Identification of functional CCAAT/enhancer-binding protein and Ets protein binding sites in the human chorionic somatomammotropin enhancer sequences

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Abstract

The human chorionic somatomammotropin (CS) A and B genes (listed as CSH1 and CSH2 in the HUGO database) are highly expressed in placenta. A 241 bp potent enhancer, nucleotides (nts) 1–241, located at the 3’ end of the CS-B gene (CS-Benh) stimulates promoter activity specifically in placental trophoblast cells in vitro. Strong activity is exerted by a 23 bp element within the CS-Benh (nts 117–139), shown to interact with transcription enhancer factor (TEF) members of the transcription enhancer activator (TEA) DNA-binding domain-containing family. An identical TEF element is present in 23 bp element within the CS-Benh (nts 117–139), shown to interact with transcription enhancer factor (TEF) members of the transcription enhancer activator (TEA) DNA-binding domain-containing family. An identical TEF element is present in the homologous (97.5%) CS-Aenh; however, a few nucleotide differences suppress its activity. Previously, we identified the transcription enhancer activator (TEA) DNA-binding domain-containing family. An identical TEF element is present in 23 bp element within the CS-Benh (nts 117–139), shown to interact with transcription enhancer factor (TEF) members of the transcription enhancer activator (TEA) DNA-binding domain-containing family. An identical TEF element is present in the homologous (97.5%) CS-Aenh; however, a few nucleotide differences suppress its activity. Previously, we identified regulatory sequences distinct from the TEF element within an 80 bp modulatory domain (nts 1–80) in the CS-Benh. Using structural and functional assays we now show that CCAAT/enhancer-binding protein (C/EBP) binding sites exist in the 80 bp modulatory domains of both enhancers, and an Elk-1 binding site exists in the modulatory domain of the CS-Aenh.

Introduction

The human chorionic somatomammotropin (CS) genes (CS-A or CSH1 and CS-B or CSH2) are expressed at very high levels in placenta throughout pregnancy. The sequence requirements for efficient in vivo CS expression have not as yet been determined (Kimura et al. 2007, Jin et al. 2009). However, a 241 bp region, nucleotides (nts) 1–241, located about 2 kb downstream of the CS-B gene has been identified, and shown to enhance homologous or heterologous promoter activity by up to 300- to 400-fold specifically in placental trophoblasts cells in vitro (Walker et al. 1990, Jacquemin et al. 1994, Jiang & Eberhardt 1994, Lytras & Cattini 1994). In addition, the ‘enhancer’ region was shown to contain hyperacetylated histone H3 and H4 in primary human placental and choriocarcinoma (BeWo) cells in culture (Kimura et al. 2007). A family of transcription factors has been identified containing an evolutionarily conserved binding domain that is referred to as the TEA (TEF-1, Tec1p, and AbaAp) or ATTS (AbaAp, TEF-1, Tec1p, and Scalloped) motif. The transcription enhancer factors (TEFs) are members, and have been shown to interact with sequences in the CS-B enhancer. Specifically, TEF-1, the first member of this family (Xiao et al. 1991), was highlighted as the major factor (Walker et al. 1990, Jacquemin et al. 1994, Jiang & Eberhardt 1994, Lytras & Cattini 1994). This was supported by its binding to the minimal 23 bp (nts 117–139) CS-B enhancer element (Fig. 1) as well as to flanking sequences (Walker et al. 1990, Jacquemin et al. 1996, Jiang et al. 1997, 2000). Subsequently TEF-5, which is relatively more abundant in the placenta, was suggested to mediate CS enhancer activity (Jacquemin et al. 1997, Jiang et al. 1999). Furthermore, TEF-1 overexpression in several cell types repressed transcriptional activity (Xiao et al. 1991, Jiang & Eberhardt 1995, 1996).

It is already apparent that additional transcription elements/factors bind to elements in the enhancer region and modify the activity of TEFs (Jiang & Eberhardt 1995, 1996, Jiang et al. 1997). At the DNA level, this notion is supported by the observation that the minimal 23 bp CS-B TEF enhancer element is not as potent as the 241 bp CS-B enhancer fragment (Walker et al. 1990, Jacquemin et al. 1994, Jiang & Eberhardt 1994, Lytras & Cattini 1994). We provided evidence for both repressor (RF-1) and derepressor (DF-1) elements within an 80 bp modulatory domain (nts 1–80, Fig. 1)
adjacent to the minimal enhancer region in the 241 bp CS-B enhancer fragment (Lytras & Cattini 1994).

