Transcriptional regulation of the human cystathionine β -synthase — 1b basal promoter: synergistic transactivation by transcription factors NF-Y and Sp1/Sp3

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Cystathionine β -synthase (CBS) catalyses the condensation of serine and homocysteine to form cystathionine, an intermediate step in the synthesis of cysteine. Human CBS encodes five distinct 5' non-coding exons, the most frequent termed CBS —1a and CBS —1b, each transcribed from its own unique GC-rich TATA-less promoter. The minimal transcriptional region (–3792 to –3667) of the CBS —1b promoter was defined by 5'- and 3'-deletions, and transient transfections of reporter gene constructs in HepG2 cells, characterized by CBS transcription exclusively from the —1b promoter. Included in this 125 bp region are 3 GC-boxes (termed GC-a, GC-b and GC-c), an inverted CAAT-box and an E-box. By gel-shift and supershift assays, binding of specificity protein (Sp)1 and Sp3 to the GC-box elements, upstream stimulatory factor 1 (USF-1) to the E-box, and both nuclear factor (NF)-Y and an NF-1-like factor

to the CAAT box could be demonstrated. By transient transfections and reporter gene assays in HepG2 and *Drosophila* SL2 cells, a functional interplay was indicated between NF-Y binding to the CAAT-box, or between USF-1 binding to the E-box, and Sp1/Sp3 binding to the GC-box elements. In SL2 cells, NF-Y and Sp1/Sp3 were synergistic. Furthermore, both Sp1 and the long Sp3 isoform transactivated the CBS —1b minimal promoter; however, the short Sp3 isoforms were potent repressors. These results may explain the cell- or tissue-specific regulation of CBS transcription, and clarify the bases for alterations in CBS gene expression in human disease and Down's syndrome.

Key words: basal promoter, chromosome 21, Down's syndrome, leukaemia.

INTRODUCTION

Homocysteine is formed from methionine as a result of various S-adenosylmethionine-dependent transmethylation reactions. When methionine levels are low, metabolism of homocysteine primarily involves the enzymes betaine-homocysteine methyltransferase and methionine synthase [1]. However, when methionine is present in excess, the transmethylation pathway predominates, resulting in the conversion of homocysteine into cysteine [1]. The first step of this pathway involves the pyridoxal 5'-phosphate-dependent enzyme, cystathionine β -synthase (CBS; EC 4.2.1.22), which catalyses the condensation of L-serine and L-homocysteine to form cystathionine [1]. Approx. 50% of the homocysteine formed is irreversibly converted by CBS and cystathionase into cysteine [1]. Cysteine is a major precursor of glutathione, the major redox metabolite in the cell.

The CBS gene product is a 63000 Da polypeptide [1]. The tetrameric protein is catalytically dependent on both haem and pyridoxal phosphate, and is allosterically regulated by S-adenosylmethionine [1]. The human CBS gene spans over 30 kb and consists of 23 exons, ranging in size from 42 to 209 bp [2]. The CBS polypeptide is encoded by exons 1–14 and exon 16 [2]. The human CBS gene encodes multiple mRNAs differing in their 5' untranslated regions (UTRs), resulting from the use of five alternative non-coding exons, designated CBS —1a–e, and a constant exon 0 [3,4]. Transcripts containing the CBS —1a and —1b exons appear to be most abundant, and are found in an assortment of adult and fetal tissues [3]. In contrast, usage

of exons CBS —1c, —1d and —1e seems to be rare [3]. There are at least two GC-rich TATA-less promoters immediately upstream of exons CBS —1a and —1b, containing numerous putative transcription elements [specificity protein (Sp)1, activator protein (AP)1 and AP2 etc.]; however, neither these elements nor their associated transactors have been confirmed. Upon transient expression in HepG2 and COS7 cells, the CBS —1b promoter is approx. 7–10-fold stronger than the CBS —1a promoter [2].

Loss of CBS gene expression and/or function have been adversely associated with human health and disease. For instance, severe CBS enzyme deficiencies result in the autosomal-recessive disorder, homocystinuria, characterized by mental retardation, lens dislocation, skeletal abnormalities, and elevated plasma and urine homocysteine and methionine levels [1,4]. Elevated homocysteine levels are associated with an increased risk of thromboembolism and the early onset of atherosclerosis. Partial deficiencies of CBS have been associated with hyperhomocysteinaemia, a major risk factor for atherosclerosis [1,5].

The CBS gene has been localized to human chromosome 21 (i.e. 21q22.3), and its overexpression is thought to be linked to certain phenotypic features of Down's syndrome (DS). Elevated CBS expression in DS results in low plasma homocysteine levels compared with non-DS individuals, and has been suggested to contribute to decreased incidence of atherosclerosis in DS patients [6]. Ueland et al. [7] proposed that increased CBS activity may alter the intracellular distribution of reduced folates,

Abbreviations used: CBS, cystathionine β -synthase; UTR, untranslated region; AML, acute myeloid leukaemia; DS, Down's syndrome; Sp, specificity protein; NF, nuclear factor; USF-1, upstream stimulatory factor 1; RT, reverse transcription; Ara-C, cytosine arabinoside; RACE, rapid amplification of cDNA ends.

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due to an accumulation of 5-methyl tetrahydrofolate, reflecting low levels of homocysteine for methionine synthase.

In our own studies of CBS and DS [8], we also found striking increases (approx. 12-fold) in CBS transcripts in DS myeloblasts from children with acute myeloid leukaemia (AML) compared with non-DS myeloblasts. Interestingly, this elevated CBS expression was associated with increased *in vitro* sensitivities to cytosine arabinoside (Ara-C) and generation of Ara-C triphosphate, possibly due to downstream effects of CBS on endogenous folate and nucleotide pools [8–11]. These effects may contribute to the high event-free survival rates (70–100%) and very low relapse rates (< 15%) when DS children with AML are treated with Ara-C-based protocols [12–14].

