The Dominant Role of Sp1 in Regulating the Cystathionine β-Synthase –1a and –1b Promoters Allows Tissue-Specific Regulation of CBS Expression By Kruppel-Like Factors.

Kenneth N. Maclean and Jan P. Kraus

University of Colorado School of Medicine, Denver, CO. 80262

Introduction

Cystathionine β-synthase (CBS) catalyzes a pyridoxal 5'-phosphate (PLP) dependent β-replacement reaction condensing homocysteine (Hcy) and serine to form cystathionine, which is subsequently converted to cysteine by the action of cystathionine γ-lyase (CGL). In addition to being essential for the synthesis of cysteine, CBS is also a key regulator of plasma Hcy levels. 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 (Mudd *et al.,* 2001). Moderate elevation of tHcy has been identified as a major risk factor for neural tube defects (Mills et al., 1995), Alzheimer's disease (Seshadri et al., 2002, Kruman et al., 2002) cardiovascular and thromboembolic disease (Selhub et al., 1997). Recent work has indicated that mild hyperhomocysteinemia promotes thromboembolic disease by causing endothelial dysfunction due to decreased synthesis and bioavailability of nitric oxide. Further studies have indicated that this effect can be abolished by the provision of extra glutathione (Eberhardt et al., 2000, Weiss et al., 2001, Weiss et al., 2002).

Despite the key role of CBS in regulating plasma tHcy levels, very little is known about the regulation of this gene. Previously we have determined the complete genomic sequence of human CBS (Kraus et al., 1998) and we have mapped the transcriptional start sites. To date, five human CBS mRNA isoforms, designated CBS –1a, b, c, d, and e respectively, have been identified. Isoforms –1a and –1b have been shown to form the vast majority of transcripts while isoforms –1c, d, and e have been shown to be relatively rare. It remains to be determined if these rare transcripts are of any biological significance (Bao et al., 1998). Two human promoter regions have been identified upstream of exons –1a and –1b. It was shown previously that the –1b promoter has approximately 10 fold greater promoter activity in both HepG2 and COS7 cells. The human CBS promoters differ in that the –1b promoter contains a classical CCAAT box at 31 bp upstream from the transcriptional start site. Both of the human CBS promoter regions are GC rich, lack the classical TATA box and contain numerous candidate transcription factor-binding sites for Sp1, c-Myb, AP2, C/EBP and NF1. However, although the human CBS gene promoter regions have the sequence characteristics of a housekeeping gene promoter, the gene is expressed in a highly tissue-specific manner and appears to be developmentally regulated (Mudd et al., 1965, Bao et al., 1998, Quere et al., 1999, Maclean et al., 2002). Recent work in our laboratory and by the group of Dr Jeffry Taub has shown that that the CBS –1b promoter is regulated by synergistic interactions between Sp1, Sp3 and possibly NF-Y. Dr Taub's group has suggested that the tissue specific expression of CBS is a reflection of altered ratios of Sp1 and Sp3 (Ge et al., 2001). We show here that Sp1 is dominant in its regulation of both CBS promoters and is responsible for the redox-sensitive and growth-specific regulation of the gene. Additionally, we show that although the relative abundance of Sp1 and Sp3 are capable of attenuating the level of CBS expression, they are not responsible for the absence of CBS from certain tissues. Instead, it appears that members of the KLF family of transcription factors negatively regulate CBS in a tissue-specific manner.

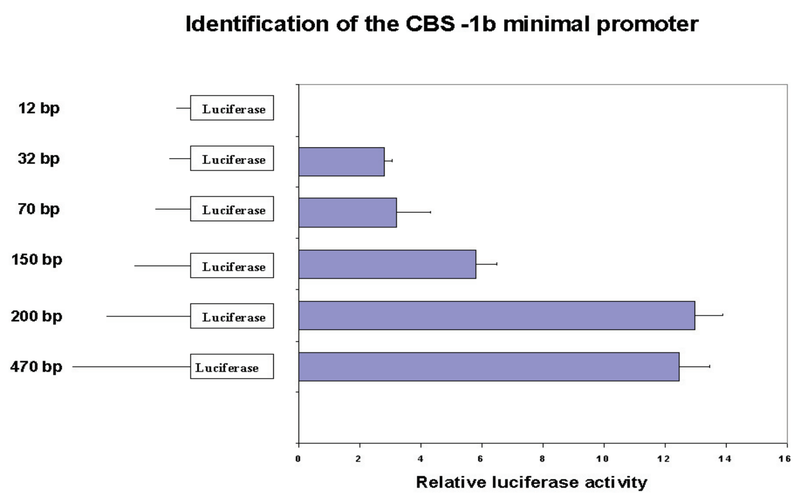


Fig.1 The CBS –1b minimal promoter.

