Transcriptional Regulation of Cell-specific Expression of the Human Cystathionine β-Synthase Gene by Differential Binding of Sp1/Sp3 to the −1b Promoter*

Yubin Ge‡, Larry H. Matherly‡‡, and Jeffrey W. Taub‡¶**

From the ‡Experimental and Clinical Therapeutics Program, Barbara Ann Karmanos Cancer Institute, and the ¶Division of Pediatric Hematology/Oncology, Children’s Hospital of Michigan and the Departments of ‡Pharmacology and ‡Pediatrics, Wayne State University School of Medicine, Detroit, Michigan 48201

Cystathionine β-synthase (CBS) catalyzes the condensation of serine and homocysteine to form cystathionine, an intermediate step in the synthesis of cysteine. We previously characterized the CBS −1b minimal promoter (−3792 to −3667) and found that Sp1/Sp3, nuclear factor Y, and USF-1 were involved in the regulation of basal promoter activity (Ge, Y., Konrad, M. A., Matherly, L. H., Taub, J. W. (2001) Biochem. J. 357, 97–105). In this study, the critical cis-elements and transcription factors in the CBS −1b upstream region (−4046 to −3792) were examined in HT1080 and HepG2 cells, which differ −10-fold in levels of CBS transcripts transcribed from the CBS −1b promoter. In DNase I footprint and gel shift analyses and transient transfections of mutant CBS −1b promoter constructs into HT1080 and HepG2 cells, transcriptionally important roles for Sp1/Sp3 binding to three GC boxes and one GT box and for binding of myeloid zinc finger 1-like proteins to two myeloid zinc finger 1 elements were indicated. In gel shift assays, very low levels of Sp1/Sp3 DNA-protein complexes were detected in HT1080 cells compared with HepG2 cells despite comparable levels of nuclear factor Y and USF-1 binding and similar levels of Sp1 and Sp3 proteins on Western blots. Mixing of HT1080 and HepG2 nuclear extracts resulted in no difference in total Sp factor binding in gel shift assays, thus excluding a role for an unknown activator or inhibitor in the disparate Sp1/Sp3 binding between the lines. Increased Sp1/Sp3 binding in gel shift assays was observed upon treatment of HT1080 nuclear extracts with protein kinase A, and decreased Sp1/Sp3 binding resulted from treatment of HepG2 nuclear extracts with calf alkaline phosphatase, suggesting a role for changes in Sp1/Sp3 phosphorylation in transcription factor binding and transactivation of the CBS −1b promoter. Characterization of CBS promoter structure and function should clarify the molecular bases for variations in CBS gene expression in genetic diseases and the relationship between CBS and Down syndrome.

* This work was supported in part by NCI Grant CA92308 from the National Institutes of Health; Grant 6203-98 from the Leukemia and Lymphoma Society; and by grants from the Children’s Leukemia Foundation of Michigan, the Children’s Research Center of Michigan, the Litvak Foundation, and Leukemia Research Life, Inc. (Detroit, MI). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence and reprint requests should be addressed: Children’s Hospital of Michigan, 3901 Beaubien Blvd., Detroit, MI 48201. Tel.: 313-745-5515; Fax: 313-745-5237; E-mail: jtaub@med.wayne.edu.

Cystathionine β-synthase (CBS; EC 4.2.1.22), a pyridoxal 5’-phosphate-dependent enzyme involved in the transsulfuration pathway, catalyzes the condensation of L-serine and L-homocysteine to form cystathionine, an intermediate step in the synthesis of cysteine. The human CBS gene product is a 63,000-Da polypeptide. The tetrameric protein is catalytically dependent on both heme and pyridoxal phosphate and is allosterically regulated by S-adenosylmethionine (1–4). The human CBS gene spans over 30 kilobases and consists of 23 exons ranging in size from 42 to 209 base pairs (5). The CBS polypeptide is encoded by exons 1–14 and 16. The human CBS gene encodes multiple mRNAs differing in their 5’-untranslated regions, resulting from the use of five alternative noncoding exons (designated −1a to −1e) and a constant exon 0. Transcripts containing exons −1a and −1b appear to be the most abundant and are found in an assortment of adult and fetal tissues (6). In contrast, use of exons −1c, −1d, and −1e appears to be rare. There are at least two GC-rich TATA-less promoters upstream of exons −1a and −1b, containing numerous putative transcription elements (Sp1-gene specificity protein 1), AP1, AP2, etc.) (5, 6). In our recent study of the CBS −1b minimal promoter (mapping between positions −3792 and −3667), we demonstrated important transactivating roles for Sp1, Sp3, NF-Y, and USF-1 (7).