Thus additional studies of CBS gene regulation are clearly warranted in order to better understand the role of alterations in CBS gene expression in human disease, and also for further characterizing the molecular bases for the disparate therapeutic responses of DS AML patients to chemotherapy, including Ara-C. In the present paper, we significantly extend earlier reports of CBS promoter structure and function [2] by identifying the critical *cis*-elements and transcription factors that regulate CBS basal transcription from the major CBS –1b promoter.

MATERIALS AND METHODS

Chemicals and reagents

[γ - 32 P]ATP (3000 Ci/mmol) and [α - 32 P]dCTP (3000 Ci/mmol) were purchased from DuPont-New England Nuclear Life Science Products (Boston, MA, U.S.A.). Synthetic oligonucleotides were purchased from Genosys Biotechnologies (The Woodlands, TX, U.S.A.). Restriction and modifying enzymes, reporter gene vectors (pGL3-Basic and pRLSV40) and other molecular biologicals were purchased from Promega. The pPacSp1 and pPacO plasmid constructs were provided by Dr Robert Tijan (University of California, Berkeley, CA, U.S.A.), and the pPacSp3 and pPacUSp3 constructs were provided by Dr Guntram Suske (Philipps-Universität, Marburg, Germany). The pPacNF-YA, pPacNF-YB and pPacNF-YC plasmid constructs were provided by Dr T. F. Osborne at the University of California at Irvine, CA, U.S.A.

Cell culture

The HepG2 human hepatocellular carcinoma cell line was obtained from the A. T. C. C. *Drosophila* SL2 cells were provided by Dr Bonnie Sloane (Wayne State University, Detroit, MI, U.S.A.). The HepG2 cell line was maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere at 37 °C in the presence of air/CO₂ (19:1). SL2 cells were maintained at 25 °C in Schneider's medium supplemented with 10% fetal bovine serum and 2 mM glutamine plus antibiotics.

Analysis of CBS promoter usage by reverse transcription (RT)-PCR

Total RNA was isolated from 1×10^7 cultured cells, as previously described [7]. For the analysis of CBS promoter usage, first-strand cDNAs were synthesized from 2 μ g of total RNA in 20 μ l of a reaction mixture containing 2.5 μ M of random hexadeoxynucleotide primers, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 0.5 mM each dNTP and 50 units of Moloney murine leukaemia virus reverse transcriptase (PerkinElmer). After 1 h incubation at 42 °C, the reaction was stopped by heating at 95 °C for 10 min. Aliquots of the cDNA product were

PCR-amplified in a reaction mixture containing 1 μ l of RT product, 10 $\times$ PCR buffer (PerkinElmer), 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.2 μ M forward primer for the CBS –1a (5'-CCAA-GTAAACAGCATCGGAACACCA-3') or CBS –1b (5'-CG-CCCCTCTTTTCCATGTATCCGTCCAG-3') 5'-UTR, and 0.2 μ M of a common reverse primer (5'-ACCTGGCATT-GGTGGGCGTCCTCACA-3'; CBS/R2) from exon 5, and 1.25 units of *Taq* DNA polymerase (Promega). PCR was performed for 35 cycles of 30 s at 94 °C, 30 s at 65 °C, and 45 s at 72 °C in a PerkinElmer GeneAmp 9600 PCR system. Aliquots (20 μ l) were analysed on an agarose gel (1.5%) with ethidium bromide staining. 18 S ribosomal RNA (localized to chromosome 13; Ambion Inc., Austin, TX, U.S.A.) was used as internal control.

Rapid amplification of 5'-cDNA ends (5'-RACE) assay

To determine the transcription start sites of the CBS gene, 5'-RACE was performed using a 5'-RACE system (version 2.0; Life Technologies Inc.) and total RNA from HepG2 cells. The cDNA was first synthesized from 2 μ g of total RNA using Superscript[®] II reverse transcriptase (Life Technologies Inc.) and an antisense primer (CBS/R1, 5'-ATCTCATCAGCGGTGGTGTCTGTAG-TG-3', based on exon 6 sequence) at 37 °C for 1 h. The cDNA was purified by a GlassMax spin cartridge (Life Technologies Inc.), and used in terminal dideoxynucleotide-transferase tailing, then PCR-amplified with an internal antisense primer (CBS/R2, 5'-ACCTGGCATTGGTGGGCGTCCTCACA-3', based on exon 5 sequence) and a 5'-sense primer ('Abridged Anchor Primer'). This was followed by nested PCR using a nested gene-specific antisense primer (CBS/R3, 5'-ATCCACAGGGGCTC-CTGGCTTCCTT-3', based on exon 1 sequence) and a nested adapter primer ('Abridged Universal Amplification Primer'). PCR products were analysed on a 2% agarose gel, isolated, subcloned into pGEM-T Easy vector (Promega), and sequenced by automated sequencing using BigDye DNA Sequencing Kit (PE Applied Biosystems, Foster City, CA, U.S.A.).