We have defined the CBS-1b minimal promoter by using deletion mutagenesis and subsequent promoter assays in HepG2 cells (fig1). This minimal promoter is significantly smaller than that which has been published previously.

CCTCTCCGCCAACATCCGCCCGCCTGCTCCCCTCG
CCAAC GC box-Sp1 Ap2

CCGT----EXON –1b (-3690 relative to the ATG)
Ap2

NF-Y was not found to bind the CCAAC sequence in DNA footprinting experiments (fig 2) but was found to bind an inverted CAAT box just upstream. Although NF-Y can activate the CBS basal promoter in Schneider cells, deletion or mutagenesis of this sequence did not decrease the observed promoter activity indicating that this transcription factor does not play a major role in regulating CBS.

Ap2 acts to negatively regulate CBS in cultured cells by inducing differentiation (Maclean *et al.,* 2002).

Sp1. This minimal promoter was shown to bind Sp1 in EMSA analysis and mutagenesis of this Sp1 completely abolished all promoter activity from the CBS-1b minimal promoter when transfected into HepG2 cells. This work and other experiments described here, indicate that Sp1 is necessary and sufficient for proliferation-specific and redox –sensitive regulation of the human CBS gene.

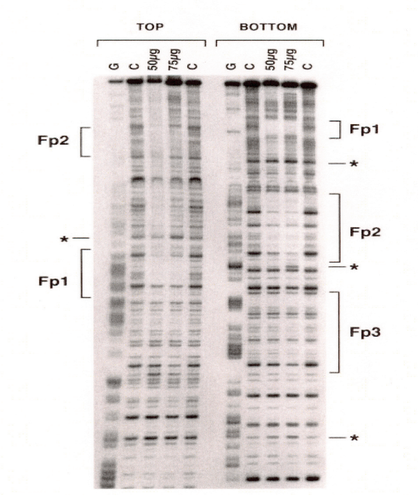


Fig 2. DNA footprinting identifies key Sp1 binding sites. A 320 bp DNA fragment representing the CBS –1b basal promoter region was 5' end-labeled on both the top and bottom strand and was used in DnaseI footprinting. Approximately 4x10^4 cpm of labeled probe was incubated with either 50 or 75 µg of crude nuclear protein (isolated from proliferating HepG2 cells) for 10 minutes at room temperature. The reaction mixture was then subjected to digestion with DnaseI (20ug/ml for 90 seconds at room temperature). Three distinct DNA regions containing Sp1 binding sequences (designated FP1, FP2 and FP3) were either partially or completely protected from DnaseI digestion. A number of DnaseI hypersensitive sites are marked (*). A guanine (G) sequencing ladder was included for sequence positioning purposes. C: control lane in which the labeled probe was incubated with DnaseI without the addition of nuclear extract. The lower case "a" refers to the top strand whereas"b" refers to the protected regions identified on the bottom strand.

Fig. 3 The CBS –1b promoter is sensitive to changes in intracellular redox

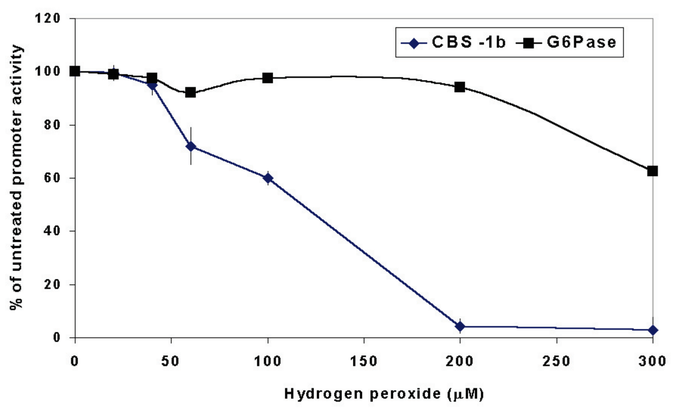
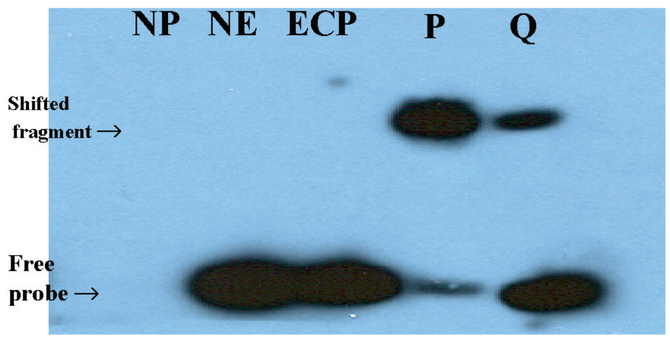