The CBS gene has been localized to human chromosome 21 (e.g. 21q22.3), and its overexpression has been suggested to be linked to certain of the phenotypic features of Down syndrome (DS). Elevated CBS expression in DS results in low plasma homocysteine compared with non-DS individuals and has been suggested to contribute to decreased atherosclerosis in DS patients (8). In our own studies of CBS and DS, we also found striking increases (~12-fold) in CBS transcripts in myeloblasts from DS children with AML compared with non-DS myeloblasts (9). Interestingly, this elevated CBS expression was associated with increased in vitro sensitivities to cytokine arabinoside (Ara-C) and generation of Ara-C triphosphate, likely due to downstream effects of CBS on endogenous folate and nucleotide pools (9–12). This may explain, in part, the remarkably high event-free survival rates (70–100%) and low relapse rates (~15%) of DS children with AML compared with non-DS children treated with Ara-C-based chemotherapy protocols. Increased CBS transcripts in DS over non-DS myeloblasts in-
volve those transcribed from the CBS – 1b promoter (13) and may arise from differences in mechanisms of transcriptional control. In this study, we significantly extend our earlier studies of CBS promoter structure and function by identifying the critical cis-elements and transcription factors in the CBS – 1b promoter upstream region.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—[γ-32P]-ATP (3000 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences. Synthetic oligonucleotides were purchased from Generon Biotechnology, Inc. The Woodlands, TX. Restriction and modifying enzymes, reporter gene vectors (pGL3-Basic and pRLSV40), and other molecular biological products were purchased from Promega (Madison, WI). The pPacSp1 and pPacO plasmid constructs were provided by Dr. Robert Tjian (University of California, Berkeley, CA), and the pPacSp3 and pPacUSp3 constructs were provided by Dr. Guntram Suske (Philips-Universität, Marburg, Germany). The pPacNF-YA, pPacNF-YB, and pPacNF-YC plasmid constructs were provided by Dr. T. F. Osborne (University of California, Irvine, CA). MZF-1 cDNA was amplified from CCRF-CEM T-cell leukemia total RNA by reverse transcription-PCR using sense (5′-AGAATGTTTTCTTTGATGCG-3′) and antisense (5′-GACAGGGGTGCCTGCGGATA-3′) primers. The full-length human CBS cDNA was a gift from Dr. Warren Kruger (Fox Chase Cancer Institute, Philadelphia, PA).

**Cell Culture**—The human HT1080 fibrosarcoma and HepG2 hepatocellular carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). Drosophila SL2 cells were provided by Dr. Bonnie Sloane (Wayne State University, Detroit, MI). The HT1080 cell line was maintained in RPMI 1640 medium containing 10% heat-inactivated iron-supplemented calf serum (Hyclone Laboratories), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37 °C in the presence of 5% CO2 and 95% air. The HepG2 cell line was maintained in minimal essential medium with 10% fetal bovine serum and the antibiotics. SL2 cells were maintained in Schneider’s insect medium supplemented with 10% fetal bovine serum and 2 mM glutamine plus antibiotics at 25 °C. 

**Northern Blot Analysis and Assay of CBS mRNA Turnover**—Total RNAs were isolated from HT1080 and HepG2 cells using Trizol reagent (Life Technologies, Inc.). Total RNA from each cell line (20 µg) was electrophoresed on 0.9% agarose gel containing 2.2 M formaldehyde and 1× MOPS and capillary-transferred to GeneScreen Plus membrane (PerkinElmer Life Sciences). The membrane was baked at 80 °C for 1.5 h in vacuo, prehybridized, and hybridized with [α-32P]dCTP-labeled CBS cDNA (labeled by random priming). The membrane was washed to a final stringency of 0.1% SSC and 0.1% SDS at 42 °C and exposed to film. Densitometry was performed on a Molecular Dynamics Storm 860 fluorescence and radioactivity imaging system with ImageQuant software.

**Electrophoretic Mobility Shift Assays (EMSAs)**—Complementary single-stranded oligonucleotides were annealed, end-labeled with 32P, and purified using Sephagel G-25 quick spin columns (Roche Molecular Biochemicals). Nuclear proteins were preincubated in a reaction solution containing 20 mM Tris-borate/EDTA (pH 8.4) at 4 °C. After 10 min, the 32P-labeled duplex oligonucleotide (2 x 10^6 cpm) was added, and the reaction was incubated for another 20 min on ice. For supershift experiments, 2 µg of rabbit polyclonal antibody (Sp1; Geneka Biotechnology, Inc.) or goat polyclonal antibody (Sp3; Santa Cruz Biotechnology) was added to the reaction mixtures and incubated for 30 min. DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gels in 0.5× Tris boric/EDTA (pH 8.4) at 4 °C and 35 mA. The gels were dried, and the complexes were visualized by autoradiography.

**Western Blot Analysis**—Nuclear protein was isolated from HT1080 and HepG2 cells as described above. 50-µg aliquots of each nuclear protein were fractionated on a 7.5% polyacrylamide gel with SDS and electroblotted onto a polyvinylidene difluoride membrane. The blot was blocked overnight at room temperature in TBB (TWEEN/Tri-based saline with 0.1% Tween 20 (pH 7.5)) containing 1% fat-free dried milk powder and was then incubated with anti-Sp3 or anti-Sp1 antibody (diluted 1:10,000 and 1:2,000, respectively) in TBB containing 0.5% fat-free dried milk powder for 2 h at room temperature. The blot was washed with TBS/T, incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:5000 in TBS

**FIG. 1. Northern blot analysis of CBS transcripts in HT1080 and HepG2 cells.** Total RNAs (20 µg) from HT1080 and HepG2 cells were electrophoresed on an agarose gel, transferred to GeneScreen Plus membrane, and hybridized to a 32P-labeled CBS cDNA probe. The 2.9-kilobase band corresponds to the major CBS transcript in these two cell lines. Ethidium bromide-stained 28S and 18S RNAs were used as references to permit normalization.
containing 0.5% milk powder) for 1 h at room temperature, and detected by Lumi-Light Western blotting substrate (Roche Molecular Biochemicals).