Construction of luciferase plasmids and site-directed mutagenesis

The CBS –1b promoter was PCR-amplified from human genomic DNA isolated from leukaemic blasts from a patient with AML using nested external (forward: PROB/F1, 5'-CCACCAG-GACGCACGTTTCAAG CTCATCAGTA-3'; reverse: PROB/R1, 5'-GAAAACCTCCATCCCCCGAACGCAACC-3') and internal (forward: PROB/F2, 5'-ACAGCATCGGAACACC-AGGTAGAAGACACCAC-3'; reverse: PROB/R2, 5'-CGG-ATCTCACCTGGACGGATACATGGAAAAGA-3') primers, based on the human CBS genomic sequence (GenBank[®] accession number AF042836). The resulting 482 bp PCR product was subcloned into pGEM-T Easy vector and, subsequently, into pGL3-basic to generate pCBSb–4046/–3565. In order to generate the 5'-deletion clones, pCBSb–3938/–3565 (position –3938 to –3565) and pCBSb–3724/–3565 (position –3724 to –3565), the antisense CBS –1b promoter insert in pGEM-T-Easy was digested by *Psh*AI and *Pst*II, and *Nar*I and *Pst*II, respectively. The larger fragments were blunt-ended and religated. The 5'-deleted CBS –1b promoter fragments were isolated by digesting the antisense CBS –1b insert in pGEM-T-Easy with *Sac*I and *Nco*I, and subcloning into the same sites in pGL3Basic. The pCBSb–3792/–3565 (position –3792 to –3565 bp) construct was generated by direct digestion of pCBSb–4046/–3565 with *Sac*I and *Apa*I, followed by religation. pCBSb–3667/–3565 (position –3667 to –3565 bp) was generated by the digestion of pCBSb–4046/–3565 with *Sac*I and *Sal*DI and religation.

All the 3'-deletion constructs were prepared by similar methods with restriction enzyme digestions. Thus the pCBSb–4046/–3624

Table 1 Summary of double-stranded oligonucleotides

DNA sequences for double-stranded oligonucleotides used as competitive oligonucleotides in gel-shift assays and in the preparation of reporter gene constructs for transient transfection experiments. Lower case letters indicate the bases mutated. mt, mutant.

Oligonucleotide	Sequence
—3766/—3725	GGGGCTGGGCGGGGGTGTCTGCCACGAGACATTGGCCG
GC-c mt	GGGGCTGGGCGGGGGTGTCTGCCACGAGACATTGGCCG
USF-1 mt	GGGGCTGGGCGGGGGTGTCTGCCAAGACATTGGCCG
—3724/—3682	CGCCGGCCTCGCCCGCCCTCTCCGCCAATCACC GCCCGCCT
GC-b mt	CGCCGGCCTCGGCaCCaGCCTCTCCGCCAATCACC GCCCGCCT
NF-Y mt	CGCCGGCCTCGCCCGCCCTCTCCGCAATCACC GCCCGCCT
GC-a mt	CGCCGGCCTCGCCCGCCCTCTCCGCCAATCACC GcGcGcCT
CAAT	GGATCTAGGGTTGGCAATCTACTCCAGGAGCTT
Sp1	ATTCGATCGGGCGGGGGCGAGC
NF-1	TTTGGATTGAAGCCAATATGATAA
USF-1	CACCGGCTACGTGGCCTACACC
NF-Y	AGACCGTACGTGATTGGTTAATCTCTT

(position —4046 to —3624) was prepared by digestion of pCBSb—4046/—3565 with *Bsm*BI and *Bgl*II and religation, whereas pCBSb—4046/—3659 (position —4046 to —3659) was prepared by digestion of the PCR-generated 482 bp full length CBS —1b fragment with *Bst*UI, blunt-ending with T4 DNA polymerase, and ligation into the *Sma*I site of pGL3Basic. The 3'-deletion clone, pCBSb—4046/—3724 (position —4046 to —3724), was prepared by digestion of pCBSb—4046/—3565 with *Nar*I and *Nhe*I, generation of blunt-ends with T4 DNA polymerase, and ligation of the 350 bp fragment into pGL3Basic at the *Sma*I site. The pCBSb—4046/—3792 construct (position —4046 to —3792) was generated by direct digestion of pCBSb—4046/—3565 with *Apa*I and *Bgl*II, and religation.

A series of luciferase plasmids harbouring nucleotide substitutions which alter or abolish consensus sequences for transcription-factor binding was also constructed by site-directed mutagenesis using an overlap-extension PCR protocol [15]. Two separate PCR fragments for each half of a final hybrid product were generated with mutagenesis primers (Table 1) and PROB/F2 or PROB/R2. The two products were mixed, and a second PCR was performed using the two outside primers. The resulting products were blunt-ended and subcloned into the *Sma*I site of pGL3Basic. All the deletion and site-directed mutagenesis constructs were sequenced to confirm the intended deletions or mutations.

Transient transfections and luciferase assay

CBS-luciferase reporter gene constructs in pGL3 Basic or the promoterless pGL3 Basic vector (1 μ g) were co-transfected with 12.5 ng of pRLSV40 (Promega) into approx. 50% confluent HepG2 cells using Lipofectin reagent (Life Technologies) in accordance with the manufacturer's protocols. Lipofectin treatments were for 24 h and, after an additional 48 h of incubation in complete medium, cells were harvested and lysates prepared. Firefly luciferase activities were assayed with a Dual-Luciferase Reporter Assay System (Promega) in a Turner TD2420 luminometer, and normalized to *Renilla* luciferase activity.

Drosophila SL2 cells were co-transfected with 1 μ g of the CBS-luciferase reporter gene constructs and 25–200 ng of Sp1 (pPacSp1) or Sp3 (pPacUSp3 and pPacSp3, for the long and short isoforms respectively) or nuclear factor (NF)-Y (pPacNF-YA, pPacNF-YB, and pPacNF-YC; 25 ng each) cDNA constructs, using Fugene[™] 6 reagent (Roche Diagnostics), according to the manufacturer's recommendations. Cells were

harvested after 48 h for luciferase assays using the Single Luciferase Assay System (Promega). Luciferase activities were normalized to cellular protein, measured by the Bio-Rad protein assay system.