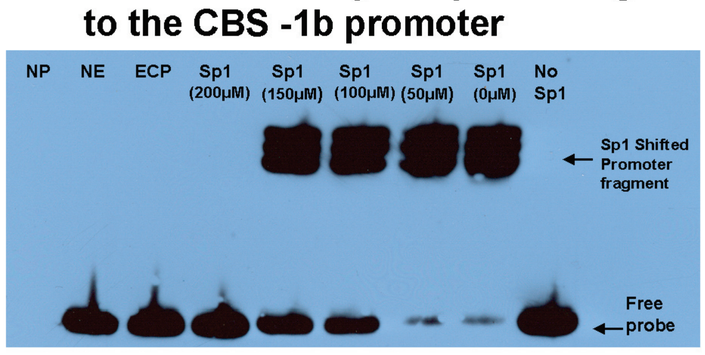
Fig. 3. The effect of hydrogen peroxide upon CBS -1b promoter activity. CBS –1b promoter (♦) reporter luciferase assays performed in sub-confluent HepG2 cells after 24 hours incubation in the presence and absence of various concentrations of hydrogen peroxide. A glucose 6-phosphatase promoter-luciferase construct (■) was used as a control. Values shown represent the mean and SEM of at least three independent experiments.

Analysis of the Effect of Proliferation Status Upon Transcription Factor Binding to the CBS -1b Promoter by EMSA



NP-no probe, NE-no extract ECP- excess cold probe, P-proliferating HepG2 cell extract Q-quiescent HepG2 extract.

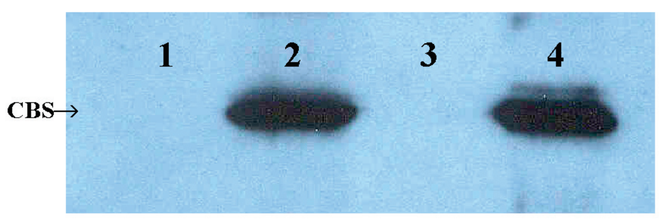
Redox sensitivity of Sp1 binding to the CBS -1b promoter



NP-no probe, NE-no extract, ECP-excess cold probe, No Sp1- Schneider cell extract, Sp1-Schneider cell extract obtained after transfection and expression of Sp1 expression Construct. Figures given in parentheses denote the concentration of hydrogen peroxide added to the cell extract prior to the binding reaction and subsequent electrophoresis.

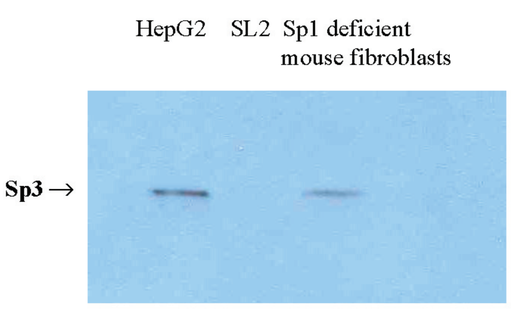
Figs 4 and Fig 5. Binding of Sp1 to the CBS-1b minimal promoter is decreased as cells become quiescent and in the presence of hydrogen peroxide. EMSA analysis was performed using biotinylated double-stranded oligonucleotides containing the CBS –1b minimal promoter sequence. Nuclear extracts were prepared according to the method of Dignam *et al.,* (1983). Probe DNA was incubated in the presence of nuclear extract prepared from either proliferating or quiescent drosophila Schneider cells that had been transfected with the Sp1 expression construct pPac-Sp1. Binding reactions were carried out in the presence of 2 µg of non-specific competitor poly(dI.dC). Reactions were incubated at room temperature for 30 minutes and were then separated on a 6% non-denaturing polyacrylamide gel pre-run at 100V for 30 min in 0.5xTAE buffer and then run for 150V for approximately 4 hours. The reaction products were transferred to a nylon membrane by Southern blotting followed by UV cross-linking. Biotin labeled DNA and DNA-protein complexes were then visualized by exposing the membrane to X-ray film after incubation with Streptavidin-Horseradish peroxidase conjugate and subsequent exposure to enhanced chemiluminescence reagents.