Phosphorylation and Dephosphorylation of Nuclear Extracts—HT1080 nuclear extracts (100 μg) were incubated with or without 40 units of protein kinase A catalytic subunit (Sigma) in 50 mM Tris (pH 7.4), 10 mM MgCl₂, and 10 μM ATP at 30 °C for 1 h in a total volume of 50 μl. 10 μg of the treated nuclear extract were used for EMSA as described above. Nuclear extracts (100 μg) from HepG2 cells were incubated with or without 20 units of calf intestinal alkaline phosphatase (Promega) for 1 h at 37 °C in a total volume of 30 μl. The reaction was stopped by the addition of a mixture of phosphatase inhibitors to final concentrations of 10 nM NaF, 10 nM okadaic acid, and 10 nM Na₃MoO₆. 10 μg of the treated nuclear extract were used for EMSA.

RESULTS

CBS Gene Expression and mRNA Turnover in HepG2 and HT1080 Cells—The levels of CBS transcripts were compared between human HT1080 fibrosarcoma and HepG2 hepatocellular carcinoma cell lines on Northern blots. For both lines, a single 2.9-kilobase transcript was detected; however, the levels were ~10-fold higher in HepG2 compared with HT1080 cells (Fig. 1).

This disparity in the levels of CBS transcripts could conceivably reflect differences in mRNA stabilities between the lines. To assess this possibility, cells were treated with 10 μg/ml actinomycin D, and changes in the levels of CBS transcripts were followed over 12 h on Northern blots. Under these conditions, CBS turnover was virtually identical (half-lives of ~10 h for both HT1080 and HepG2 cells) (data not shown). This suggests that the difference in the levels of CBS transcripts between the lines likely arises at the transcriptional level. Thus, the HT1080 and HepG2 cell lines appeared to be appropriate models for a systematic study of the transcriptional regulation of cell-specific expression of the human CBS gene.

Since two promoters were previously identified in the human CBS gene (GenBank™/EBI Data Bank accession number...
AF042836 (4), designated −1a (positions −4467 to −4093) and −1b (positions −4055 to −3576), it was essential to establish whether the differential gene expression between the HT1080 and HepG2 lines on Northern blots was due to differences in promoter usage. To test this possibility, a 5′-RACE assay was performed. Essentially identical results for HT1080 and HepG2 cells were obtained. For both lines, all the transcripts contained 5′-untranslated regions with exon −1b sequence. Four of the five 5′-RACE subclones sequenced from HT1080 cells were identical and began at position −3637, or 28 base pairs downstream from the reported 5′-end of exon −1b. One clone contained 58 additional nucleotides upstream from this position (position −3695) (data not shown). For the HepG2 cells, all four 5′-RACE clones were identical and began at position −3634 (7). For all of the 5′-RACE clones from both lines, there were no differences from the published GenBank™/EBI Data Bank sequence for human CBS (accession number AF042836). These results establish that both HT1080 and HepG2 cells exclusively use the −1b promoter and suggest that transcription of CBS −1b transcripts initiates at multiple sites.

In Vitro Analysis of the Upstream Region of the CBS −1b Promoter—In our previous study (7), we localized the basal CBS −1b promoter to positions −3792 to −3666 (relative to ATG) and identified Sp1/Sp3, NF-Y, and USF-1 as important transactivating factors (Fig. 2, lower panel) shows a schematic of the CBS −1b promoter region. Our results also suggested an important transcriptional role for the CBS −1b promoter region upstream of position −3792 since progressive 5′-deletions from the full-length promoter construct (positions −4046 to −3565) in pGL3-Basic resulted in a 70% loss of luciferase activity in HepG2 cells, accompanying deletions from positions −4046 to −3792 (7). However, upon 3′-deletion of the basal CBS −1b promoter region from positions −3565 to −3792, promoter activity was completely lost. This shows that the region between positions −4046 and −3792 is by itself incapable of driving luciferase activity.

To localize the major regulatory elements in the CBS −1b promoter upstream region, DNase I footprint analysis was performed using two overlapping probes (positions −4046 to −3792 and positions −3565 to −3659) and nuclear extracts prepared from HT1080 and HepG2 cells. Four clearly protected regions were demarcated with nuclear extracts from HepG2 cells (Fig. 2, upper panel, designated A, B, C, and D) that, from data base analysis, contained numerous transcription elements, including GC boxes (designated GCe, GCf, and GCg), a GT box (GTd), MZF-1 (MZF-1a and MZF-1b), and Ikaros-2 (IK2) (Fig. 2, lower panel). Conversely, protected regions with equivalent amounts of nuclear extracts from HT1080 cells were far less obvious (Fig. 2, upper panel).

### Table I

| Oligonucleotide | Sequence |
|----------------|---------|
| FPA (−3994 to −3967) (a) | GTTGAAACCCGGGGCGCCGCGCTCAAGG |
| FPA-GGc mt (b) | GTTGAAACCCGGGGCGCCGCGCTCAAGG |
| FPA-MZF-1a mt (d) | AGGGCGGGAGGGCGCGCGCCCG |
| FPC-GGC mt (e) | AGGGCGGAGGGCGCGCGCCCG |
| FPC (−3876 to −3845) (f) | CATGCGGCAGTCGGGGAGGCGCGGTTCCG |
| FPC-GCc mt (g) | CATGCGGCAGTCGGGGAGGCGCGGTTCCG |
| FPD (−3816 to −3787) (h) | GGGTGAGAGGGGGGAGGGGGAGGCC |
| FPD-MZF-1b mt (i) | GGGTGAGAGGGGGGAGGGGGAGGCC |
| FPD-GTdT mt (j) | GGGTGAGAGGGGGGAGGGGGAGGCC |
| Sp1 (k) | ATTCTAGGCGGCGGGGACG |
| ZF1–4 (l) | GATCTAAAAGTGGAGGAGAAA |
| ZF5–13 (m) | GATCCTGGTGTAGGGGGAGATCG |
| IK2 (n) | TCAGCTTTTGGGATACCTGTC |