An auxiliary region (AF-1, Fig. 1) was also identified that on mutation eliminated CSB enhancer activity (Lytras et al. 1996). A comparison of CS-A and CS-B ‘enhancer’ regions also provides evidence for additional regulatory elements/factors. The 241 bp CS-A ‘enhancer’ region failed to stimulate promoter activity in an efficient manner, even though it contains the identical minimal 23 bp enhancer element and high affinity TEF binding site as found in equivalent CSB sequences (Rogers et al. 1986, Jacquemin et al. 1996, Lytras et al. 1996). The difference in activity between the CS-A and CS-B ‘enhancer’ regions was traced to one and two nucleotide differences in the DF-1 and AF-1 regions, respectively, which lie outside the minimal 23 bp TEF enhancer element (Fig. 1; Rogers et al. 1986, Lytras & Cattini 1994, Lytras et al. 1996).

With regard to the mechanism of CSB enhancer action, it has been suggested that the AF-1 and DF-1 regions of CS-B (i.e. AF-1B and DF-1B, respectively) contain lower affinity TEF sites that act in combination with additional TEF sites present in the CS-B enhancer (Fig. 1; Jiang & Eberhardt 1994, Jiang et al. 1997). In this study, we examined the alternative possibility that members of other transcription factor families participate in complexes formed on the AF-1 region of CSA (AF-1A) and/or AF-1B sequences. Our data demonstrate binding by members of the CCAAT/enhancer-binding protein (C/EBP) and Ets families of transcription factors. In addition, we show that these factors are capable of modifying relative CS promoter activity under the influence of the CSA versus CS-B enhancers. Furthermore, we provide evidence that factors interacting directly or indirectly with the minimal TEF enhancer element can also interact with the AF-1A or AF-1B regions. Finally, we provide evidence that C/EBPβ associates with CSA and CS-B sequences in human term placental chromatin in situ, including differential interaction of C/EBPβ with the CSAenh versus the CSBenh. These results support the notion that factors other than TEs, specifically members of the C/EBP and Ets families, can differentially modulate CSA and CS-B enhancer activities, and that these enhancer regions are of regulatory significance in vivo.

## Materials and methods

### Materials

Chemicals and reagents were obtained from Sigma, St Louis, MO, USA; Gibco-BRL, Burlington, ON, Canada; ICN/FLOW, Cleveland, OH, USA; Pharmacia, Baie d’Urfé, QC, Canada; Mallickrodt, Paris, KY, USA; Fisher Fair Lawn, Fair Lawn, NJ, USA; Bio-Rad, Missisauga, ON, Canada; Aldrich Chem. Comp., Inc., Milwaukee, WI, USA and DuPONT, Boston, MA, USA. Monoclonal anti-FLAG antibodies (M2) were from Sigma, anti-FGF receptor monoclonal antibodies (anti-FGFR) from QED Bioscience, Inc. (San Diego, CA, USA) and anti-human retinoblastoma monoclonal antibodies (anti-Rb) from Pharmingen (San Diego, CA, USA).

### Plasmid construction

A 1022 bp fragment of the CS-B gene containing placental enhancer sequences was provided by Dr G F Saunders (M.D. Anderson Cancer Center, Houston, TX, USA). A 241 bp AccI/PvuII fragment containing the 22 bp TEF enhancer element was isolated from the 1022 bp fragment and subcloned into PUC19 as described previously (Lytras & Cattini 1994). The isolation of CSA and CS-B 265 bp enhancer regions from genomic DNA by PCR and their subcloning to PUC119 vectors has been previously described (Lytras et al. 1996).

A PUC19 vector that contained the 241 bp CS-B enhancer fragment (Lytras & Cattini 1994) was digested with HindIII and BamHI. A 2-2 kb fragment containing the CS promoter (CSp) fused immediately upstream of the chloramphenicol acetyl transferase (CAT) gene was excised after HindIII/BamHI digestion from a vector containing CSp.CAT as described previously (Lytras & Cattini 1994) and ligated to HindIII and BamHI digested CS-B enhancer-containing PUC19, placing the CS-B enhancer downstream of the CAT gene. To generate a similar CS-A enhancer construct, a PUC119 vector containing a 265 bp CS-A enhancer fragment was digested by HindIII/BamHI and the HindIII/BamHI 2-2 kb CSp.CAT fragment was inserted upstream of the CSA enhancer.

