The Dominant Role of Sp1 in Regulating the Cystathionine $\beta$-Synthase $-1a$ and $-1b$ Promoters Facilitates Potential Tissue-specific Regulation by Kruppel-like Factors*  

Kenneth N. Maclean‡, Eva Kraus, and Jan P. Kraus  

From the Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado 80262

Cystathionine $\beta$-synthase (CBS) catalyzes the condensation of serine with homocysteine to form cystathionine and occupies a crucial regulatory position between the methionine cycle and transsulfuration. The human cystathionine $\beta$-synthase gene promoters $-1a$ and $-1b$ are expressed in a limited number of tissues and are coordinately regulated with proliferation through a redox-sensitive mechanism. Site-directed mutagenesis, DNase I footprinting and deletion analysis of 5276 bp of 5' proximal $-1b$ flanking sequence revealed that this region does not confer tissue-specific expression and that 210 bp of proximal sequence is sufficient for maximal promoter activity. As little as 32 bp of the $-1b$ proximal promoter region is capable of driving transcription in HepG2 cells, and this activity is entirely dependent upon the presence of a single overlapping Sp1/Egr1 binding site. Co-transfection studies in Drosophila SL2 cells indicated that both promoters are transactivated by Sp1 and Sp3 but only the $-1b$ promoter is subject to a site-specific synergistic regulatory interaction between Sp1 and Sp3. Sp1-deficient fibroblasts expressing both Sp3 and NF-Y were negative for CBS activity. Transfection of these cells with a mammalian Sp1 expression construct induced high levels of CBS activity indicating that Sp1 has a critical and indispensable role in the regulation of cystathionine $\beta$-synthase. Sp1 binding to both CBS promoters is sensitive to proliferation status and is negatively regulated by Kruppel-like factors in co-transfection experiments suggesting a possible mechanism for the tissue specific regulation of cystathionine $\beta$-synthase.

Cystathionine $\beta$-synthase (EC 4.2.1.22, CBS)$^1$ catalyzes a pyridoxal 5'-phosphate-dependent $\beta$-replacement reaction condensing homocysteine (Hcy) and serine to form cystathionine, which is subsequently converted to cysteine by the action of cystathionine $\gamma$-lyase. In addition to being essential for the synthesis of cysteine by transsulfuration, CBS is also a key regulator of plasma total Hcy (tHcy) levels (1). CBS deficiency is the most common cause of homocystinuria, an inherited autosomal recessive metabolic disease, which if untreated, causes skeletal abnormalities, dislocated optic lenses, mental retardation, and a dramatically increased incidence of vascular disorders particularly thromboembolic disease (1). Moderate elevation of plasma tHcy has been identified as a major risk factor for Alzheimer’s disease (2, 3), neural tube defects (4), and cardiovascular disease (5). Because of the key role of CBS in regulating plasma tHcy levels, there is a need for a greater understanding of the mechanisms and principles that govern its regulation.

Previously, we have determined the complete genomic sequence of human CBS (6) and mapped the transcriptional start sites of five human CBS mRNA isoforms, designated CBS $-1a$, $-1b$, $-1c$, $-1d$, and $-1e$, respectively (7). Of these, we found that isoforms $-1a$ and $-1b$ form the vast majority of transcripts, whereas isoforms $-1c$, $-1d$, and $-1e$ are relatively rare. We identified two promoter regions upstream of exons $-1a$ and $-1b$ and found that the $-1b$ promoter has $-10$-fold greater promoter activity than that of the $-1a$ promoter in both HepG2 and COS7 cells (6). The CBS promoter regions constitute an interesting paradox in that they have multiple sites of transcriptional initiation, are GC-rich and lack the classic TATA box sequence. Yet, despite having all of the usual characteristics of a ubiquitously expressed “housekeeping” gene, CBS is expressed in a highly tissue-specific manner with a wide range of tissues completely negative for CBS activity. The temporal and spatial expression patterns of CBS appear to be developmentally regulated (8–10). Recently, we have shown that both human and yeast CBS are coordinately regulated with proliferation and that the human CBS $-1b$ promoter is serum- and fibroblast growth factor-responsive and is down-regulated by growth arrest due to nutrient depletion or the action of differentiation-inducing reagents (10, 11). Another group has recently investigated the CBS $-1b$ promoter and defined the minimal promoter region as a 125-bp region of sequence proximal to exon $-1b$. These investigators also found that the $-1b$ promoter is activated by a synergistic interaction between NF-Y and either Sp1 or Sp3 (12). CBS expression levels in HepG2 and HT1080 cells were directly correlated with differences in the level of Sp1/Sp3 binding to the CBS $-1b$ promoter region in these cells possibly as a consequence of phosphorylation by protein kinase A. This finding was proposed as a possible mechanism for the tissue-specific regulation of CBS (13).

In this report, we examine $-5$ kbp of CBS 5'-flanking sequence for the ability to modulate CBS expression and to confer tissue-specific expression. We demonstrate that as little as 32 bp of the $-1b$ proximal promoter region is capable of driving transcription in HepG2 cells and that a single Sp1/Egr1 binding site is essential for this activity. Both the CBS $-1b$ and $-1a$

* This work was supported by National Institutes of Health Grants PO1HD0805 and HL65217 (to J. P. K.). The costs of publication of 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 should be addressed: Dept. of Pediatrics, C-233, University of Colorado School of Medicine, Denver, CO 80262. Tel.: 303-315-3619; Fax: 303-315-8080; E-mail: ken.macleane@uchsc.edu.