Preparation of nuclear extracts and electromobility shift assays

Nuclear extracts from HepG2 cells were prepared by standard methods [16], with slight modifications. Final protein concentrations were determined by the Bio-Rad protein assay. Complementary single-stranded oligonucleotides were annealed, end-labelled with ³²P, and purified using Sephadex G-25 quick spin columns (Boehringer Mannheim). Nuclear proteins were pre-incubated in a reaction solution containing 20 mM Tris/HCl, pH 7.9, 2 mM MgCl₂, 1 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P40 and 2 μ g of poly(dI-dC). Oligonucleotide competition analysis was conducted by pretreating with 100-fold molar excess of commercial competitor oligonucleotides [Sp1, NF-Y, upstream stimulatory factor 1 (USF-1) and NF-1; Santa Cruz Biotechnology] or 100-fold molar excess of unlabelled wild-type or mutant Sp1, USF-1 and NF-Y oligonucleotides. For supershift experiments, 2 μ g of rabbit polyclonal antibodies [Sp1 (Geneka Biotechnology, Inc., Montreal, QC, Canada); USF-1 and NF-1 (Santa Cruz Biotechnology)] or goat polyclonal antibodies [CAAT-binding factor-A, CAAT-binding factor-B and Sp3 (Santa Cruz Biotechnology)] were added to the reaction mixtures and incubated for 30 min. DNA-protein complexes were separated on 5% or 6% non-denaturing polyacrylamide gels in 0.5 $\times$ Tris/borate/EDTA (pH 8.4) at 4 $^{\circ}$ C and 35 mA. The gels were dried and the complexes were visualized by autoradiography.

RESULTS

CBS promoter usage analysis by RT-PCR and 5'-RACE in HepG2 cells

The human HepG2 hepatoma cell line expresses high levels of the major 2.9 kb CBS transcript on Northern blots (results not shown). Since two unique promoters were previously identified in the human CBS gene [2] (GenBank[®] accession number AF042836), designated —1a and —1b (Figure 1, upper panel), it was essential to identify which promoter(s) was being used. Accordingly, total RNAs were prepared from HepG2 cells and reverse transcribed, then PCR amplified with upstream primers specific for the CBS —1a or CBS —1b exons and a common downstream primer (CBS/R2; to exon 5). Our RT-PCR assay detected only the 701 bp product derived from the CBS —1b transcript (Figure 2, upper panel). An identical result was obtained by 5'-RACE analysis of the CBS 5'-UTRs. Four 5'-RACE clones were sequenced, and all were identical and began at position —3634 (Figure 2, lower panel). There were no sequence differences between the 5'-RACE products and the published GenBank[®] sequence for exon CBS —1b of the human CBS gene. These results establish that HepG2 cells exclusively use the —1b CBS promoter.

5'- and 3'-deletion analysis of the CBS —1b promoter: identification of the minimal promoter region

By database analysis (Transfac 3.2, MatInspector V2.2; Gesellschaft für Biotechnologische, Braunschweig, Germany), the —1b CBS promoter region contains numerous putative transcription elements (Figure 1, lower panel; [2]). In previous studies [2], promoter activity could be detected by transient transfections of CBS —1b reporter gene constructs (in the pGL3 enhancer plasmid) into COS7 and HepG2 cells. However, neither

Figure 1 Schematic of the upstream region of the human CBS gene and DNA sequence of the CBS -1b promoter

Upper panel: the upstream structure of the human CBS gene, including the CBS -1a and CBS -1b promoters and non-coding exons -1a-e and exon 0, and exon 1 including the translation start site (ATG). Numbering is relative to the translation start site (+1). Lower panel: the DNA sequence of the CBS -1b. Potential *cis*-regulatory elements on the plus (+) and minus (-) DNA strands (determined by Transfac 3.2, MatInspector V2.2) are underlined and shown in *italics*. The transcription start site, determined by 5'-RACE (Figure 2, lower panel), is shown by an asterisk. The boxed regions represent exon CBS -1a and CBS -1b sequences. Numbering is relative to the CBS translation start site. MZF1, myeloid zinc finger; IK2, Ikaros 2.

the regions of regulatory significance have been experimentally confirmed, nor have the major transcription factors that regulate CBS -1b basal transcriptional activity been identified to date.

Accordingly, progressive 5'-deletions were introduced into the CBS -1b promoter. The 5'-deletion constructs in pGL3Basic were transiently expressed in HepG2 cells and luciferase activities measured (Figure 3). A high level of promoter activity was detectable for the full-length (pCBSb -4046/-3565) construct. Deletion of 108 bp from the 5'-end of the full-length construct (e.g. pCBSb -3938/-3565) resulted in an approx. 70% loss of luciferase activity, which was essentially unchanged upon removal of an additional 146 bp (pCBSb -3792/-3565). Activity was completely abolished upon further removal of 125 bp (pCBSb -3724/-3565 and pCBSb -3667/-3565) from the 5'-end of pCBSb -3792/-3565.

To further delineate the CBS -1b minimal promoter region, progressive 3'-deletions were performed from positions -3565 to -3792 (Figure 3). 3'-deletions of 94 bp (to pCBSb -4046/-3659) were accompanied by only approx. 30% loss of activity; however, upon removal of an additional 133 bp (pCBSb -4046/-3792), luciferase activity was essentially undetectable (Figure 3). In combination with our 5'-deletion data, these results localize

the basal CBS -1b promoter to within 125 bp, between positions -3792 to -3667.

Gel-shift analysis of the CBS -1b minimal promoter region

The 125 bp CBS -1b minimal promoter region is GC rich (approx. 80%) and lacks a TATA-box (Figure 1, lower panel; [2]). By database analysis, 3 GC boxes [positions -3760 to -3749 (GC-c); -3715 to -3705 (GC-b); and -3691 to -3683 (GC-a)], an inverted CAAT-box (position -3698 to -3694), and a potential E-box (-3741 to -3736) were identified in this region. To explore the protein-binding associations with these potentially transcriptionally important regions, we used two double-stranded oligonucleotide probes, -3724/-3682 (includes GC-a and GC-b, and the CAAT-box) and -3766/-3725 (includes GC-c and one potential E-box), in gel-shift assays.