A hybrid CAT gene SV40p35 containing the 22 bp high affinity TEF site inserted directionally at the BamHI/Sall sites downstream of the CAT gene directed by the SV40 promoter (pCAT-Basic Vector; Promega) has been described (Nachtigal et al. 1993). For hybrid CAT genes containing AF-1 oligonucleotides, a 25 bp
AF-1A or AF-1B oligonucleotide (nts 33–57) was inserted in the forward orientation at the BamHI site immediately upstream of the TEF element maintaining the 5′–3′ positioning of the two elements within the 241 bp enhancer fragment.

Expression vectors for: Elk-1 (with C-terminus FLAG) was provided by Dr Sharrocks; for pCMV-Elf-R and pCMV-Elf-I were provided by Dr Kovar; Rcmv-C/EBPα (N-terminal FLAG-tagged), Rcmv-C/EBPβ (N-terminal FLAG-tagged), CMV-C/EBPα, and CMV-C/EBPβ were provided by Dr Schaufele; and pcDNA3/ets-1 and pcDNA3/ets-2 were provided by Dr Papas.

**Cell culture and DNA transfer**

Human choriocarcinoma JEG-3 cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA and grown in monolayer in DMEM supplemented with 8–10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO₂. Cells were grown on 60–100 mm culture dishes and harvested when they reached about 80% confluence. For plasmid DNA transfer, JEG-3 cells were plated in 60–100 mm culture dishes at a density of 1–2 × 10⁶ (20–40% confluence) and transiently transfected by calcium precipitation, unless otherwise stated. The culture medium containing the DNA precipitate was replaced by fresh medium 20–24 h after transfection with an individual DNA precipitation, unless otherwise described (Cattini & Eberhardt 1987). Each plate was transfected with an expression vector or control vector as described previously (Andrews & Faller 1991). Extracts in buffer C (20 mM HEPES–KOH pH 7.9, 25% glycerol, 0.42 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT)) in the presence of 2 µg of poly dI-dC in a final volume of 15–18 µl. Incubation of the reaction on ice for 20 min and at room temperature for 15 min was followed by electrophoresis in non-denaturing 4% polyacrylamide gels with 1:60 bis to acrylamide cross-linking ratio. For competition, 10–250 ng double-stranded synthetic oligonucleotides were added to nuclear extracts on ice before the addition of the radiolabeled DNA. For ‘supershift’ experiments, 3–5–6 µg of nuclear extracts (in 10–10.5 µl buffer C) were incubated on ice with 2 µg of M2 anti-FLAG antibody or, as controls, 2 µg anti-FGFR1 or 2 µg anti-Rb, monoclonal antibodies for 40 min. Subsequently, radiolabeled double-stranded AF-1A or AF-1B oligonucleotides (~1 ng; 40–50 × 10⁻⁵ c.p.m.) and 2 µg of poly dI-dC in buffer D were added per sample to a final volume of 15–16 µl, incubated on ice for 45 min and at room temperature for 15 min before loading on non-denaturing 5% polyacrylamide gels (1:60 bis to acrylamide cross-linking ratio). For autoradiography Kodak XAR films were exposed for 18 h to 10 days at −70 °C in a cassette with intensifying screens (DuPONT).

**Electrophoretic mobility shift assays**

For electrophoretic mobility shift assays (EMSAs), JEG-3 extracts (2.5–12 µg) in 6–13 µl buffer C (see above) were incubated with 3²P-end-labeled DNA fragments or synthetic oligonucleotides (0.2–2.0 ng; 2–5 × 10⁴ c.p.m. diluted in 4–6 µl buffer D (10 mM HEPES–NaOH pH 7.9, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT)) in the presence of 2 µg of poly dI-dC in a final volume of 15–18 µl. Incubation of the reaction on ice for 20 min and at room temperature for 15 min was followed by electrophoresis in non-denaturing 4% polyacrylamide gels with 1:60 bis to acrylamide cross-linking ratio. For competition, 10–250 ng double-stranded synthetic oligonucleotides were added to nuclear extracts on ice before the addition of the radiolabeled DNA. For ‘supershift’ experiments, 3–5–6 µg of nuclear extracts (in 10–10.5 µl buffer C) were incubated on ice with 2 µg of M2 anti-FLAG antibody or, as controls, 2 µg anti-FGFR1 or 2 µg anti-Rb, monoclonal antibodies for 40 min. Subsequently, radiolabeled double-stranded AF-1A or AF-1B oligonucleotides (~1 ng; 40–50 × 10⁻⁵ c.p.m.) and 2 µg of poly dI-dC in buffer D were added per sample to a final volume of 15–16 µl, incubated on ice for 45 min and at room temperature for 15 min before loading on non-denaturing 5% polyacrylamide gels (1:60 bis to acrylamide cross-linking ratio). For autoradiography Kodak XAR films were exposed for 18 h to 10 days at −70 °C in a cassette with intensifying screens (DuPONT).