1 The abbreviations used are: CBS, cystathionine $\beta$-synthase; AdoMet, S-adenosyl-l-methionine; EMSA, electrophoretic mobility shift assay; Hcy, homocysteine; tHcy, total homocysteine; KLF, Kruppel-like factor; LKLF, long Kruppel-like factor; BKLF, basic Kruppel-like factor; CMV, cytomegalovirus.
promoters are regulated in a proliferation-sensitive manner by Sp1 and that, although this transcription factor is involved in a previously unreported synergistic interaction with Sp3 in specifically regulating the 1b promoter, neither Sp3 nor NF-Y are able to substitute for Sp1 in vivo. Additionally, we present evidence to show that members of the KLF family of transcription factors have the potential to play a role in the tissue-specific regulation of the CBS gene by acting to block transcriptional activation of the CBS promoters by Sp1.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO). Reagents for enhanced chemiluminescence (ECL) were obtained from Amersham Biosciences (Piscataway, NJ). Restriction and modifying enzymes were purchased from New England Biolabs (Beverly, MA). All reagents for mammalian tissue culture were obtained from Life Sciences Technologies (Rockville, MD) except for fetal calf serum, which was obtained from HyClone (Logan, UT). Synthetic oligonucleotides were obtained from Integrated DNA Technologies (Corvalis, IA).

Media and Mammalian Cell Culture—HepG2 hepatocellular carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as described previously (10). Drosophila SL2 (Schneider) cells were a generous gift from Dr. Joan Horvitz (Department of Cellular and Structural Biology, University of Colorado Health Sciences Center). SL2 cells were maintained in Schneider's insect medium supplemented in the dark at room temperature with heat-inactivated 10% fetal bovine serum and 2 mM glutamine plus 100 units of penicillin and 100 µg of streptomycin/ml. SL2 fibroblasts were obtained from Dr. Jeremy Efron (Emory University) and were passaged as described previously (14).

Plasmids, Transfections, and Luciferase Assays—The sequence and construction of the CBS 1b promoter luciferase reporter construct pCBS47 and the CBS 1a promoter luciferase reporter construct pCBS57 has been described previously (6). A series of extended CBS 1b plasmids reporter constructs containing progressively larger regions of 5' flanking sequence were generated by cleaving pCBS47 with Sp1l and either XbaI, SacI, BglII, BamHI, or XhoI and subsequently sub-cloning in the adjacent Sp1-XbaI (pCBS47 - 8236 to -3576), Sp1l-Sacl (pCBS474 - 7241 to -3576), Sp1l-BglII (pCBS474 - 5189 to -3576), Sp1l-BamHI (pCBS474 - 4518 to -3576), or XhoI-Sp1l restriction fragments of 5' flanking DNA that were isolated from a previously described P1 cosmid containing the CBS genomic DNA (6). The largest reporter construct CBS476 (-8943 to -3576), was generated by sub-cloning the EcoRI-XbaI fragment from the P1 cosmid into pZERO-2 (Invitrogen). This region was then excised from this vector as a KpnI-XbaI fragment and ligated into pCBS476, which had been cut with the same enzymes. In this manner we generated a range of larger CBS promoter luciferase constructs with up to 5276 kbp of 5' flanking sequence. To investigate the minimal sequence region of the 1b promoter capable of driving transcription, we made progressive deletion derivatives of our base construct pCBS47. Two constructs, pCBS474 (-3877 to -3576) and pCBS475 (-3972 to -3576) were made by end filling and subsequent blunt-end ligating after digesting pCBS47 with KpnI and Sp1 and ApaI, respectively. The pCBS473 construction (-3737 to -3576) was generated by subcloning of a PCR fragment amplified from a pCBS 47 template using 5' CTGATGACTTCTGCTTGGCAGGAC- GAGCAT-3' and antisense 5' TACGAGCCTTCTGACGATTACAGAAGGACGG-3' primers. Nucleotides shown in boldface represent the KpnI (sense) and HindIII (antisense) sites introduced to facilitate directional cloning of the amplification product. The sequence of this clone was verified by DNA sequencing. The pCBS472 (-3699 to -3576) and pCBS471 (-3679 to -3576) were generated by sub-cloning double-stranded synthetic oligonucleotides containing the desired sequences. This strategy was also used to generate mutant forms of pCBS472 with the CCAAT sequence mutated to CCGTG (pCBS472-1) or the Sp1 core CGGC sequence mutated to CTTG (pCBS472-2). All of the pCBS471 1b reporter construct derivatives tested had a common 3' end that included the transcription start sites defined previously upstream of exon 1b (17).

The pIsoNF-Y, pActNF-Y, and pActNF-Y/C plasmid constructs were kindly provided by Dr. T. F. Osborne (University of California, Irvine, CA). The mammalian expression construct D4 NF-YA-m29 expressing the dominant negative NF-YA29 subunit, capable of forming an inactive NF-Y complex by trimmerization with NF-YB and NF-YC, was obtained from Dr. Roberto Mantovani (University of Milan, Italy).

The Sp1 mammalian and Drosophila expression constructs pActSp1, pPAcUSp1, pPAcCMV/Sp1, and pCGN-Sp1 and their relevant empty parent vectors that were used as negative controls were all provided by Dr. Paul Gardner (Brudnick Neuropsychiatric Research Institute, Worcester, MA) and were used with the kind permission of Drs. Edward Seto (University of South Florida, FL), Thomas Shenk (Princeton University, NJ), and Guntram Suske (Philippi-University, Marburg, Germany). The LKLF mammalian expression construct pBCKMVwLKLFL was a generous gift from Dr. Jerry Lingrel (University of Cincinnati College of Medicine, OH) and the KLF8 and BKLFL expression constructs pMT2.BKLF, pMT2.KLF8, and pPac.BKLF were kindly provided by Dr. Merlin Crossley (University of Sydney, New South Wales, Australia). Transient transfections and subsequent luciferase reporter assays in all proliferating cells were performed as described previously (10).