Five distinct complexes (numbered 1-5; Figure 4, lanes 2, 7 and 10) could be detected with the -3724/-3682 oligonucleotide and a nuclear extract prepared from HepG2 cells. All the complexes were completely abolished by a 100-fold excess of unlabelled -3724/-3682 oligonucleotide (Figure 4, lanes 3, 8

Figure 2 Analysis of CBS promoter usage by RT-PCR and 5'-RACE

Upper panel: total RNA was isolated from 1×10^7 HepG2 cells as described in the Materials and methods section. For the analysis of CBS promoter usage by RT-PCR, first-strand cDNAs were synthesized by random priming. Aliquots of the cDNA product were PCR-amplified using CBS exon -1a- or -1b-specific primers and a common reverse primer. The ethidium bromide-stained 1.5% agarose gel shows only products of the expected sizes for the CBS -1b (701 bp) and 18 S rRNA (488 bp) internal standard. None of the CBS -1a product (700 bp) was detected. Lower panel: for 5'-RACE analysis, first-strand cDNAs were synthesized with a CBS-specific primer (CBS/R1). After purification and polycytidylylated tailing, the CBS 5'-end cDNA was amplified with semi-nested PCR. PCR products were subcloned into pGEM-T-Easy vector. Four bacterial clones were picked and sequenced to determine the transcription initiation site and to confirm promoter usage. The Figure shows that the 5'-ends of the RACE products map identically to position -3634.

Figure 3 5'- and 3'-deletion analysis of the CBS -1b promoter

A series of 5'- and 3'-deletion constructs in pGL3-Basic were prepared as described in the Materials and methods section and transiently transfected into HepG2 cells. Numbering is based on the CBS -1b promoter sequence in Figure 1 (lower panel). Firefly luciferase activities were normalized to transfection efficiencies by co-transfection with *Renilla* luciferase plasmid. Luciferase activities for the deletion constructs were expressed relative to the activity for the full-length pCBSb-4046/-3565. The data are presented as the means $\pm$ S.D. from at least three experiments carried out in duplicate. Luc., luciferase.

and 11). Complexes 1 and 2 were competed by 100-fold molar excess of a commercial Sp1 consensus oligonucleotide (Figure 4, lane 4), and were supershifted by antibodies to Sp3 and Sp1 (Figure 5, lanes 7 and 9 respectively). Complexes 3 and 4 were competed by CAAT and NF-Y consensus oligonucleotides (Figure 4, lanes 5 and 6 respectively). Interestingly, the addition of CAAT and NF-Y consensus oligonucleotides also appeared to result in partial competition of complexes 1 and 2 (Figure 4, lanes 5 and 6), suggesting an effect on Sp1/Sp3 binding by CAAT-box-binding proteins. Supershifts of complexes 3 and 4 were observed with antibodies to NF-YA and NF-YB (Figure 5, lanes 4 and 5 respectively). Although complex 5 appeared to be partially competed with a 100-fold excess of the nuclear factor-

Figure 4 Gel-shift assays with the -3724/-3682 CBS -1b probe

Gel-shift assays were performed with HepG2 nuclear extracts and the 32 P-labelled -3724/-3682 probe in the absence and presence of commercial consensus oligonucleotides or mutant -3724/-3682 oligonucleotides. DNA sequences for the wild-type and mutant -3724/-3682 oligonucleotides and commercial competitive oligonucleotides are summarized in Table 1. The specific DNA-protein complexes are numbered (1-5). NA, no additions.

1 (NF-1) consensus oligonucleotide (Figure 4, lane 9), the complex could not be supershifted with NF-1 antibody (Figure 5, lane 6). Thus confirmation of its identity remains uncertain.

Although competition with Sp1 consensus oligonucleotides and supershifts with Sp1/Sp3 antibodies implied a role for Sp-factor binding in complexes 1 and 2, 100-fold excess of GC-a or GC-b mutant oligonucleotide (GC-a mutant or GC-b mutant; Table 1) effectively competed with complexes 1-5 nearly as well as the wild-type -3724/-3682 oligonucleotide (Figure 4, lanes 14 and 12 respectively). This may, in part, reflect cross-competition with the non-mutated GC-box in either the -3724/-3682 GC-a mutant or GC-b mutant oligonucleotide.

Figure 5 Supershift assays with the -3724/-3682 CBS -1b probe

Gel-shift assays were performed with HepG2 nuclear extracts and 32 P-labelled -3724/-3682 probe. For the supershifts, specific antibodies to Sp1 (Sp1-ab), Sp3 (Sp3-ab), NF-1 (NF-1-ab) and NF-Y (NFYA-ab and NFYB-ab) were added to the reaction mixtures and incubated for 30 min prior to separating the DNA-protein complexes. The specific DNA-protein complexes are numbered (1-5), and the supershifted (SS) complexes for Sp1, Sp3, NF-YA and NF-YB are noted. NA, no additions.

Figure 6 Gel-shift assays with the $-3766/-3725$ CBS $-1b$ probe

Gel-shift assays were performed with HepG2 nuclear extracts and the ^{32}P -labelled $-3766/-3725$ probe in the absence and presence of 100-fold molar excess of commercial consensus oligonucleotides or mutant $-3766/-3725$ oligonucleotides. DNA sequences for the wild-type and mutant $-3766/-3725$ oligonucleotides and commercial competitive oligonucleotides are summarized in Table 1. The specific DNA-protein complexes are numbered (1–3). NA, no additions; mt, mutant.

Figure 7 Supershift assays with the $-3766/-3725$ CBS $-1b$ probe

Gel-shift assays were performed with HepG2 nuclear extracts and ^{32}P -labelled $-3766/-3725$ probe. For the supershifts, specific antibodies to Sp1 (Sp1-ab), Sp3 (Sp3-ab) and USF-1 (USF-1-ab) were added to the reaction mixtures and incubated for 30 min prior to separating the DNA-protein complexes. The specific DNA-protein complexes are numbered (1–3), and the supershifted (SS) complexes for Sp1, Sp3, USF-1, NF-YA and NF-YB are noted. NS, non-specific complex; NA, no additions.