**ChIP on chip tiling array assays**

‘ChIP on chip’ tiling array assays were performed by Active Motif (Carlsbad, CA, USA). Human term placenta tissue was fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Collection of term placental tissue was carried out under the...
approval of the Bannatyne Campus Research Ethics Board of the University of Manitoba. Term placentas were collected from routine births at the Women’s Hospital (Winnipeg, MB, Canada) as discarded tissue with no connection to the identity or family history of mother or newborn. Chromatin was isolated by adding lysis buffer, followed by dissection with a motorized pestle. Lysates were sonicated and the DNA sheared to an average length of 300–500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield.

An aliquot of chromatin (20–30 μg) was pre-cleared with protein A or G agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using an antibody against C/EBPβ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After incubation at 4°C overnight, protein A or G agarose beads were used to isolate the immune complexes. Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Cross-links were reversed by incubation overnight at 65°C, and ChIP DNA was purified by phenol–chloroform extraction and ethanol precipitation.

ChIP DNA was amplified by whole genome amplification (WGA) using the GenomePlex WGA Kit (Sigma). The amplified DNA was fragmented and labeled using the DNA Terminal Labeling Kit from Affymetrix (Santa Clara, CA, USA), and then hybridized to Affymetrix GenChip Tiling arrays at 45°C overnight. Arrays were washed and scanned, and the resulting CEL files were analyzed using Affymetrix TAS software. Thresholds were selected, and the resulting BED files were analyzed using Genpathway software (proprietary) that provides comprehensive information on genomic annotation, peak metrics and sample comparisons for all peaks (intervals). To verify peaks, the results for ChIP DNA were normalized against existing input DNA array data (Active Motif database) from three different human genomes.

Statistical analysis

Unpaired t-tests and (ANOVA) or nonparametric ANOVA (Kruskal–Wallis) were used to assess the statistical significance of differences observed in these studies. When the results of the ANOVA indicated the presence of a significant difference, a Bonferroni post hoc test or Dunn’s Multiple Comparisons Test was applied (InStat). Significance was defined by P<0.05.

Results

The AF-1A region interferes with TEF enhancer activity on a heterologous SV40 promoter

Previously, we had observed a 65% decrease in the activity of the minimal TEF enhancer element in the presence of the AF-1 region from the CS-B gene (AF-1B) in the context of a SV40 promoter-directed CAT reporter gene (Lytras et al. 1996). We have now expanded this experiment to include the analogous region from CSA (AF-1A) by comparing the activities of the SVp.CAT.AF-1B.TEF and SVp.CAT.AF-1A.TEF constructs, relative to the basic SVp.CAT and SVp.CAT.TEF (Fig. 2). The activity observed with SVp.CAT.AF-1A.TEF was comparable to that of the enhancerless SVp.CAT construct (Fig. 2). Thus, while AF-1B showed significant repression of TEF-induced activity (n=23–30, P<0.05), AF-1A completely abolished it (n=6–23, P<0.001).

The AF-1A and AF-1B regions show different EMSA patterns, and TEF oligonucleotides compete with varying affinities for some AF-1A and AF-1B complexes

Nuclear extracts from placental choriocarcinoma (JEG-3) cells were used to compare the EMSA patterns obtained with the AF-1A and AF-1B regions. Multiple complexes of various mobilities were observed with both regions (C1, C2, and C3; Fig. 3). In addition, multiple