**Differential Binding of Sp1/Sp3 to the CBS −1b Promoter in HT1080 and HepG2 Cells**—To characterize transcription factors that interact with the binding sites in the upstream region of the CBS −1b promoter, EMSAs and supershift assays were performed. Synthetic FPA (labeled a in Table I), FPB (labeled c), FPC (labeled f), and FPD (labeled h) oligonucleotides (containing wild-type CBS −1b promoter sequence) were designed from the protected regions on DNase footprints (Fig. 2) and database analysis of potential transcription factor-binding sequences. Double-stranded FPA, FPB, FPC, and FPD oligonucleotides were labeled with [γ-32P]ATP and incubated with nuclear extracts prepared from HT1080 and HepG2 cells. In HepG2 cells, 11 major DNA-protein complexes were detected (numbered 1–11 in Fig. 3, A–D, lanes 4, 12, 26, and 36) that were effectively competed by a 100-fold excess of unlabeled nucleotides (lanes 5, 13, 27, and 37), establishing specificity. The identities of the bound transcription factors were assessed by competitions with unlabeled competitor oligonucleotides (Table I). Complexes 1, 3, 5–8, 10, and 11 were nearly completely abolished by competition with a commercial Sp1 consensus oligonucleotide (labeled k Table I; Fig. 3, A–D, lanes 6, 14, 28, and 38). With the exception of complex 11, these were all supershifted by antibodies to Sp1 and/or Sp3 (lanes 8, 9, 21, 22, 31, 32, 44, and 45). Complex 2 was partially competed by an Sp1 consensus oligonucleotide (lane 6) and partially supershifted by anti-Sp1 antibody (lane 8). For anti-Sp3 antibody, the loss of signal is consistent with an effect on a DNA-Sp3 protein complex. Thus, complex 2 appears to represent two DNA-protein complexes migrating together, only one of which involves Sp1 and Sp3.

The addition of the CBS −1b FPA, FPB, FPC, and FPD oligonucleotides including mutations in the GC/GT boxes (FPA-GGc mt, FPB-GCf mt, FPC-GCc mt, and FPD-GTdT mt) (Table 1) abolished competition for binding of DNA-protein complexes 1, 3, 5; 6 and 7; and 8, 10, and 11, respectively, as reflected in the reappearance of the radiolabeled complexes (Fig. 3, A–D, lanes 7, 19, 29, and 41). Competition for complex 2 was partially abolished (lane 7). Complexes 4 and 9 were competed by a 100-fold molar excess of the unlabeled wild-type CBS −1b oligonucleotides (FPB and FPD, respectively) (lanes 13 and 37) or an MZF-1 consensus oligonucleotide (lanes 16 and 40). However, competition was lost when the FBP/FPD MZF-1-binding sites were mutated (FPB-MZF-1a mt and FPD-MZF-1b mt, respectively) (lanes 18 and 42).

These results establish that DNA-protein complexes 1, 3, 5–8, and 10 and part of complex 2 involve Sp1 and/or Sp3. Complexes 4 and 9 appear to be MZF-1-like proteins. The identity of the additional factor comprising complex 2 is unclear. Since complex 11 could be completely competed by an
Fig. 3. Gel shift and supershift assays with FPA, FPB, FPC, and FPD. Gel shift assays were performed with HT1080 and HepG2 nuclear extracts (NE) and the $^{32}$P-labeled FPA, FPB, FPC, and FPD oligonucleotide probes (A–D, respectively) in the absence and presence of a 100-fold molar excess of commercial consensus or mutant CBS−1b oligonucleotides. The oligonucleotides used for the gel shift assays are summarized in Table I. For the supershift assays, specific antibodies to Sp1 (Sp1-ab) and Sp3 (Sp3-ab) were added to the reaction mixtures and incubated for 30 min prior to electrophoretically separating the DNA-protein complexes. The major DNA-protein complexes are numbered 1–11, and the supershifted complexes for Sp1 (Sp1-SS) and Sp3 (Sp3-SS) are also noted. In E are shown the results from a gel shift assay of the −3724/−3682 and −3766/−3725 oligonucleotides comprising the basal CBS−1b promoter region (7) using nuclear extracts prepared from HepG2 and HT1080 cells. The major complexes, including Sp1/Sp3, NF-Y, NF-1, and USF-1, are indicated. NS designates a nonspecific complex.
Sp1 consensus oligonucleotide, but not supershifted by antibody to Sp1 or Sp3, this might be another member of the Sp family, such as Sp4. The DNA-protein complexes identified in gel shift assays in the FPA, FPB, FPC, and FPD regions of the CBS –1b promoter with HepG2 nuclear extracts are summarized in Table II.