Interestingly, mutation of the CAAT-box in the full-length $-3724/-3682$ oligonucleotide (NF-Y mutant; Table 1) resulted in decreased competition for complexes 1 and 2 (Figure 4, lane 13) as well. The NF-Y mutant oligonucleotide also decreased binding to complex 5 (Figure 4, lane 13), consistent with the possibility of a DNA-protein complex involving binding of an NF-1-like factor at the CAAT-box.

With the upstream $-3766/-3725$ oligonucleotide, three specific DNA-protein complexes (numbered 1–3) were identified in a nuclear extract prepared from HepG2 cells (Figure 6, lanes 2 and 6). Complexes 1 and 3 were completely abolished by a 100-fold molar excess of Sp1 consensus oligonucleotide (Figure 6, lane 5). When the GC-box at position -3760 to -3749 was mutated in the $-3766/-3725$ oligonucleotide (i.e. GC-c mutant; Table 1), competition with complexes 1 and 3 was significantly abolished (Figure 6, lane 8). Complex 1 could be further resolved by supershifting with antibodies to Sp1 and Sp3 (Figure 7, lanes 7 and 5 respectively); complex 3 was also supershifted by Sp3

antibody (Figure 7, lane 5). Complex 2 was effectively competed by a USF-1 oligonucleotide (Figure 6, lane 4). When the USF-1 consensus element at position -3741 to -3736 was mutated (USF-1 mutant; Table 1), binding was abolished, as indicated by decreased competition on gel shifts (Figure 6, lane 9). This was accompanied by increased binding (decreased competition) for complexes 1 and 3. When complex 2 was supershifted with USF-1 antibody (Figure 7, lane 4), decreased signals were observed for complexes 1 and 3 as well.

These results demonstrate that an assortment of transcription factors can bind to the 125 bp CBS minimal promoter region, including Sp1, Sp3, NF-Y and USF-1. The finding that addition of CAAT and NF-Y consensus oligonucleotides, and that mutation of the CAAT element are all accompanied by apparent decreases in the relative binding of Sp1 and/or Sp3 to GC-a and GC-b sites in the $-3724/-3682$ oligonucleotide, suggests a possible functional interplay between these factors in the regulation of CBS $-1b$ promoter activity. Likewise, an interplay between E-box binding of USF-1 and Sp1/Sp3 binding to the GC-c element is implied.

Functional analysis of transcription-factor-binding sites by site-directed mutagenesis

Experiments were performed to confirm further the functional significance of the DNA-protein complexes detected by gel-shift analyses in Figures 4–7 in the transactivation of the CBS $-1b$ minimal promoter. The GC-a-c, CAAT and E-box consensus elements in the -3792 to -3667 basal region were mutated individually or in combination, and the basal mutant constructs in pGL3-Basic were transiently transfected into HepG2 cells. The mutant constructs are summarized in Table 1. Luciferase activities for the mutant constructs were compared with that of the wild-type basal promoter construct, pCBSb- $3792/-3565$ (Figure 8).

Although protein binding to the three conserved GC-boxes and the E-box element in the minimal CBS $-1b$ promoter was demonstrated on gel shifts, the inhibitory effects of mutations of these elements on CBS $-1b$ promoter activity were variable (Figure 8). Thus mutation of the GC-boxes at position -3691 to -3683 (GC-a mutant) or -3760 to -3749 (GC-c mutant) individually resulted in nearly identical losses of CBS $-1b$ promoter activity (approx. 30–35%), and only slightly greater than that (approx. 15%) resulting from mutation of GC-b (GC-b mutant; -3715 to -3705). Simultaneous mutation of all three GC-boxes resulted in approx. 40% decrease of the CBS $-1b$ promoter activity. A similar loss (approx. 30%) of CBS $-1b$ promoter activity accompanied mutation of the E-box element at -3741 to -3736 (USF-1 mutant; Table 1).

Mutation of the CAAT-box (NF-Y mutant) at position -3698 to -3694 completely abolished binding of NF-Y and an NF-1-like factor, as suggested by loss of competitive binding on gel shifts (Figure 4); this was accompanied by a potent (approx. 80%) loss of promoter activity (Figure 8). This inhibitory effect was not appreciably affected by the presence of mutated GC-b, and only slightly diminished with the mutated GC-a or -c in the same promoter construct (Figure 8). These results confirm an important transactivating role for the CAAT-box element at position -3698 to -3694 in the CBS $-1b$ promoter, and to a lesser extent, the GC-a-c and the E-box elements.

Functional analysis of GC-a-c, E-box and CAAT-box in *Drosophila* SL2 cells

Both Sp1 and Sp3 can exert activating or inhibiting effects on transcription, depending on the cell or promoter context. Fur-

Figure 8 Functional analysis of mutated regulatory sequences in the CBS $-1b$ minimal promoter in HepG2 cells

HepG2 cells were transfected with the wild-type and mutant CBS $-1b$ reporter constructs as described in Materials and methods section. The mutant constructs are summarized in Table 1. Luciferase activities of the mutant constructs are shown relative to the wild-type (unmutated) CBS $-1b$ basal construct (pCBSb-3792/-3565). The data are presented as means $\pm$ S.D. from three independent experiments in duplicate.

thermore, Sp3 exists as three isoforms with different translational starts and characterized by distinct transactivating/inhibiting properties [17]. NF-Y (also known as CAAT-binding factor) is composed of three subunits, NF-YA, NF-YB and NF-YC, and typically binds to a CAAT-box in the proximal region (-60 to -80 upstream of initiation) of a large number of promoters, where it participates in transcriptional activation [18].