Analysis of CBS Expression—CBS enzyme activity was determined by a previously described radioisotope assay using [14C]serine as the labeled substrate (15). One unit of activity is defined as the amount of CBS that catalyzes the formation of 1 µmol of cystathionine in 1 h at 37 °C. SDS Western blots were performed after running 10- to 75-µg samples of total protein on 10% polyacrylamide gels. Transfer, primary, and secondary antibody incubation conditions and subsequent detection using ECL chemiluminescent reagents were performed as described previously (16).

Nuclear Extracts and EMSA—Nuclear extracts were prepared from actively proliferating HepG2 cells (60% confluent) as described previously (17). Electrophoretic mobility shift assays (EMSA) were performed using a lightshift chemiluminescent EMSA kit according to the manufacturer's standard protocol. The minimally active 1b promoter region was generated for EMSA by synthesizing oligonucleotides representing the proximal 32-bp probe of sequence.

The oligonucleotide 5' GCCAAATCCGCGCCTGGTCCTCCCTGCGCCCTGCTCCAGC-3' and its reverse complement, 5' ACCGGAGGGGGAGGACGG-3' were labeled using a biotin 3'-end labeling kit according to the manufacturer's instructions (Pierce, Rockford, IL).

Standard EMSA reactions contained 20 µg of cell extract and 60 fmol of biotin end-labeled DNA in a 20-µl volume binding reaction in the presence of 2.5% glycerol, 5 mM MgCl2, 50 µg/ml of poly(dI-dC), and 0.05% nonionic detergents. EMSA reactions were incubated at room temperature for 30 min, terminated by adding 2 µl of 10× loading buffer (0.2% (v/v) bromophenol blue and 0.2% xylene cyanol containing 10% (v/v) glycerol), and then separated on 6% polyacrylamide gels pre-run at 100 V for 30 min at 0.5× TAE buffer (0.02 M Tris-acetate, 0.001 M EDTA) and subsequently run at 150 V for 4 h after sample loading. Reaction products were then transferred to a Biodyne B membrane (Pierce) by capillary transfer and fixed by UV cross-linking. The biotin-labeled reaction products were then visualized by incubation with streptavidin horseradish peroxidase conjugate and subsequent incubation with ECL chemiluminescent reagents.

DNase I Footprinting—The Sp1-HindIII DNA fragment bearing 210 bp of the 5' proximal sequence was excised from pCBS474 as an Sp1-HindIII fragment into the high copy number vector pUC19. This region was then excised as a BamHI-HindIII fragment, gel-purified, 5'-end labeled with 32P on either the top (at the BamHI site) or bottom strand (at the HindIII site), and used as a probe in DNase I footprinting as described previously (18). Approximately 4 × 106 cpm of labeled probe was incubated with either 50 or 75 µg of crude nuclear extract derived from actively proliferating (60% confluent) HepG2 cells for 10 min at room temperature in DNase I digestion buffer (100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 3 mM MgCl2, 0.15 mM spermine, and 0.5 mM spermidine). The reaction mixture was then subjected to digestion with DNase I (20 µg/ml) for 90 s at room temperature. Reaction products were analyzed on a 6% polyacrylamide gel by nuclease probe that had been incubated with DNase I in the absence of nuclear extract and a guanine sequencing ladder derived from the fragment for sequence positioning purposes. After electrophoresis, the gel was dried and visualized by autoradiography.

RESULTS

Defining the CBS 1b Basal Promoter—In our first study of the CBS 1b promoter we examined the 385-bp region between the end of exon 1a and the start of exon 1b for promoter activity (6). Subsequent reports regarding the CBS basal promoter activity have been essentially confined to this 481-bp region (12, 13, 19). However, it is not unusual for sequence elements several kb upstream of the transcriptional start sites to contain sequence elements that significantly modulate

Basal and Tissue-specific Regulation of CBS

8559
basal activity or confer tissue-specific expression. To fully define the CBS −1b basal promoter and to search for both enhancer and repressor sequence elements, we examined a series of CBS −1b promoter reporter constructs with up to 5276 kbp of 5′-flanking sequence in promoter assays after transient transfection into HepG2 cells. We used our previously reported 479-bp CBS −1b luciferase reporter construct pCBS47 to −3756 as a reference for comparative purposes. The results of these experiments (Fig. 1) indicated that the presence of an additional 4787 kbp of flanking sequence did not lead to any significant change in promoter activity relative to the original pCBS47 CBS −1b base construct. Deletion of an additional 259 bp from the pCBS47 construct did not adversely affect promoter activity. Further deletion of the CBS −1b promoter region was accompanied by significant reductions in the resultant promoter activity allowing us to define the 210 bp proximal to exon −1b as the basal promoter region. The observation that deletion of the region between the Sp1 and Apal sites is accompanied by an approximate 50% reduction in promoter activity indicates that the 84-bp region between −3877 and −3792 contains sequence elements that contribute significantly to basal expression of the CBS −1b promoter.

As Little As 32 bp of Proximal CBS −1b Promoter Sequence Is Capable of Driving Transcription in HepG2 Cells—To define the minimal portion of the CBS −1b promoter sequence capable of driving transcription, we generated a series of progressive 5′ deletion derivatives of pCBS47 and assayed them for promoter activity after transient transfection into HepG2 cells (Fig. 1). We were able to delete down from 82 to 32 bp (−3699 to −3576) before losing all detectable promoter activity. This definition of the minimal promoter differs significantly (4-fold smaller) from the previously published version, and, consequently, we examined the function of this region in detail. The pGL-enhancer vector used to make our pCBS472 expression construct contains an SV40 enhancer sequence located downstream of luc+ and the poly (A) signal. To assess the possible contribution of this downstream enhancer element to the sensitivity of our promoter assays, the −1b minimal promoter region was subcloned into pGL-basic, which lacks this SV40 enhancer sequence but is otherwise identical. When this 32-bp minimal promoter region was examined in the absence of the enhancer sequence in HepG2 cells it was found to have essentially identical promoter activity as was observed previously.