**Table II**

| Probe | DNA-protein complex* | Probable transactors |
|-------|----------------------|----------------------|
| FPA   | 1                    | Sp1/Sp3              |
| FPA   | 2                    | Sp1/Sp3              |
| FPB   | 3                    | Sp1/Sp3              |
| FPB   | 4                    | MZF-1-like factor    |
| FPC   | 5                    | Sp1/Sp3              |
| FPC   | 6                    | Sp1/Sp3              |
| FPD   | 7                    | Sp1/Sp3              |
| FPD   | 8                    | Sp1/Sp3              |
| FPD   | 9                    | MZF-1-like factor    |
| FPD   | 10                   | Sp1/Sp3              |
| FPD   | 11                   | Sp3                  |

* The DNA-protein complexes are numbered 1–11, as shown in Fig. 3 (A–D).
* A second unidentified transcription factor was detected along with Sp1/Sp3.
* This complex was identified as an Sp family member since it could be competed by Sp1 consensus oligonucleotide; however, it could not be supershifted by either anti-Sp1 or Sp3 antibody. Its identity is unclear.

In contrast to HepG2 cells, the levels of the DNA-protein complexes in HT1080 nuclear extracts were nearly undetectable (Fig. 3, A–D, lanes 2, 11, 24, and 34). Gel shift assays were also performed with probes including the CBS –1b minimal promoter (CBS –1b –3729/–3682 and –3766/–3725 oligonucleotides) (7) and nuclear extracts from HT1080 and HepG2 cells (Fig. 3E). Although Sp1 and Sp3 did not bind appreciably to either the upstream or the minimal promoter regions in extracts prepared from HT1080 cells (lanes 47 and 52), binding of NF-Y, USF-1, and NF-1 was detected to the same extent as in HepG2 cells (i.e. compare lanes 47 and 49 and lanes 52 and 54).

Functional Analysis of Transcription Factor-binding Sites by Site-directed Mutagenesis—To further confirm the functional significance of the DNA-protein complexes detected by gel shift and supershift analyses in Fig. 3, the Sp1/Sp3 and MZF-1 consensus elements in the CBS –1b promoter upstream region were mutated individually using the mutant oligonucleotides in Table I, and the mutant CBS-reporter gene constructs were transiently transfected into HT1080 and HepG2 cells. Luciferase activities of the mutant constructs were compared with that of the wild-type full-length promoter construct pCBSb–4040/–3565 (Fig. 4).

Qualitatively similar results were obtained with HT1080 and HepG2 cells despite striking differences in promoter activities. Although 4-fold more reporter plasmid (with a constant 4 μg of plasmid) was used for the HT1080 transient transfections, maximal luciferase activity (normalized to Renilla luciferase activity) was still ~6-fold less than in the HepG2 cells (Fig. 4). Since cotransfections with the pRLSV40 plasmid resulted in nearly identical levels of Renilla luciferase activity in both HT1080 and HepG2 cells, this disparity was not due to differences in transfection efficiencies between the lines.

The effects of mutations of the assorted cis-elements implicated by EMSAs were variable despite similar losses of factor binding in gel shift assays. Thus, mutation of GCg (FPA-GCg mt) (Table I) resulted in an ~30% loss of CBS –1b promoter activity in both lines. Greater losses of CBS –1b promoter activity accompanied mutation of the MZF-1b (FPD-MZF-1b mt; decreases of 59 and 45% for HT1080 and HepG2 cells, respectively) and GTd (FPD-GTd mt; decreases of 73 and 55% for HT1080 and HepG2 cells, respectively) elements. Mutation of the MZF-1a site (FPB-MZF-1a mt) only slightly suppressed the promoter activity (~15% in both lines). Interestingly, mutation of GCf (FPB-GCf mt) was accompanied by a potless loss of promoter activity (~90% in both lines). These results confirm an important transactivating role for the GCf element in the CBS –1b promoter and, to a lesser extent, for other GC/GT boxes (GCg, GCe, and GTd) and the MZF-1 elements.

Collectively, these results demonstrate that an assortment of transcription factors, including Sp1, Sp3, and MZF-1-like proteins, can bind to the CBS –1b promoter upstream region, resulting in transactivation. The differential binding of Sp1/Sp3 to the CBS –1b promoter for HepG2 versus HT1080 cells may explain, in part, the differences in the levels of CBS transcripts and CBS –1b promoter activity between the lines.

**Cell-specific Transactivation of the CBS –1b Promoter by MZF-1**—The role of MZF-1 in the regulation of the CBS –1b promoter was assessed through transient cotransfections of the CBS –1b promoter-reporter gene construct pCBSb–4040/–3565 and an MZF-1 cDNA expression vector (pcDNA3-MZF-1) in HT1080 and HepG2 cells. Cotransfections with pcDNA3-MZF-1 resulted in a stimulation of CBS –1b promoter activity in HT1080 cells (1.7- and 2.3-fold at 50 and 100 ng, respectively). Conversely, the effect of overexpressing MZF-1 on CBS –1b promoter activity in HepG2 cells was insignificant (Fig. 5). These studies suggest that MZF-1 binds to the CBS –1b promoter and transactivates promoter activity and that this occurs in a cell-specific manner.

Functional Analysis of the GC and GT Boxes in the Upstream Region of the CBS –1b Promoter in Drosophila SL2 Cells—To further explore the transcriptional roles of the upstream GC and GT boxes and their relationships to NF-Y binding to the CAAT box located in the CBS –1b minimal promoter region, transient cotransfection experiments were performed in Dro-
sophila SL2 cells, which provide a null background for the Sp family of transcription factors and NF-Y (16, 17). The pCBSb−4046/−3565 reporter gene construct including the full-length CBS −1b promoter was cotransfected with expression vectors for Sp1 (pPacSp1) (16) and/or NF-Y (pPacNF-Y), each under control of the Drosophila-specific promoter. Parallel transfections were performed with mutant CBS −1b promoter constructs in which each of the GC/GT box elements was mutated (Table I).