To explore further the roles of the Sp family of transactors and NF-Y in CBS $-1b$ promoter activity, transient transfection experiments were performed in *Drosophila* SL2 cells, which provide a null background for these transcription factors [19,20]. The pCBSb-3792/-3565 reporter gene construct, including the CBS $-1b$ minimal promoter, was co-transfected with expression vectors for Sp1 (pPacSp1), NF-Y (pPacNF-Y) or the long (pPacSp3) isoforms of Sp3, all under the control of a *Drosophila*-specific promoter. Luciferase activities were compared with those obtained with the empty pPacO expression vector.

Co-transfection of pCBSb-3792/-3565 with pPacSp1, pPacSp3 or pPacNF-Y, individually (each at 25 ng), transactivated the basal promoter by 14-fold, 6-fold and 2-fold respectively (Figure 9A). However, pPacSp3, encoding the short Sp3 isoform, was transcriptionally inert (up to 200 ng; results shown for 25 ng). When added together, transactivation by Sp1 and the long Sp3 isoform (Usp3) was completely additive (Figure 9A); the short Sp3 isoform slightly repressed transactivation by Sp1 or Usp3 (20 and 30% respectively; Figure 9A). When either Sp1 or the long Sp3 isoform was co-transfected with NF-Y, a striking synergistic activation of promoter activity was observed (35-fold for Sp1 and 11-fold for Usp3 respectively; Figure 9A).

In SL2 cells co-transfected with pPacSp1, promoter activity was nearly completely abolished (approx. 95%) for the CBS $-1b$ basal construct with a mutated GC-a (GC-a mutant) and, to a

Figure 9 Activation of the CBS $-1b$ basal promoter by Sp1/Sp3 and NF-Y in *Drosophila* SL2 cells

(A) *Drosophila* SL2 cells were co-transfected with 1 μ g of the CBS $-1b$ basal reporter gene construct (pCBSb-3792/-3565) with or without 25 ng of pPacSp1, pPacSp3 or pPacUSp3, and/or 25 ng each of pPacNF-YA, pPacNF-YB and pPacNF-YC. For all transfections, constant plasmid was maintained (at 100 ng of the pPac series) with 25–100 ng of empty pPacO vector. Data from three experiments are presented as the means $\pm$ S.D. fold-increases in luciferase activity relative to a control for which pCBSb-3792/-3565 was co-transfected with 100 ng of pPacO vector. (B) Wild-type CBS $-1b$ basal construct pCBSb-3792/-3565, the GC-box (GC-a-c), E-box and CAAT-box mutants (1 μ g each) were co-transfected in SL2 cells along with 25 ng of pPacSp1. At 48 h post transfection, the cells were lysed and assayed for luciferase activity. Luciferase activity was expressed relative to that of the wild-type construct pCBSb-3792/-3565. Data presented as means $\pm$ S.D. from at least three independent experiments carried out in duplicate. WT, wild type.

lesser degree (approx. 50%), for the GC-c mutant construct (Figure 9B). Interestingly, mutation of GC-b (i.e. GC-b mutant) effected a 3.1-fold activation of luciferase activity compared with the wild-type pCBSb-3792/-3565 construct, suggesting a transrepressive role for this GC-box element in SL2 cells. Finally, mutation of either the E-box (USF-1 mutant) or the CAAT box (NF-Y mutant) potentially and nearly completely suppressed CBS $-1b$ basal activity (Figure 9B). Since the latter was done without any pPacNFY, the loss of promoter activity in these experiments would seem to have a spatial (i.e. conformational) basis rather than involve protein-protein interactions.

These results suggest the Sp1, in combination with the long and short isoforms of Sp3, can potentially enhance or repress the transactivation of the CBS $-1b$ minimal promoter, apparently by binding to the same GC-box elements. Furthermore, they demonstrate a potent synergism between Sp-factor binding to GC-box elements and NF-Y binding to the inverted CAAT box in the minimal CBS $-1b$ promoter. Finally, they imply an important role for the E-box in transactivation of the CBS $-1b$ minimal promoter.

DISCUSSION

Multiple transcripts have been reported for CBS in a variety of human tissues [2]. Five distinct 5'-non-coding exons have been described, the most frequent termed CBS $-1a$ and CBS $-1b$, each encoded by its own unique GC-rich TATA-less promoter. In the present paper, we defined the minimal transcriptional region (-3792 to -3667) of the CBS $-1b$ promoter by 5'- and 3'-deletions, and transient transfections in HepG2 cells,

characterized by high levels of CBS transcription exclusively from the $-1b$ promoter. Included in this 125 bp minimal CBS $-1b$ region are three GC-boxes (termed GC-a-c), an inverted CAAT-box and an E-box. By gel-shift and supershift assays, binding of Sp1 and Sp3 to the GC-box elements, USF-1 to the E-box, and both NF-Y and an NF-1-like factor to the CAAT box could be demonstrated.

The functional roles of all these elements in CBS $-1b$ promoter activity were assessed by mutating these sequences and transfecting the mutated basal constructs into HepG2 cells. For the Sp family of factors and NF-Y, additional experiments were performed in Sp- and NF-Y-null *Drosophila* SL2 cells co-transfected with Sp1/Sp3 and/or NF-Y expression constructs. A critical role for the inverted CAAT-box was confirmed, since mutation of this element resulted in nearly a complete loss of promoter activity in both HepG2 and SL2 cells. Mutations of the GC-a-c and the E-box element also effected changes in promoter activity; however, this was somewhat variable and strongly dependent on the transfection model. Thus mutations of GC-a-c and the E-box, individually, or all three GC-boxes together, resulted in low (15–40%), but consistent, losses of CBS $-1b$ promoter activity in HepG2 cells. In SL2 co-transfections, the effect ranged from a moderate (GC-c mutant, approx. 50%) to potent (GC-a mutant, approx. 95%) suppression of promoter activity, or involved a strong activation response (GC-b, 317%). Most notably, when the GC-a-c elements were each mutated in combination with the CAAT-box, the final luciferase activities achieved for the double mutant constructs in HepG2 cells were only slightly different from that for the singly mutated CAAT-box construct. This demonstrates that any transactivation/repression effects from Sp1/Sp3 binding to the three GC-boxes, or from USF-1 binding to the E-box, are clearly secondary to that resulting from NF-Y binding to the inverted CAAT-box.