Analysis of the minimal promoter sequence (Fig. 2A) shows one candidate Sp1 binding site that overlaps with a binding site for the zinc-finger transcription factor early growth-response protein (Egr1), one CCAAT box, and putative binding sites for Ap2, MZF1, IK2, and Gfi1. Ap2 is unlikely to contribute to the promoter activity of this construct, because it is not expressed in HepG2 cells (20) and has previously been shown to down-regulate CBS when heterologously expressed in these cells (10). The tissue distribution of Gfi1, IK2, and MZF1 (21) (22–24) effectively precludes any role for these transcription factors in the regulation of CBS in tissues such as liver, kidney, pancreas, and brain, which are the main sites of transsulfuration in humans (8). Consequently, the contribution of these transcription factors to CBS −1b promoter activity was not investigated. To assess the relative importance of the CCAAT box and the Sp1 site, we generated derivatives of the pCBS472 construct where these binding sites had been mutated. The effect of these mutations was examined by measuring their relative promoter activities in HepG2 cells (Fig. 2B).
Mutagenesis of the CAAT box (pCBS472-1) did not affect the level of basal promoter activity in HepG2 cells indicating that this sequence does not play a crucial role in directing transcription from the −1b promoter in these cells. Mutagenesis of the Spi1/Egr1 site in the pCBS472-2 construct lead to the complete abolition of promoter activity indicating that the promoter activity of the CBS −1b minimal promoter sequence is dependent upon the Spi1-Egr1 binding site.

DNA Footprinting Reveals Key Areas of the CBS −1b Minimal Promoter Region—DNase I footprinting analysis was performed using double-stranded fragments corresponding to nucleotides −3877 to −3786. This Sphl-HindIII restriction fragment from pCBS474 contains 210 bp of −1b proximal sequence and was used as a probe in DNase I footprinting using a HepG2 cell extract generated as described under “Experimental Procedures” (Fig. 3, A and B). Scoring and interpretation of protected regions was performed blind by two independent observers with no prior knowledge of the CBS promoter or the results of our deletion and mutagenesis study described above. Three distinct regions designated Fp1 (−3808 to −3787 on the top strand and −3810 to −3783 on the bottom strand), Fp2 (−3715 to −3681 on the bottom strand), and Fp3 (−3664 to −3639 on the bottom strand) were found to be either partially or totally protected from DNase I digestion. Examination of the sequence contained within the protected Fp1 region revealed two adjacent Sp1 binding sites separated by a MZF1 site. Interestingly, these binding sites are present at the 3′ end of the 85-bp region that was shown in the previous section to contribute significantly to basal transcription indicating that these Sp1 sites are of considerable importance for maximal promoter activity. The vast majority of the Fp1 region was also found to be protected in a DNase I footprinting analysis of the −1b promoter region performed previously where it was described as region D (13).

The second protected region (Fp2) was not previously reported to be protected (13) and contains the Sp1/Egr1 overlapping binding site that was shown to be essential for the minimal promoter function in the previous section and serves as an independent confirmation of the functional importance of this binding site. This protection is relatively weak, but it is part of the nature of the DNase I footprinting methods that the relative binding affinities of different sites afford different levels of protection from the DNase I treatment. The lower degree of protection of this site is presumably due to a relatively reduced level of Sp1 binding that is entirely consistent with the level of promoter activity that this site was shown to confer in the deletion analysis described above. Interestingly, the inverted CAAT box-NF-Y binding site 40 bp upstream of the −1b minimal promoter sequence that has previously been identified as playing a key role in regulating the CBS −1b promoter (12, 13) was not protected. We have independently found that NF-Y can weakly transactivate the CBS −1b promoter (but not −1a) in SL2 cells. However, this site is close to a DNase I-hypersensitive site, which is typically indicative of conformational changes induced by protein binding and often occurs at sites where only a small fraction of the probe molecules are bound by protein. This finding suggests that NF-Y may bind to this sequence but is not present at a sufficiently high concentration in our cell extract to confer complete protection.

A third protected fragment (Fp3) was located within the −1b exon itself. This region was not investigated in previous DNase I footprinting experiments (13). Examination of this sequence using the MatInspector version 2.2 program with the TRANSFAC 5.0 matrices (25) revealed binding sites for NF1 and MZF1. The ability of NF1 to transactivate the CBS −1b promoter was previously investigated, but the results gained were inconclusive (12) and clearly merit further investigation.

K. N. Maclean, unpublished results.
SP1 and SP3 Interact Synergistically to Up-regulate the CBS –1b Promoter but Not the –1a Promoter—Simultaneous co-transfection of SL2 cells with pCBS47, Sp1, and Sp3 dramatically increased the observed promoter activity of the –1b promoter to between 70- and 100-fold over background (Fig. 4A). Interestingly, the scale of this synergistic up-regulation was found to be influenced by growth status and was significantly reduced when the luciferase assays were performed in late confluence (data not shown). Synergistic Sp1 and Sp3 activation was not observed for the CBS –1a promoter, indicating that the observed interaction between Sp1 and Sp3 is not common to all functional Sp1 binding sequences in the CBS promoter regions.

To further investigate the synergistic activation of the CBS –1b promoter, we repeated this experiment using pCBS47 (388 bp of proximal sequence), pCBS474 (210 bp of proximal sequence), pCBS475 (125 bp of proximal sequence), pCBS473 (82 bp of proximal sequence), and pCBS472 (32 bp of proximal sequence).