Figure 2 Comparison of the effects of the AF-1A and AF-1B regions on TEF-1 enhancer activity. Placental choriocarcinoma cells (JEG-3) were transiently transfected with CAT reporter gene constructs, directed by the SV40 promoter and containing the 22 bp high affinity TEF site in combination with the AF-1 regions from either the CS-A (AF-1A) or the CS-B (AF-1B) gene. While AF-1B showed significant repression of TEF-induced activity (n=23–30, P<0.05), AF-1A completely abolished it (n=6–23, P<0.001). A diagram of the basic structure of the SVp.CAT.AF-1.TEF-1 construct has been published previously (Lytras et al. 1996).
additional lower mobility complexes were readily detected with the AF-1A alone (C4, C5, and C6). However, a lower mobility complex migrating similarly to C6 was also observed in prolonged exposures of AF-1B EMSAs (not shown). We used competition assays to examine the specificity and affinities of the observed complexes as well as to assess the possibility that the absence of lower mobility complexes from the AF-1B region may reflect different affinities of the AF-1A and AF-1B regions for some of the lower mobility complexes (Fig. 4). For AF-1B complexes, AF-1A competed for C2 and weakly for C3. For AF-1A complexes, AF-1B competed efficiently for C2, and, with lower efficiency, also for the lower mobility C5 and C6. In contrast, the AF-1B region was unable to compete for C3 and C4 formed on the AF-1A region.

We showed previously that the TEF oligonucleotide, corresponding to nts 117–139, competes for some low but not high affinity complexes formed on the AF-1B region (Lytaras et al. 1996). In this study, we compared competition of complexes formed on AF-1A versus AF-1B regions by the TEF oligonucleotide. For AF-1A complexes, C2 and C5 were competed for most effectively by AF-1A, but also by TEF, which was in turn a more efficient competitor than AF-1B. TEF oligonucleotides were also able to compete, but less effectively, for C6 but did not compete for C1, C3, and C4. For AF-1B complexes, TEF competed weakly for C2 but not for C3.

Putative C/EBP and Ets sites are present in the AF-1B and/or the AF-1A region

Using available transcription factor databases, we searched the modulatory domains of the CSA and CS-B enhancers for putative transcription factor recognition sites (core 100%, overall similarity >75%; Fig. 5). A putative Elk-1 site (100% core, 93% overall similarity), an Ets family member, was identified in the reverse orientation in the AF-1A region. Because of a single nucleotide discrepancy in the AF-1B region relative to AF-1A, the core of the Elk-1 site present in AF-1A (CGGA) is absent in the AF-1B region (CtGA). A putative site for C/EBP was identified in both the AF-1A and AF-1B regions. The C/EBP site overlaps the Elk-1 site in AF-1A and, because of the same single nucleotide difference, is not identical in AF-1B (100% core, 79 and 88% overall similarity for the AF-1A and AF-1B regions respectively).

Elk-1 overexpression results in modification of DNA–protein interactions on the AF-1A but not the AF-1B region

Following the identification of the putative Elk-1 binding site in AF-1A, nuclear extracts were prepared from JEG-3 cells transfected with expression vectors carrying the cDNAs for various Ets family members (Ets-1, Ets-2, Elf-R, Elf-1, and Elk-1), and were examined in EMSAs using the AF-1A and, as a comparison, the AF-1B region (Fig. 6). A high mobility complex (HMC) and a lower mobility complex with similar mobility to C4 were observed in Elk-1 extracts but not with any of the other Ets family members on the AF-1A region (Fig. 6A). In contrast, no modification of the mobility pattern was observed with the AF-1B region. In parallel, a reduction of C5 on the AF-1A region was also observed with Elk-1 overexpression. The specificity and affinity of the Elk-1-induced complexes on AF-1A but not AF-1B sequences, was further characterized by competition with either unlabeled AF-1A or AF-1B oligonucleotide competitors (Fig. 6B). Both Elk-1-induced complexes (HMC, C4) were readily competed by AF-1A but not AF-1B competitor.

To confirm the participation of Elk-1 in the C4-like complex formed on the AF-1A region, nuclear extracts from JEG-3 cells transfected with the Elk-1 expression vector (Elk-1 is FLAG-tagged), were preincubated with monoclonal antibodies against the FLAG epitope (Fig. 7). A ‘supershift’ of C4 was observed with nuclear extracts from JEG-3 cells that were transfected with but not without the Elk-1 expression vector. No distinct ‘supershift’ or competition of HMC was observed with anti-FLAG antibodies. This complex might be induced by Elk-1 but not comprised of Elk-1, and thus may or may not be important for the formation of other AF-1 complexes. It is also possible that conformational changes may interfere with accessibility of the FLAG...
tag, and thus allow for differential recognition of Elk-1-FLAG associated with HMC versus C4. Finally, it could be a simpler complex resulting from dissociation of a larger complex after association of Elk-1 with AF-1A; for example, Elk-1 displaced a factor that can still associate with AF-1 as a monomer.