In cotransfections with pPacSp1, CBS −1b promoter activity for the wild-type construct was stimulated (Fig. 6A). Activity for the mutant GTd construct was nearly as strong as for the wild-type construct. Interestingly, mutation of the GCg and GCe boxes effected an activation (2.0- and 1.3-fold, respectively) of luciferase activity in these experiments, suggesting a trans-repressive role for these GC box elements. As with the HT1080 and HepG2 cells, mutation of the GCf box potently suppressed CBS −1b promoter activity in SL2 cells (80%) (Fig. 6A).

Sp1 transactivation of the full-length CBS −1b promoter (positions −4046 to −3565) exceeded that of the basal promoter (positions −3792 to −3565) by −5-fold (Fig. 6B). When both Sp1 and NF-Y were cotransfected together with the full-length promoter construct, a striking synergism was observed (−7-fold over that in cotransfections with Sp1 alone) (Fig. 6B). This synergistic activation far exceeded that with the basal promoter construct (2.3-fold) (Fig. 6B) (7). Thus, deletion of the CBS −1b promoter upstream region significantly decreases transactivation by Sp1 alone and the extent of synergistic transactivation by Sp1 in combination with NF-Y.

These results further suggest important transcriptional roles for the Sp family of factors via their binding to the upstream GC or GT box elements in the CBS −1b promoter. Furthermore, they demonstrate that the transactivating effects of Sp factors and NF-Y are highly synergistic.

**Differential Binding of Sp1/Sp3 to the CBS −1b Promoter Is Not Due to Differences in Levels of Sp1/Sp3 or to Specific Cofactor(s)**—Since Sp1 and Sp3 levels were nearly identical in HT1080 and HepG2 cells (Fig. 7), other explanations are necessary to explain the differences in Sp factor binding to the CBS −1b promoter upstream region in EMSAs and, potentially, transcriptional activity between the lines. This could reflect the presence of an Sp1/Sp3 binding inhibitor in HT1080 cells or an activator in HepG2 cells. To test these possibilities, 10 μg of a nuclear extract from HepG2 cells were mixed with different amounts (from 2.5 to 10 μg) of nuclear extract from HT1080 cells, and Sp1/Sp3 binding was analyzed in gel shift assays with 32P-labeled FPD probe. As shown in Fig. 8, binding of Sp1 and Sp3 in the HepG2 nuclear extract to 32P-labeled FPD was unaffected by the addition of increasing amounts of HT1080 nuclear extract. Thus, the presence of an Sp1/Sp3 binding inhibitor or activator is unlikely.

**Phosphorylation of Sp1 Modulates Its Binding to the CBS −1b Promoter in Vitro**—Sp1 phosphorylation has been reported to increase trans-factor binding to GC box elements (18) and to facilitate promoter activation for a number of genes (18–20). A similar mechanism may be operative for CBS. Consistent with this possibility, treatment of nuclear extracts from HT1080 cells with protein kinase A catalytic subunit prior to EMSA resulted in an increase in Sp1/Sp3 binding to the 32P-labeled FPD oligonucleotide by EMSA (Fig. 9A, lane 4). Likewise, pretreatment of HepG2 nuclear extracts with calf intestinal phosphatase significantly decreased Sp1/Sp3 binding to the FPD probe (Fig. 9B, lane 12). Thus, the differential Sp1/Sp3 binding to the CBS −1b promoter in HT1080 and HepG2 cells appears to be at least partially due to differences in the phosphorylation status of Sp1/Sp3 between these two lines. The lack of a complete response to protein kinase A treatment may reflect a requirement for additional post-translational modifications (phosphorylation by other protein kinases or glycosylation) of Sp1/Sp3 or other mechanisms altogether.

**DISCUSSION**

CBS plays a very important role in human disease. Deficiencies of the enzyme result in the genetic disorders homocystinuria and hyperhomocystinemia (40, 41), whereas increased expression of the CBS gene may contribute to some of the phenotypic features of DS (9). Our previous studies suggested an important role for variations in CBS gene expression as a determinant of the enhanced Ara-C sensitivities of DS myeloblasts and CBS-transfected leukemia cell line models (9, 12).
A wide range of CBS gene expression has been described among assorted human tissues (6). Furthermore, multiple transcripts have been reported for CBS (6). Five distinct 5’-noncoding exons have been described, the most frequent termed –1a and –1b, each encoded by its own unique GC-rich TATA-less promoter (5). In our previous study, we defined a minimal transcriptional region (positions –3792 to –3667) of the CBS –1b promoter by 5’- and 3’-deletions and transient transfections in HepG2 cells, a cell line characterized by high levels of CBS transcription exclusively from the –1b promoter (7). Included in this 125-base pair CBS –1b minimal promoter region are three GC boxes (termed GCa, GCb, and GCc), an inverted CAAT box, and an E box. In 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. Synergism between Sp1/Sp3 and NF-Y in CBS transactivation was also observed (7). The functional significance of the region upstream of the CBS –1b minimal promoter (positions –3792 to –3565) was suggested by 5’-deletion analysis (7).