Expression of CBS has an obligate requirement for Sp1

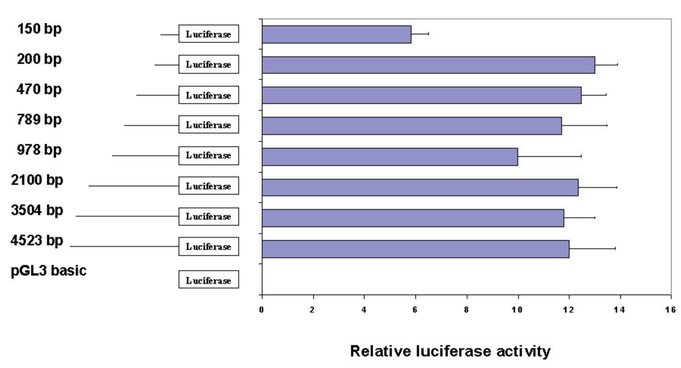


1. CBS negative a23 cells.
2. Purified 63 kDa CBS.
3. Sp1 deficient mouse fibroblasts.
4. Sp1 deficient mouse fibroblasts transfected with an Sp1 expression construct.

Sp3 does not activate the mouse CBS promoter in the absence of Sp1



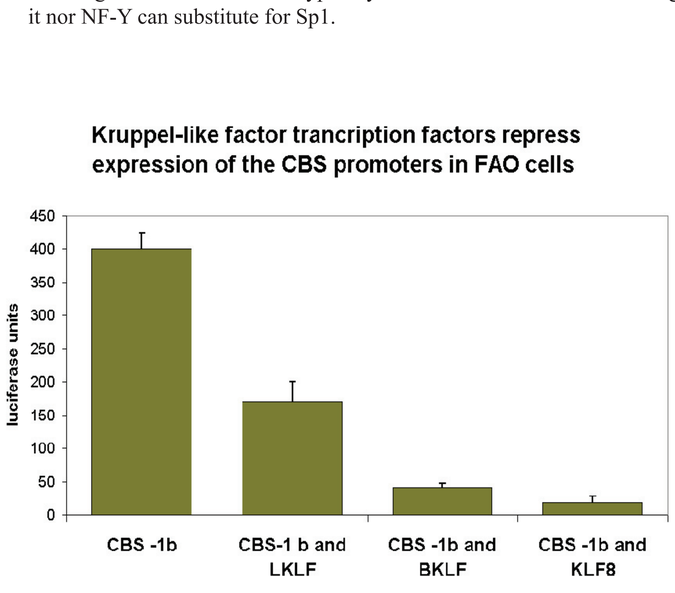
Expression of the CBS –1b promoter in the CBS negative Lung epithelial line E10