**C/EBPα overexpression results in the formation of a ‘novel’ complex on the AF-1B region**

Nuclear extracts were prepared from JEG-3 cells transfected with control vector (RcCMV) and expression vectors carrying the cDNAs for C/EBPα or C/EBPβ (FLAG-tagged) and were examined in EMSAs using the AF-1A or the AF-1B region as a probe. The patterns obtained with both AF-1 regions appeared modified by C/EBP overexpression (Fig. 8A). C/EBPα overexpression resulted in the formation of a ‘novel’ complex on AF-1B, migrating similarly to C5 formed on AF-1A with control extracts. This suggested that C/EBPα might physically participate in the formation of the novel C5 complex on AF-1B. To directly determine the presence of C/EBPα in this complex, monoclonal antibodies against the FLAG epitope were used. A ‘supershift’ of the novel complex was observed (Fig. 8B). No ‘supershift’ was produced in the absence of C/EBPα overexpression or when an anti-Rb monoclonal antibody was used as control.

We tested the effectiveness of the AF-1B and AF-1A unlabeled regions to compete for the novel AF-1B C5 complex (Fig. 9). Indeed C5 was readily competed by both AF-1B and AF-1A oligonucleotides.

**Elk-1 overexpression in JEG-3 cells modifies the relative CS-A versus CS-B enhancer activity**

We examined the possible functional significance of these new complexes on the basis of differential Elk-1 effects on the CS-A versus the CS-B enhancers. Initially, we examined the effects of Elk-1 overexpression on the activity of Csp.CAT constructs containing either the CS-A or the CS-B enhancer (Fig. 10A). Interestingly, Elk-1 repressed the activity of the CS promoter alone
(CSp.CAT) resulting in a remaining activity of 44.8\% (n=5, P<0.001). A CSp.CAT:CS-Benh construct was also repressed in an identical manner with a remaining activity of 43.6\% (n=5, P<0.005). However, Elk-1 overexpression repressed more strongly the CSp.CAT:CS-Aenh resulting in a remaining activity of 26.4\% (n=5, P<0.001). The difference between the percentage of the remaining activity of the CSp.CAT or the CSp.CAT:CS-Benh constructs and the CSp.CAT:CS-Aenh construct was statistically significant (n=5, P<0.001). Thus, in this context, relative to CSp.CAT, the CS-A, but not the CS-B, enhancer demonstrated a negative effect in response to Elk-1 overexpression.

**Figure 5** Putative transcription factor recognition sites in the modulatory domains of the CS-A and CS-B enhancers. Putative transcription factor recognition sites were identified using transcription factor databases (core 100\%, overall similarity >75\%). TEF-related sites, as reported by others (Jacquemin et al. 1997, Jiang et al. 1997) are highlighted in the CS-B enhancer sequence. A putative Elk-1 site is present in the CS-A enhancer (highlighted) but not in the CS-B enhancer sequence. Binding sites common to both regions are indicated between the two sequences. Nucleotide discrepancies between CS-B and CS-A are indicated in lower case.

**C/EBP\(\alpha\) and C/EBP\(\beta\) overexpression in JEG-3 cells**

C/EBP\(\alpha\) and C/EBP\(\beta\) overexpression in JEG-3 cells represses the activity of the CSp, but increases CS-B and CS-A enhancer activities resulting in a net increase in the activity of a CSp.CAT:CS-Benh construct but not of a CSp.CAT:CS-Aenh construct.

Given the identification of C/EBP binding sites, the effect of C/EBP\(\alpha\) or C/EBP\(\beta\) overexpression on the activity of the 496 bp CSp fragments was examined in the presence and absence of either the CS-A or the CS-B 241 bp enhancers (Fig. 10B). Both C/EBP\(\alpha\) and C/EBP\(\beta\) resulted in increased CSp.CAT:CS-Benh construct activity (by 64.3 and 71.7\%, respectively, n=4,
of magnitude (from 7.9-fold under control conditions to 81.9- and 87-fold in the presence of C/EBPβ or C/EBPβ respectively).