In this study, we used DNase I footprint analysis of the –4046/–3792 fragment, immediately upstream of the basal promoter region, to identify protected regions in HT1080 and HepG2 cells, designated FPA (positions –3992 to –3970), FPB (positions –3955 to –3935), FPC (positions –3874 to –3849), and FPD (positions –3813 to –3790). Four potential GC or GT box elements, two MZF-1 sites, and an IK2-binding site were identified by data base analysis. In gel shift and supershift assays with a HepG2 nuclear extract, binding of Sp1 and Sp3 to the GC and GT box elements (designated GCg, GCF, GCe, and GTd) and binding of MZF-1-like proteins to MZF-1-binding sites in FPB and FPD (designated MZF-1a and MZF-1b, respectively) were demonstrated. Interestingly, in nuclear extracts prepared from HT1080 cells, Sp1 and/or Sp3 DNA-protein complexes were detected in only trace amounts despite comparable binding of CAAT-binding factors (NF-Y and NF-1) and USF-1 and nearly identical levels of Sp1 and Sp3 proteins on Western blots.

The functional roles of all these upstream elements in CBS –1b promoter activity were further evaluated by mutating these sequences and transfecting the mutant constructs into HT1080 and HepG2 cells. These mutations all effected changes in promoter activities; however, this was somewhat variable and was strongly dependent on the expression model. Thus, mutations of GCg, GCF, GTd, and the two MZF-1-binding sites (MZF-1a and MZF-1b) individually resulted in losses (15–73%) of CBS –1b promoter activity in HT1080 and HepG2 cells. Mutation of GCg resulted in a striking loss of promoter activity (90%) in both lines (Fig. 4), suggesting a critical transcriptional role for this GC box element.
In Drosophila SL2 cells, the effects of these mutations ranged from moderate (GTd mt, −15%) to potent (GCF, −80%) inhibition to a strong activation response (GCG, −200%). Sp1 and Sp3 have been previously reported to function as either transcriptional activators or repressors, depending on the cell and promoter context (21–24). Interactions between NF-Y binding to the CAAT box and Sp1/Sp3 binding to the GC boxes were indicated since in cotransfections of Sp1 and NF-Y with the full-length construct, a potent synergistic transactivation of the CBS−1b promoter was detected (7-fold), exceeding that obtained with the basal CBS−1b promoter (2.3-fold) (7). There is ample precedent for interactions between Sp1 and NF-Y in the functional transactivation of a number of genes (30–39).

MZF-1 is a myeloid zinc finger protein that is essential for granulopoiesis (25). MZF-1 contains 13 zinc finger domains, divided into two groups (26). The amino-terminal group has four zinc fingers, and the carboxyl-terminal group has nine zinc fingers. MZF-1 is a bifunctional transcription factor, capable of repressing transcription in non-hematopoietic cells and activating transcription in cells of hematopoietic origin (25). In our gel shift assays, we demonstrated binding of MZF-1-like proteins to the MZF-1a−1 and MZF-1b-binding elements in the CBS−1b promoter upstream region. Whereas mutagenesis of the MZF-1 sites resulted in −15–59% losses of promoter activity in both HepG2 and HT1080 cells, only HT1080 cells showed CBS−1b transactivation in cotransfections with MZF-1 cDNA and CBS−1b promoter-reporter gene constructs, suggesting a cell-specific response.

Our finding that significantly decreased CBS transcripts and −1b promoter activity in HT1080 cells were accompanied by very low levels of transcription factor binding compared with HepG2 cells in gel shift assays was of particular interest since similar differences in CBS expression were previously seen in AML specimens, including those from children with DS (9). Furthermore, levels of CBS were implicated as a critical determinant of sensitivities to Ara-C. Likewise, they could potentially clarify the link between chromosome 21 and the increased risk of leukemia in DS children or the relationships 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, hyperhomocysteinemia, and atherosclerosis.

Acknowledgments—We thank Drs. Guntram Suske and Robert Tijan for providing the pPacSp3, pPacSp3p, pPacOPl, and pPacO plasmids. Additional thanks are extended to Dr. T. F. Osborne for providing the pPacNF-YA, pPacNF-YB, and pPacNF-YC vectors and to Dr. Warren Kruger for providing the full-length CBS cDNA.