Interestingly, although Sp1 and Sp3 are capable of transactivating all of these CBS –1b constructs, the synergistic interaction of Sp1 and Sp3 was only observed for pCBS47 and pCBS474. This finding indicates that not all of the functional Sp1 sites in the –1b promoter are equivalent and that Sp1 and Sp3 interact synergistically at a site within the 84-bp region that was shown by our deletion analysis to contribute significantly to basal promoter activity. Our DNA footprinting analysis showed only two protected overlapping Sp1 sites in this region, and it is likely that one or both of these sequences are the site of the Sp1/Sp3 synergistic interaction. Similar co-transfection experiments in SL2 cells by another group were limited to only one reporter construct (–3792 to –3565) that lacked this critical region and thus the synergistic interaction between Sp1 and Sp3 described here was missed (12). Further experiments are currently in progress in our laboratory to investigate if these Sp1 sites function as a focal operator site for modulating CBS expression levels.

Endogenous Sp1/Sp3 Levels in HepG2 Cells Preclude Further Activation by Exogenously Added Sp1/Sp3—To further investigate the regulation of the CBS –1b promoter by Sp1 and Sp3, we co-transfected proliferating HepG2 cells with the CBS –1b reporter construct pCBS47 alone and in combination with mammalian expression constructs for either Sp1 (pCGN-Sp1) or Sp3 (pRC/CAMV/Sp3). Additionally, to assess the contribution of NF-Y to basal CBS expression in a mammalian host, we co-transfected HepG2 cells with pCBS47 and a mammalian expression construct for the NF-Y dominant negative mutant NF-YA29. The results of these experiments (Fig. 4B) showed very modest activation of the –1b promoter as a consequence of overexpression of Sp1 or Sp3, indicating that endogenous levels of Sp1 and Sp3 are sufficient to fully activate the CBS –1b promoter under the conditions tested. Co-expression of the NF-YA29 dominant negative mutant form of NF-Y lead to an approximate 30% decrease in basal activity further indicating that NF-Y plays a role in the regulation of CBS. However, the scale of the decrease suggests that NF-Y is not indispensable for CBS expression.

The Role of Sp1 Appears to Be Dominant in Vivo—In an attempt to further investigate the relative roles of Sp1, Sp3, and NF-Y in regulating the expression of CBS, we assayed a
previously described Sp1-deficient fibroblast line (14) for CBS activity. We could detect no CBS activity or protein in this cell line indicating that Sp1 has a critical and indispensable role in the regulation of CBS. Transfection of this cell line with the mammalian Sp1 expression construct pCGN-Sp1 caused the induction of high levels of CBS activity (60 milliunits/mg of protein) (Fig. 5). Subsequent Western blotting of cell extracts of the Sp1-deficient fibroblasts indicated that the ubiquitous transcription factors Sp3 and NF-Y are present in this cell line (data not shown) but are clearly unable to drive CBS expression in the absence of Sp1. These findings indicate that the synergistic co-regulation of CBS by Sp1, Sp3, and possibly NF-Y may involve an interaction between these transcription factors prior to binding to the CBS promoter and that, without this interaction, neither Sp3 nor NF-Y are capable of activating the CBS promoter in vivo.

Sp1 Is Necessary and Sufficient for Growth-specific Regulation of the CBS –1b Promoter—Sp1 binding has previously been shown to be sensitive to proliferation status and intracellular redox (30–32). We have previously shown that growth arrest and oxidative stress (in the form of exogenously added hydrogen peroxide) can negatively influence transcription of the CBS –1b basal promoter in HepG2 cells (10). To investigate the mechanisms that underlie these responses, we investigated the influence of growth status upon the ability of Sp1 to bind to the 32-bp –1b minimal promoter region using EMSAs. Because the Sp1 binding sites in the –1b promoter have the potential to bind to up to 4 members of the Sp1/KLF family, we simplified the interpretation of our experiments by using cell extracts derived from Drosophila SL2 cells that had been transfected with the Sp1 expression construct pAct-Sp1. This approach allowed us to be absolutely certain that we were analyzing the binding of Sp1 specifically and not another related transcription factor or complex thereof. The ability of Sp1 to bind the CBS –1b promoter was assessed using Sp1-expressing SL2 cell extracts derived from actively proliferating SL2 cells expressing Sp1 and identical cells that had growth-arrested at confluence due to nutrient depletion. It can be seen that the ability of Sp1 to bind the CBS –1b minimal promoter, although not abolished, is significantly reduced concomitant with growth arrest at confluence (Fig. 6). Western blotting of these cell extracts indicated that they contained essentially identical levels of Sp1 protein (data not shown) thus reinforcing the point that it is not necessarily the intracellular concentration of Sp1 per se that dictates CBS expression levels but rather it is the degree of Sp1 binding that is important. Taken together, these results indicate that the dominant role of Sp1 in regulating the CBS promoters is instrumental in the growth-specific and differentiation status-sensitive regulation of CBS expression.