**C/EBPβ associates with the CS-A and CS-B genes and enhancer regions, in situ**

An unbiased ChIP on chip tiling array for chromosome 17 was used to assess whether C/EBPβ associates with CS-A and CS-B sequences in the context of human term placenta chromatin. C/EBPβ was targeted for examination based on its high level of placental expression, particularly during late pregnancy (Bamberger et al. 2004). C/EBPβ ‘intervals’, indicating transcription factor binding in situ, were detected in both the CS-A and CS-B genes (Fig. 11). The modulatory domains of both the CS-A and CS-B enhancer regions were found at the borders of C/EBPβ ‘intervals’ that extended from the enhancer regions to ~3312 and 3311 nts downstream of their respective transcription initiation sites (Table 1). In both cases ‘peak’ regions were found a few hundred bp upstream of these 3′ enhancers more proximal to the coding region of the gene.

In addition, differential spreads of the CS-A and CS-B C/EBPβ intervals over the enhancer regions were observed. In the case of the CS-A gene, the C/EBPβ interval starts at the core of the enhancer modulatory domain and extends upstream toward the coding region of the gene, and in the opposite direction relative to the main TEF-1 site which lies downstream of the modulatory domain (Fig. 11). Indeed, in the CS-A gene, the C/EBPβ interval spans from nts 59323740 to 59325870 (Table 1) extending upstream toward the CS-A gene-coding region. As the overlapping Elk-1/C/EBPβ site in the modulatory domain of the CS-A enhancer spans nts 59323721–59323739, the detected C/EBPβ interval starts just upstream of this binding site (Fig. 11). Putative C/EBP sites are present upstream of the Elk-1 site (not shown) of which the most proximal overlaps with the c-myb site shown in Fig. 5. In contrast to CS-A, for the CS-B gene, the C/EBPβ interval spans from nts 59300535 to 59303012 (Table 1) and includes the full length 241 bp enhancer, and thus the ‘main’ TEF-1 site, and similarly extends upstream toward the CS-B coding region.

**Discussion**

In previous studies, we assessed the functional differences between the CS-A and CS-B enhancers to gain insight into the mechanism by which placental enhancer function is regulated (Lytras & Cattini 1994, Lytras et al. 1996, Jin et al. 2009). In particular, in comparison to the CS-B enhancer, the CS-A enhancer bears nucleotide substitutions that render it inactive in
transfected choriocarcinoma cells in vitro (Lytras et al. 1996). These substitutions are found in sequences outside the minimal enhancer region that is identical in the two enhancers (nts 117–139) and has been shown to interact with the TEF-1 and TEF-5 members of the TEA domain family. We have examined the structural and functional implications of these few nucleotide differences with an aim to identifying proteins different from TEFs that may have a regulatory role, having differential effects on CS-A versus CS-B enhancer function. We have now identified binding sites for the C/EBP and Ets families of transcription factors, in areas distinct from the minimal enhancer element, and with small nucleotide differences between them. Furthermore, we show that members of these families can differentially modify CS-A and CS-B enhancer activities. Finally, observations made from ChIP on chip tiling array assay of human term placenta chromatin, are consistent with C/EBP binding to the CS ‘enhancer’ regions, and even suggest differential occupancy of the CS-A and CS-B sequences. This clearly increases confidence that the CS ‘enhancer’ regions are sites of regulatory significance in vivo.

There are only six nucleotide differences between the 241 bp CS-A and CS-B enhancer regions, with three in the modulatory domain, including two in AF-1 and one in DF-1 (Lytras et al. 1996). These can be likened to point mutations, and thus highly specific indicators of the sequences and by extension the elements that confer the CS-Aenh versus CS-Benh differences, as suggested for AF-1 in response to Elk-1 and C/EBP in this study. Insertion of AF-1A upstream of the TEF element conferred a stronger repression than AF-1B resulting in complete loss of minimal TEF enhancer activity (Fig. 2). This difference in the effects of AF-1A versus AF-1B is consistent with the generation of enhancer activity by the mutational ‘repair’ of AF-1A to resemble AF-1B in the context of the 241 bp CS-A enhancer (Lytras et al. 1996). It has been suggested that the combined AF-1 and DF-1 regions may represent lower affinity TEF binding sites (Jiang & Eberhardt 1994, 1996). If so, this would provide an explanation for