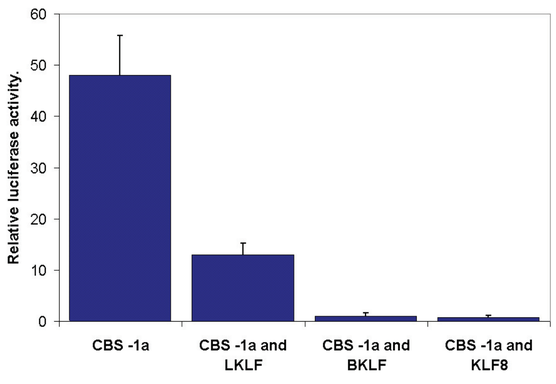


Figs. 6 and 7. Sp1 is dominant in the regulation of CBS. Co-transfection experiments indicate that both Sp3 and NF-Y are capable of activating the CBS –1b promoter in the absence of Sp1. However, these experiments use unnaturally high numbers of the target sequence in the presence of over-expressed transcription factors. In order to see if either Sp3 or NF-Y is capable of directing CBS expression in the absence of Sp1 *in vivo*, we assayed Sp1 knockout fibroblasts for CBS activity. These cells were found to be completely devoid of CBS activity and protein (Fig 6) despite having abundant levels of Sp3 (Fig 7.) and NF-Y (data not shown). Transfection of the Sp1 deficient cell line with a mammalian Sp1 expression construct restored CBS Expression (Fig.6). The level of CBS activity observed in these cells after heterologous expression of Sp1 was 80 mU/mg of protein, which is far higher than that which is typically observed in fibroblasts. Taken together, these results indicate that although Sp3 can interact synergistically with Sp1 to regulate CBS, neither it nor NF-Y can substitute for Sp1.

Fig. 8.The CBS 5'-flanking region does not confer tissue specificity and tissue-specific expression of CBS is not due to variance in the ratio of Sp1 and Sp3. The CBS promoter constitutes an interesting paradox in that with its high GC content, multiple sites of transcription initiation and lack of a TATA box it looks like an archetypal house-keeping gene promoter. Despite this, CBS clearly has a defined tissue distribution that appears to change during development, possibly as a consequence of cellular differentiation. (Mudd et al., 1965, Bao et al., 1998, Quere et al., 1999, Maclean et al., 2002). One tissue that is consistently negative at all stages of development is the lung. E10 cells are a model of non-transformed lung epithelial cells that can be grown in culture. 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 (Smith et al., 1984). When these cells were assayed for CBS activity they were found to be completely devoid of CBS activity and protein (results not shown). In order to investigate if any region of the CBS promoter confers tissue specific expression we transfected this cell line with a range of CBS –1b promoter constructs containing up to 4.523 kb of 5' flanking sequence. It can be seen that all of these constructs demonstrate promoter activity in E10 cells (Fig. 8). Further experiments in a range of CBS deficient cell lines found essentially identical results indicating that the proximal 4.523 kb of CBS 5'-flanking sequence does not confer tissue specific expression**.** This result is not surprising as all human cells appear to contain Sp1, Sp3 and NF-Y but it prompts the question as to how a promoter that is clearly controlled by ubiquitous transcription factors can be switched off in tissues that contain high levels of those factors? One possible mechanism is indicated by the results shown in figures 9, 10 and 11.

Kruppel-like factor trancription factors repress expression of the CBS promoters in FAO cells





Figs. 9 and 10. KLF transcription factors negatively regulate the human CBS gene promoters. A number of possible mechanisms that could allow tissue-specific regulation of an Sp1-driven promoter have been investigated in our laboratory. During the course of this investigation, we observed that transfection of HepG2 or FAO cells with various KLF mammalian expression constructs acts to repress the CBS –1b (Fig. 9) and –1a (fig.10) promoters. Lung kruppel-like factor (LKLF), Basic kruppel-like factor (BKLF) and KLF8 are not ubiquitously expressed and have a relatively limited tissue distribution. The distribution of LKLF appears to be specifically limited to tissues that do not express CBS (Cookwright et al., 2001, Turner and Crossley 1998).

BKLF competitively inhibits the activation of the CBS promoters by Sp1 and Sp3 in Drosophila Schneider cells.

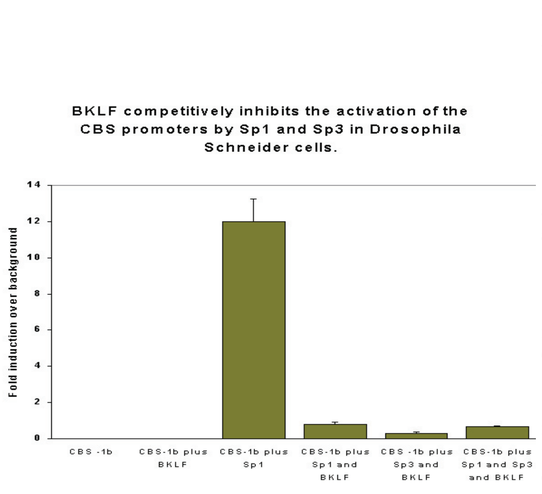


Fig. 11. BKLF acts to repress the CBS –1b promoter by competitively inhibiting Sp1/ Sp3 activation. KLF transcription factors have been reported to function by binding to GC box sequence elements with high affinity and thus competitively displace activating transcription factors such as Sp1 and Sp3 from their cognate binding sites. From co-transfection studies in drosophila Schneider cells (lacking all members of the SP1/KLF family) it can be seen that BKLF is capable of abolishing activation of the CBS-1b minimal promoter by Sp1 and /or Sp3. This finding indicates that the KLF related repression of CBS promoter activity is a consequence of competitive binding to the CBS promoters to block activation by endogenous Sp1/Sp3.