The CBS 5′-Flanking Sequence Does Not Confer Tissue-specific Expression—Although clearly regulated by the ubiquitously expressed transcription factors Sp1, Sp3, and NF-Y, CBS is expressed in a restricted number of tissues with many cell types completely negative for transsulfuration. A number of GC-rich TATA-less promoters regulated by Sp1 and Sp3 have previously been shown to contain sequence elements that confer tissue-specific expression (33, 34). Additionally, the CBS 5′-flanking sequence contains numerous binding sites for delta EF1 and HNF-3 beta, which have previously been shown to regulate gene expression in a tissue-specific manner (35, 36). Although our investigation of the –1b basal promoter indicated that the proximal 5 kbp of 5′-flanking sequence neither significantly enhances nor represses CBS –1b promoter activity in HepG2 cells, it is possible that this region contains sequence elements that act to repress promoter activity in CBS-deficient tissues. To investigate this possibility, we examined the relative expression levels of our series of CBS–1b reporter constructs in a number of CBS-negative cell lines. Previous work has shown that CBS is not expressed in the lung at any stage of development or in adult tissues (8, 9). We used the previously described E10 cell line as a model system for a CBS-deficient tissue. This cell line was originally established from normal lung explants and has alveolar type II cell features such as lamellar bodies and surfactant apoprotein immunoreactivity at early passage (37). To assess the suitability of this cell line, we assayed E10 cells for CBS activity. We could detect absolutely no CBS activity in this cell line, and Western blots could find no evidence of CBS protein (data not shown). We individually transfected batches of E10 cells with our full range of CBS–1b reporter constructs to see if any portion of the –1b 5′-flanking sequence confers tissue-specific expression. All of the CBS promoter constructs were active in E10 cells and showed a level of promoter activity essentially identical to that observed in HepG2 cells (Fig. 1) indicating that the 5276 kbp of flanking sequence does not contain any repressor sequence elements that confer tissue specificity upon CBS expression. These findings were confirmed in two other previously identified CBS-negative cell lines, a23 (38) and Buffalo rat liver cells (39). Taken together, these findings indicate that the complete absence of CBS in numerous tissues is not likely to be due to
BKLF Acts to Repress the CBS −1b Promoter by Inhibiting Sp1 Activation—Some negatively acting KLF transcription factors have been reported to function by binding to GC box sequence elements with high affinity and thus act to competitively displace activating transcription factors such as Sp1 from their cognate binding sites (40). Alternatively it has been reported that KLF factors can actively bind Sp1 itself and thus prevent it from binding to a given promoter (41). In mammalian cells, as many as 40 members of the Sp1/KLF family and splice variants thereof may be expressed at any given time depending upon the growth and differentiation status of the cell (42). This complexity of possible interactions can obscure efforts to determine specific regulatory mechanisms for a given promoter. To investigate the mechanism by which the KLF factors may be repressing expression of the CBS promoters, we used co-transfection studies in SL2 cells, which provide an Sp1/KLF null background (Fig. 7B). When the CBS −1b base expression construct pCBS47 (1 μg) was transfected into SL2 cells alone, no promoter activity was detected. Co-transfection of this construct with either pACT-Sp (1 μg) or pPACUSp3 (1 μg) leads to transactivation of the −1b promoter as described above. However, when pCBS47 and either of the Sp1/3 expression constructs was accompanied by the BKLF Drosophila expression construct pBac. BKLF, the transactivation of the CBS −1b promoter by either Sp1 or Sp3 was almost completely abolished. Essentially identical results were observed when this experiment was repeated using the CBS −1a promoter reporter construct pCBS37 (data not shown). The implication of these results is that the BKLF-based inhibition of the CBS −1b and −1a promoters in HepG2 cells proceeds by inhibiting activation by Sp1. Taken together, these results indicate that KLF inhibition of CBS expression functions by blocking activation of the CBS promoters by Sp1. The expression of many of the various KLF family members is highly tissue-specific and constitutes a possible mechanism by which the CBS promoter can be regulated by the ubiquitous transcription factor Sp1 in a tissue-specific manner.

Heterologous Expression of LKLF Abolishes CBS Activity in HepG2 Cells—To investigate the possible negative regulation of CBS by KLF family members in a more natural chromosomal setting, we examined CBS activity levels in HepG2 cells in the presence and absence of heterologously expressed LKLF. HepG2 cells (60% confluent) were transfected with the mammalian LKLF expression construct pBKCMVwtLKLF. Cells were allowed to grow for a further 24 h post-transfection and were then harvested for CBS enzyme assay as described previously (15). As a control, this experiment was repeated using HepG2 cells (60% confluent), which were individually transfected with empty vector. These control cells showed normal levels of CBS activity (15 milliunits/mg of total protein, S.E. = 0.473, n = 3), whereas expression of LKLF effectively abolished CBS activity (0.066 milliunit/mg of total protein, S.E. = 0.033, n = 3). These results further illustrate the potential of negative regulation by multiple members of the KLF family of transcription factors to contribute to the tissue-specific expression of the CBS gene in mammals.

**DISCUSSION**

Binding of the Sp1 transcription factor has been shown previously to be serum-inducible (43) and to mediate proliferation-dependent and redox-sensitive regulation of numerous promoters (44–47, 48). Additionally, the binding activity of Sp1 has been shown to be down-regulated upon growth arrest and terminal differentiation of the liver (30, 49). Consequently, the dominant role of Sp1 in regulating CBS expression is in perfect agreement with all of our previously published observations regarding the coordinate regulation of this gene with prolifer-
Basal and Tissue-specific Regulation of CBS

Our findings with regard to the CBS –1b minimal promoter region and the Fp2 region that is protected therein in our DNase I footprinting analysis differ significantly from previously published investigations (12, 13). Previously, another group defined the minimal promoter region as the proximal 125-bp region, and they did not observe protection of the Fp2 region from DNase I digestion in HepG2 cell extracts. Interestingly, the 125-bp region defined by this group to be the minimal promoter, which we both agree is perfectly capable of driving transcription, does not contain any protected regions in their footprinting analysis. It is hard to imagine that this minimal promoter functions without some transcription factor binding, indicating that some more subtle interactions such as our Fp2 region were lost in their analysis.

We have recently shown that CBS promoter activity is critically linked to growth state (10), and the role of proliferation status in regulating CBS promoter activity offers a possible explanation for both of these discrepancies. The previously published work did not provide any details of the degree of confluence the HepG2 cells were at when harvested for luciferase assays or for making nuclear extracts. The failure of this group to detect promoter activity in constructs with less than 125 bp of –1b proximal sequence or protection of the Fp2 region could easily be due to harvesting cells at a sub-optimal level of confluence where promoter activity is dramatically reduced.

This work is the first report of the site-specific synergistic interaction between Sp1 and Sp3 in regulating the CBS –1b promoter. Previously, another group examined the effect of co-transfecting Sp1 and Sp3 upon the expression of a 125 bp (–3792 to –3667) promoter fragment in SL2 cells but found no evidence of a synergistic interaction within this sequence (12). This finding is consistent with our observation that only constructs containing the tandem Sp1 binding sites immediately upstream of this region are capable of synergistic up-regulation. Although there are numerous functional Sp1 sites in the –1b promoter that contribute to basal activity, they are clearly not all equivalent in their relative contribution.