REFERENCES

1. Skovby, F., Kraus, J. P., and Rosenberg, L. E. (1984) J. Biol. Chem. 259, 588–593
2. Skovby, F., Kraus, J. P., and Rosenberg, L. E. (1984) Am. J. Hum. Genet. 36, 452–459
3. Bukowska, G., Kery, V., and Kraus, J. P. (1994) Protein Expression Purif. 3, 122–148
4. Roper, M. D., and Kraus, J. P. (1992) Arch. Biochem. Biophys. 298, 514–521
5. Kraus, J. P., Olivieriusová, J., Sokolová, J., Kraus, E., Vlek, C., de Franchis, R., Maclean, K. N., Ban, L., Bukowska, G., Patterson, D., Paces, V., Anzorge, W., and Kozich, V. (1998) Genomics 52, 312–324
6. Bao, L., Vlek, C., Paces, V., and Kraus, J. P. (1998) Arch. Biochem. Biophys. 350, 95–103
7. Goff, K., Konrad, M. A., Matheley, L. H., and Taub, J. W. (2001) Biochem. J. 375, 97–105
8. Chafedaux, B., Ceballos, I., Hamet, M., Coude M., Puisonnier, M., Kamosun, P., and Allard, D. (1988) Lancer 2, 741
9. Taub, J. W., Huang, X., Matheley, L. H., Stout, M. L., Buck, S. A., Massey, G. V., Becton, D. L., Chang, M. N., Weinstein, H. J., and Ravindranath, Y. (1999) Blood 94, 1393–1400
10. Taub, J. W., Matheley, L. H., Stout, M. L., Buck, S. A., Gurney, J. G., and Ravindranath, Y. (1996) Blood 87, 3395–3403
11. Taub, J. W., Stout, M. L., Buck, S. A., Huang, X., Vega, R. A., Becton, D. L., and Ravindranath, Y. (1997) Leukemia (Baltimore) 11, 1594–1595
12. Taub, J. W., Huang, X., Ge, Y., Dutcher, J. A., Stout, M. L., Mohammad, R. M., Ravindranath, Y., and Matheley, L. H. (2000) Cancer Res. 60, 6421–6426
13. Taub, J. W., Ge, Y., Huang, X., Stout, M. L., Dutcher, J. A., Mohammad, R. M., Ravindranath, Y., and Matheley, L. H. (1999) Blood 94, 382a (abstr.)
14. Pan, J., and McElver, R. P. (1993) J. Biol. Chem. 268, 22600–22608
15. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
16. Dooley, K. A., Bennett, M. K., and Osborne, T. F. (1999) J. Biol. Chem. 274, 5295–5299
17. Courey, A. J., Holtzman, D. A., Jackson, S. P., and Tijan, R. (1988) Cell 50, 827–836
18. Bohill, C., Ahmad, S., Borellini, F., Lei, J., and Glazer, R. I. (1997) J. Biol. Chem. 272, 21317–21441
19. Alroy, I., Soussan, L., Segei, R., and Yarden, Y. (1999) Mol. Cell. Biol. 19, 1961–1972
20. Chen, J. F., Semmes, O. J., Neuveut, C., and Jeang, K. T. (1998) J. Biol. Chem. 273, 1625–1629
21. Birnbaum, M. J., van Wijnen, A. J., Odgren, P. R., Last, T. J., Suske, G., Stein, G. S., and Stein, J. L. (1990) Biochemistry 34, 16505–16508
22. Liang, Y., Robinson, D. F., Dennig, J., Suske, G., and Fahl, W. E. (1996) J. Biol. Chem. 271, 11792–11797
23. Majello, B., De Luca, P., Hagen, G., Suske, G., and Lania, L. (1994) Nucleic Acids Res. 22, 4914–4921
24. Prowse, D. M., Bolgan, L., Molnar, A., and Dotto, G. P. (1997) J. Biol. Chem. 272, 1308–1314
25. Hromas, R., Davis, B., Rauscher, P. J., Klemm, M., Tenen, D., Hoffman, S., Xu, D., and Morris, J. F. (1996) Curr. Top. Microbiol. Immunol. 211, 159–164
26. Hromas, R., Collins, S. J., Hickenstein, D., Raskind, W., Deaven, L. L., O’Hara, P., Hagen, F. S., and Kaushansky, K. (1991) J. Biol. Chem. 266, 14180–14187
27. Jackson, S. P., MacDonald, J. J., Lees-Miller, S., and Tijan, R. (1990) Cell 63, 155–165
28. Armstrong, A. S., Barry, D. A., Leggett, R. W., and Mueller, C. R. (1997) J. Biol. Chem. 272, 13489–13495
29. Leggett, R. W., Armstrong, A. S., Barry, D., and Mueller, C. R. (1995) J. Biol. Chem. 270, 25579–25584
30. Yamada, K., Tanaka, T., Miyamoto, K., and Noguchi, T. (2000) J. Biol. Chem. 275, 18129–18137
31. Roder, K., Wolf, S. S., Beck, K. K., and Schweizer, M. (1997) J. Biol. Chem. 272, 26161–26164
32. Roder, K., Wolf, S. S., Larkin, K. J., and Schweizer, M. (1999) Gene (Amst.) 234, 61–69
33. Wright, K. L., Moore, T. L., Vålen, B. J., Brown, A. M., and Ting, J. P.-Y. (1995) J. Biol. Chem. 270, 20708–20706
34. Inoue, T., Kaminaka, J., and Saka, T. (1999) J. Biol. Chem. 274, 32309–32317
35. Zhao, Z.-D., Hammani, K., Bae, W. S., and DeClerck, Y. A. (2000) J. Biol. Chem. 275, 18602–18610
36. Sugiura, N., and Takishima, K. (2000) *Biochem. J.* **347**, 155–161
37. Murakami, Y., Ikeda, U., Shimada, K., and Kawakami, K. (1997) *Biochim. Biophys. Acta* **1352**, 311–324
38. Wang, W., Dong, L., Saville, B., and Safe, S. (1999) *Mol. Endocrinol.* **13**, 1373–1387
39. Liang, F., Schaufele, F., and Gardner, D. G. (2000) *J. Biol. Chem.* **276**, 1516–1522
40. Födinger, M., Buchmayer, H., and Sunder-Plassmann, G. (1999) *Miner. Electrolyte Metab.* **25**, 269–278
41. Mudd, S. H., Levy, H. L., and Skovby, F. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 1279–1327, McGraw-Hill Book Co., New York
42. Haidweger, E., Novy, M., and Rotheneder, H. (2001) *J. Mol. Biol.* **306**, 201–212
43. Du, X., Edelstein, D., Rossetti, L., Fantus, I. G., Goldberg, H., Ziyadeh, F., Wu, J., and Brownlee, M. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97**, 12222–12226