NF-Y is a ubiquitous transcription factor which binds to CAAT elements in the proximal promoters of a wide variety of mammalian genes [18]. NF-Y consists of three subunits, termed A, B and C (molecular masses of 42 kDa, 36 kDa and 40 kDa respectively), all of which are essential for DNA binding [21–24]. Whereas NF-Y binding to the CAAT-box is frequently essential for gene transcription, particularly for TATA-less genes, NF-Y by itself is largely unable to activate transcription. Rather, its transcriptional role primarily relates to its capacity to enhance transactivation from nearby activating elements and/or to facilitate the positioning of key transcriptional factors at the transcriptional start site.

In our gel-shift and supershift analyses, we identified two NF-Y complexes bound to the CAAT-box in the CBS $-1b$ basal promoter (possibly corresponding to the short and long NF-YA isoforms; [18]) which competed with CAAT and NF-Y consensus competitors and were supershifted by NF-YA and NF-YB antibodies. Competitive binding by CAAT/NF-Y also extended to Sp1/Sp3 binding to the proximal GC-boxes (GC-a and GC-b), and mutation of the CAAT-box appreciably abolished Sp1/Sp3 binding to GC-a and GC-b. Thus interactions between NF-Y binding to the CAAT motif and Sp1/Sp3 binding to the GC-a and GC-b elements are likely. In co-transfections of Sp1/Sp3 and NF-Y into SL2 cells, a striking synergistic transactivation of the minimal CBS $-1b$ promoter occurred, confirming the functional importance of these binding interactions. There is ample precedence for a functional synergism between NF-Y and Sp1, having already been described for rat pyruvate kinase M [25], fatty acid synthase [26,27], major histocompatibility complex class II-associated invariant chain [28], p27Kip1 [29], human tissue inhibitor of metalloproteinases-2 [30], extracellular signal-regulated protein kinase 2 [31], Na,K-ATPase $\alpha 3$

subunit [32], E2F [33] and type-A natriuretic peptide receptor [34]. Although the mechanism(s) is not entirely certain, this may occur via effects of NF-Y binding in introducing distortions in DNA structure essential to the recruitment of critical transcription factors, by direct protein–protein interactions between NF-Y and other factors, or through its association with histone deacetylases, which maintain chromatin in an open configuration. In at least one report [35], NF-Y binding stabilizes both NF-Y and Sp1, resulting in prolonged half-lives. For the present study, we obtained direct evidence for a conformational basis for CAAT-mediated transactivation of CBS $-1b$ promoter activity since, in SL2 cells, mutation of the CAAT-box abolished promoter activity independent of the presence of NF-Y.

Superimposed on the apparent interplay between NF-Y binding to the CAAT-box and Sp-factor binding to GC-boxes GC-a-c, are the inter-relationships between Sp1 and the long and short isoforms of Sp3. We found that both Sp1 and the long Sp3 isoform were potent stimulators of basal CBS $-1b$ promoter activity in SL2 cells, and that their effects were completely additive in co-transfection experiments. Conversely, the short Sp3 isoforms were transcriptionally inactive and potently repressed the transactivating effects of either Sp1 or the long Sp3 isoform.

Co-operative interactions between the USF-1 binding to the E-box at position -3741 to -3736 and Sp1/Sp3 binding to GC-c were also suggested by the decreased levels of Sp1/Sp3 binding to GC-c on gel shifts, upon mutation of the nearby E-box or pretreatment with USF-1 antibody. This was further implied by our Sp1 co-transfection experiments in *Drosophila* SL2 cells, in which mutation of the E-box potently suppressed CBS $-1b$ basal promoter activity. A functional synergism has been described between USF-1 and other transcription factors, including Sp1 [36], ETS1 [37], HNF4 [38], and TFII-I [35].

To summarize, our results demonstrate important roles for an assortment of transcription factors in the transcriptional regulation of the 125 bp CBS $-1b$ minimal promoter, including Sp1, Sp3, NF-Y and USF-1, all ubiquitously expressed factors. A functional interplay was suggested, involving NF-Y binding to the inverted CAAT-box at position -3698 to -3694 and Sp1/Sp3 binding to GC-a and GC-b, USF-1 binding to the E-box at position -3741 to -3736 and Sp1/Sp3 binding to GC-c, and between Sp1 and the long and short isoforms of Sp3. While these interactions likely result in cell- or tissue-specific transcriptional control of CBS $-1b$ in response to external stimuli, the extent of CBS $-1b$ transcription achieved involves numerous factors, including the levels of individual transcription factors, the presence of upstream activating or inhibitory elements and overall CBS $-1b$ promoter architecture.

Our previous studies [7–10] documented an important role for CBS as a determinant of the enhanced Ara-C sensitivities of DS myeloblasts and CBS-transfected leukaemia cell line models. The results of the present study should eventually provide insights into the bases for variations in CBS gene expression between DS and non-DS myeloblasts, and for the remarkable responsiveness of DS AML patients treated with chemotherapy, including Ara-C. Likewise, they could potentially clarify the link between chromosome 21 and the increased risk of leukaemia in DS children, or the relationship between CBS and certain phenotypic features of DS. Finally, our findings should promote a better understanding of the molecular bases for variations in CBS gene expression in assorted human diseases including homocystinuria, hyperhomocysteinaemia and atherosclerosis.

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