In our SL2 co-transfection experiments, and those of others, it was observed that Sp3 or to a lesser extent, NF-Y could substitute for Sp1 in activating the CBS –1b promoter. The failure of these transcription factors to substitute for Sp1 in the Sp1-deficient fibroblasts cells is therefore very interesting particularly, because transfection of this cell line with an Sp1 expression construct was sufficient to induce relatively high levels of CBS expression. Co-transfection studies along with EMSA and DNase I footprinting are very useful and widely used tools for the functional analysis of promoter regions but a potential problem associated with these approaches is that they use cloned fragments that have been produced in Escherichia coli. As a result, these DNA fragments lack the cell-specific methylation, histone acetylation, and general chromosomal architecture that is present in vivo. Additionally, the interpretation of binding experiments using artificially high concentrations of overexpressed transcription factors is complicated when analyzing the role of different transcription factors that bind to very similar cognate recognition sequences such as Sp1 and Sp3. The ability of Sp3 to substitute for Sp1 in SL2 cell co-transfection experiments but not in the Sp1-deficient fibroblasts highlights the need for in vivo correlates to support conclusions derived from these kinds of studies.

One model to explain our findings is that Sp1 and Sp3 form a ternary complex with each other prior to binding the CBS –1b promoter region. Alternatively, because Sp1 binding has previously been shown to be capable of inducing conformational changes in DNA (50, 51), it is conceivable that one of the roles of Sp1 is to induce conformational changes in the –1b promoter region that facilitate concomitant binding of Sp3 with a resultant synergistic increase in CBS promoter activity when cellular growth conditions require. Either of these models would explain why Sp3 is unable to substitute for Sp1 in the Sp1-deficient fibroblasts and why heterologous expression of Sp1 in this line is accompanied by an induction of CBS.

We have confirmed the previous observation that NF-Y plays a role in regulating the CBS –1b promoter (12, 13). However, our observation that transfection with the NF-Y dominant negative mutant NF-YA29 results in only a 30% decrease in CBS promoter activity in HepG2 cells and that NF-Y is unable to drive transcription in the absence of Sp1 indicate that the role of NF-Y in regulating CBS is not dominant. The relative importance of NF-Y in promoters varies considerably. Relatively simple TATA-less promoters have been shown to have an absolute requirement for an intact CCAAT box for function. Other stronger promoters have been shown to be less dependent. It is clear from the work presented in this report that the CBS –1b promoter is not dependent upon NF-Y. This finding is consistent with previous reports that NF-Y by itself is a relatively weak activator of transcription and that its main function is to enhance transactivation from adjacent sequence elements or to enhance the binding of other transcription factors (52).

Previous work has shown that the level of CBS expression in different cell types is directly associated with the degree of binding by Sp1/Sp3, which is possibly influenced by phosphorylation (13). This finding is consistent with our observation that CBS regulatory factors such as proliferation status and intracellular redox clearly influence the degree of binding of Sp1 to the CBS –1b promoter. These authors proposed that the relative ratios of Sp1 and Sp3 and their phosphorylation by protein kinase A could be a mechanism for the tissue-specific regulation of CBS. However, CBS is not simply expressed at low levels in some tissues and high levels in others. It is completely absent from a wide range of tissues all of which express Sp1 and protein kinase A. Consequently, it is highly unlikely that differences in either the phosphorylation status or relative abundance of two ubiquitously expressed positively acting transcription factors could be responsible for the complete absence of CBS from specific tissues. In this context, the finding that other members of the Sp/KLF family such as BKLF, LKLF, and KLF8 are capable of repressing both CBS promoters by competing for Sp1 binding sites offers a novel insight on how the CBS gene might be regulated in a tissue-specific fashion. In contrast to Sp1 and Sp3, these genes are not ubiquitously expressed. Interestingly, LKLF has a tissue distribution that is essentially restricted to CBS-negative tissues (53). Perhaps most revealing is the relative expression patterns of LKLF and CBS in resting and proliferating lymphocytes. In quiescent lymphocytes, LKLF is readily detectable and CBS is completely absent. When these cells are induced to proliferate by mitogenic stimuli, expression of LKLF is rapidly extinguished and CBS is induced (54, 55).

However, although our KLF co-transfection experiments and the expression patterns of some members of the KLF family are consistent with a role in regulating CBS, several lines of evidence indicate that the suppression of CBS expression may be more complicated. First, none of the KLF expression constructs was capable of completely abolishing CBS expression in the co-transfection experiments. Characterization of the regulatory interactions of KLF family members with other transcription factors is still at a very early stage, so it is conceivable that regulators like LKLF could require the presence of other transcription factors and/or regulatory processes such as methyla-
tion to completely block CBS expression in a cell-specific manner. A more fundamental problem arises when one considers the obvious activity of CBS – 1a and –1b promoter expression constructs after transfection into the lung-derived CBS-negative E10 cell line. Whatever mechanisms are repressing expression of the chromosomal CBS promoters in these cells, they are clearly not repressing the expression of the CBS – 1a and –1b promoter constructs.

One obvious difference between the silenced chromosomal promoters and the plasmid-based promoters is the absence of the normal chromosomal architecture, including chromatin, histones, and methylation. Our work in this report has shown with Sp1 and Sp3 that this can significantly impact the behavior of transcription factors when compared with plasmid-based transfection experiments. In this report we have shown that overexpression of KLF1 in HepG2 cells is capable of downregulating CBS expression in a natural chromosomal context, and it is thus conceivable that differences in the architecture of the CBS promoter in the chromosomal context impact upon the binding affinities of KLF family members and/or other accessory proteins. Alternatively, it remains possible that a sequence element outside of the 5' flanking sequence that we investigated is required to confer tissue-specific expression. Further investigations are currently in progress in our laboratory to characterize the mechanisms that control tissue-specific regulation of CBS.

