In conclusion, we have identified two promoters of the human *CIDE-B* gene, which drive the expression of two transcripts of the gene respectively. Sp1 and Sp3 are key regulators of the basal transcriptional activity of both the Pu and the Pi. The expression of the long transcript is regulated by an epigenetic mechanism that involves DNA methylation of CpG sites in the Pu region. We also identified *CIDE-B* as a novel target for HNF4 α , and demonstrated a possible role for this transcription factor in the cell-specific expression of the short transcript. Our results suggest a complex model for cell-specific transcriptional regulation of the human *CIDE-B* gene, which involves epigenetic and genetic control of two different respective promoters.

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