Discussion

Previous work involving HepG12 cells and the HT1080 line that expresses low levels of CBS was presented as a model of CBS tissue specificity (Ge et al., 2001). These authors stated that tissue specific expression of CBS is a consequence of differences in the relative abundance of Sp1 and Sp3. This hypothesis is unlikely to be true as it is difficult to envisage how the ratio of two positively acting transcription factors could result in no expression and CBS is completely absent from many tissues where Sp1 and Sp3 are present in some abundance. In this work, we have used co-transfection experiments to show that the CBS promoters work very well in numerous cell lines where CBS expression is completely absent. The expression of these promoter constructs in these cells illustrates the presence of ample amounts of Sp1 and Sp3 to drive CBS promoter expression. Thus the absence of CBS from these cell lines cannot be explained solely in terms of Sp1 and Sp3. Our findings indicate that changes in the relative levels and extent of binding of Sp1 and Sp3 can act to modulate the level of CBS expression up or down in various cell types but they are not responsible for the "on or off" type of regulation typically seen in various tissues at different stages of development. The absence of CBS from tissues such as lung or smooth muscle clearly involves other mechanisms. We show here that members of the KLF family of transcriptional regulators are capable of switching off the CBS promoter in the presence of normal levels of Sp1 and Sp3 by competitively blocking the binding of these activating transcription factors. Members of the KLF family are not expressed ubiquitously and have a very specific tissue distribution. In this context, lung KLF (LKLF) is particularly interesting as its tissue distribution seems to be specifically targeted to tissues where CBS is absent (J. Lingrel personal communication,). The expression pattern of LKLF also provides an interesting explanation for the previously observed pattern of CBS expression in resting and activated lymphocytes. Previous work has shown that CBS activity is completely absent from lymphocytes unless the cells are induced to proliferate by treatment with a mitogenic agent [Goldstein, 1972 #207]. This "on" or "off" manner of CBS expression differs subtly from the pattern of proliferation-specific regulation we have previously reported in fibroblast, hepatoma and neuroblastoma cells (Maclean et al., 2002). In these cells, CBS expression is down regulated but not abolished, by approximately 60 to 70% concomitant with growth arrest. The EMSA experiments presented here shows that this kind of proliferation specific attenuation of CBS expression is a function of decreased (but not abolished) binding of Sp1 to the CBS promoters. In the case of peripheral lymphocytes, LKLF is developmentally up-regulated in mature quiescent thymocytes and T-cells and is rapidly extinguished when the cells are induced to proliferate by mitogenic stimul (Buckley et al., 2001). Thus the total absence of CBS in quiescent lymphocytes is likely to be a combination of decreased abundance and binding of Sp1/Sp3 combined with the expression of LKLF (and possibly other KLF factors) which act to switch off the CBS gene completely.

Conclusions.

- Synergistic interaction between Sp1 and Sp3 only occurs at certain key sites in the -1b promoter and is absent from the –1a promoter.
- The minimal CBS promoter is contained with the proximal 35 bp of sequence adjacent to exon –1b. This promoter contains one Sp1 site. Deletion or mutagenesis of this site abolishes all detectable promoter activity.
- Synergistic interaction between Sp1 and Sp3 only occurs at certain key sites in the –1b
- Redox and growth-specific regulation of CBS is effected by changes in the level Sp1 binding to the CBS promoter.
- NF-Y and Sp3 cannot activate CBS gene expression in the absence of Sp1 *in vivo*.
- Although changes in the ratio of Sp1 and Sp3 can attenuate the level of CBS expression they are not responsible for the absence of CBS from many different tissues.
- Kruppel-like Factor (KLF) transcription factors negatively regulate the human CBS gene promoters by competitively inhibiting activation by Sp1 and Sp3.
- The CBS promoters serve as a paradigm for tissue-specific expression by ubiquitous transcription factors.

Acknowledgements.

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