Acknowledgments—We express our gratitude to Dr. Paul Gardner, Dr. Jeremy Boss, Dr. Jerry Lingrel, and Dr. Merlin Crossley for useful discussions.

REFERENCES

1. Mudd, S. H., Levy, H. L., and Kraus, J. P. (2001) in The Metabolic and Molecular Bases of Inherited Disease (Scrivener, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Childs, B., Kinzler, K., and Vogelstein, B., eds) 8th Ed., pp. 2967–2986. McGraw-Hill, New York

2. Seshadri, S., Beiser, A., Selhub, J., Jacques, P. F., Rosenberg, I. H., and D’Agostino, R. B., Wilson, P. W., and Wolf, P. A. (2002) *J. Clin. Investig.* 110, 1752–1762

3. Shao, J., McPartlin, J. M., Kirke, P. N., Lee, Y. J., Conley, M. R., Wei, D. G., and Scott, J. M. (1996) *Anat. Rec.* 245, 149–151

4. Selhub, J. (1997) *Hematologica* 82, 129–132

5. Kraus, J. P. (1987) *Annu. Rev. Immunol.* 5, 301–343

6. Mudd, S. H., Levy, H. L., and Kraus, J. P. (000) *J. Biol. Chem.* 275, 1752–1762

7. Shao, J., McPartlin, J. M., Kirke, P. N., Lee, Y. J., Conley, M. R., Wei, D. G., and Scott, J. M. (1996) *Anat. Rec.* 245, 149–151

8. Selhub, J. (1997) *Hematologica* 82, 129–132

9. Kraus, J. P. (1987) *Annu. Rev. Immunol.* 5, 301–343

10. Smith, G. J., Le Mesurier, S. M., de Montfort, M. L., and Lykke, A. W. (1984) *Pathology* 16, 401–405

11. Skovby, F., Krasnikoff, N., and Frangione, V. (1984) *Human Genet.* 65, 291–294

12. Maccioni, R., Gaustadnes, M., Oliveira-jus, J., Janosik, M., Kraus, E., Velve, C., de Franchis, R., Maclean, K. N., Bao, L., Bukovskova, G., Patterson, D., Paves, V., Ansoncell, W., and Kozich, V. (1998) *Genomics* 52, 312–324

13. Bao, L., Velve, C., Paves, V., and Kraus, J. P. (1998) *Arch. Biochem. Biophys.* 350, 95–103

14. Mudd, S. H., Finkelstein, J. D., Ireverre, F., and Laster, L. (1965) *J. Biol. Chem.* 240, 4382–4392

15. Quere, I., Paul, V., Boulc, C., Jonz, C., London., J., Demaille, J., Kamoun, P., Daffler, J., Abihal, M., and Chasse, J. P. (1996) *Biochem. Biophys. Res. Commun.* 234, 127–137

16. Maclean, K. N., Janosik, M., Kraus, E., Kozich, V., Allen, R. H., Raab, B., and Kraus, J. P. (2002) *J. Cell. Physiol.* 192, 81–92

17. Maclean, K. N., Janosik, M., Oliveira-jus, J., Kery, V., and Kraus, J. P. (2000) *J. Inorg. Biochem.* 81, 161–171

18. Ge, Y., Matherly, L. H., and Taub, J. W. (2001) *Biochem. J.* 357, 97–105

19. Ge, Y., Matherly, L. H., and Taub, J. W. (2001) *J. Biol. Chem.* 276, 43570–43579

20. Ping, D., Boekhoudt, G., Zhang, F., Morris, A., Philipson, S., Warren, S. T., and Boss, J. M. (2000) *J. Biol. Chem.* 275, 1708–1714

21. Kraus, J. P. (1987) *Methods Enzymol.* 143, 388–394

22. Janesik, M., Oliveira-jus, J., Janosik, M., Sokolova, J., Kraus, E., and Kraus, J. P., and Kozich, V. (2001) *Am. J. Hum. Genet.* 68, 1506–1513

23. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* 11, 1475–1489

24. Wade, D. P., Lindahl, G. E., and Lawn, R. M. (1994) *J. Biol. Chem.* 269, 19575–19576

25. Ge, Y., Jensen, T. L., Matherly, L. H., and Taud, J. W. (2003) *Blood* 101, 1551–1557

26. Zeng, Y. X., Somasundaram, K., and el-Deiry, W. S. (1997) *Nat. Genet.* 15, 78–82

27. Kasrunk, H., Zeng, H., Schmidt, T., Zevnik, B., Kluge, R., Schmid, K. W., Duhrensen, U., and Morey, T. (2002) *Nat. Genet.* 30, 295–300

28. Georgopoulos, K., Winandy, S., and Avital, N. (1997) *Annu. Rev. Immunol.* 15, 155–176

29. Hromas, R., Boswell, S., Shen, R. N., Burgess, G., Davidson, A., Curnetta, K., Sutton, J., and Robertson, K. (1996) *Leukemia* 10, 1049–1050

30. Gaboli, M., Kotsi, P. A., Gurrieri, C., Cattoretti, G., Ronchetti, S., Cordon-Cardo, C., Bruynmeyer, E. H., Hromas, R., and Pandolfo, M. P. (2001) *Genes Dev.* 15, 1625–1630

31. Wingender, E., Chen, X., Fricke, E., Geffers, R., Hehl, R., Liebich, I., Kruil, M., Mays, V., Michael, H., Ohnhauser, R., Pruss, M., Schacherer, F., Thiele, S., and Urich, S. (2001) *Nucleic Acids Res.* 29, 283–283