Figure 8 Effects of C/EBPα and C/EBPβ overexpression on the DNA–protein interactions on the AF-1 regions and electrophoresis mobility supershift assays for the detection of C/EBPα-related complexes on AF-1A. (A) Nuclear extracts (5 µg) from JEG-3 cells transfected with control expression vector (RcCMV) or expression vectors for C/EBPα or C/EBPβ (as indicated) were examined in EMSAs using the AF-1A or the AF-1B radiolabeled probe (indicated below the figure). Binding reactions were resolved on a non-denaturing 4% polyacrylamide gel. Complexes (C1–C6) are labeled to correspond to previous figures. (B) Nuclear extracts from control (−) and C/EBPα-overexpressing (+) JEG-3 cells were preincubated with monoclonal antibodies against the FLAG epitope (anti-FLAG) or with commercial anti-human monoclonal antibodies (anti-Rb) as a control. Subsequent binding reactions using radiolabeled AF-1B were then resolved on a non-denaturing 5% polyacrylamide gel.
the synergistic effect of combining the AF-1/DF-1 region and the minimal TEF element in the context of the 241 bp CS-B enhancer (Jiang & Eberhardt 1994, 1996, Lytras & Cattini 1994). However, in our studies the AF-1 region, without DF-1, represses TEF effects. It is possible that the AF-1 region alone does not allow TEFs to bind or make a functional interaction with the high affinity TEF element.

An alternative possibility is that additional factors interact with the AF-1 and inhibit TEF action. This is supported by the distinct EMSA patterns observed with AF-1A and AF-1B elements (Fig. 3), and by incomplete overlap between these DNA fragments in competition assays (Fig. 4). A putative C/EBP binding site was identified in both regions (Fig. 5) and evidence of binding was obtained by EMSA (Figs 8 and 9). In the case of AF-1B, a new C/EBPα complex (C5) was generated with C/EBPα overexpression and was competed by both AF-1A and AF-1B oligonucleotides. In contrast, only minor changes were observed with AF-1A, however, this is to be expected as a band with the same mobility as C5 was observed with control (JEG-3) nuclear protein (Fig. 8). Thus, the structural data are consistent with C/EBP interaction with AF-1B as well as AF-1A, which is naturally ‘mutated’ at two nucleotides in the context of the CS-A enhancer (Fig. 5) and, notably, differs functionally from AF-1B. This functional difference could be explained by the binding of different factors in the AF-1A and AF-1B sites. As a result of a single bp difference that disrupts the Ets core, AF-1A but not AF-1B binds Elk-1, which can be competed by AF-1A but not AF-1B oligonucleotides in an EMSA (Figs 6 and 7). The presence of this Ets site may result either in facilitation or in competition of C/EBP-related complexes formed on AF-1A, establishing an ‘Ets-directed’ modulatory switch on CS enhancer function. Alternatively, differential affinity for C/EBP binding, differential conformation of C/EBP complexes or even binding of factors other than Ets could explain the functional difference between the AF-1A and AF-1B sites.

**Figure 9** Competition assays for the novel C/EBPβ-induced complex formed on the AF-1B region with AF-1B and AF-1A unlabeled competitors. Nuclear extracts from JEG-3 cells transfected with control vector (RcCMV) or with expression vectors for C/EBPβ or C/EBPα (as indicated) were used in competition EMSA with radiolabeled AF-1B. The nanogram amounts of cold AF-1A or AF-1B competitor oligonucleotides are indicated. The migrating positions of complexes C1–C6 are also labeled to correspond to previous figures.

**Figure 10** Effects of Elk-1 and C/EBP overexpression on the activity of constructs containing either the CS-A or the CS-B enhancer. (A) Elk-1 overexpression repressed the activity of the CS promoter alone (CSp.CAT) resulting in a remaining activity of 44-8% (n=5, P<0.001), and the activity of CSp.CAT constructs containing either the CS-A or the CS-B enhancer. A CSp.CAT.CS-Benh construct was repressed in an identical manner (remaining activity 43-6%) (n=5, P<0.005). However, the CSp.CAT.CS-Aenh construct was repressed more strongly by Elk-1 overexpression resulting in a remaining activity of 26-4% (n=5, P<0.001). The difference between the percentage of the remaining activity of the CSp.CAT or the CSp.CAT.CS-Benh constructs and the CSp.CAT.CS-Aenh construct was statistically significant (n=5, P<0.001). (B) C/EBPα (α) or C/EBPβ (β) co-transfection (5 μg) resulted in a strong negative effect on independent CS promoter activity (approximately eightfold or 88-5 and 87-1% repression of control activity, C/EBPα (n=4) and C/EBPβ (n=4) respectively (P<0.001)). However, the presence of the CS-B enhancer reversed this negative effect and the CSp.CAT.CS-Benh construct activity increased by 64-3 and 71-7%, in the presence of C/EBPα (n=4) and C/EBPβ (n=4) respectively (P<0.001). In contrast, the CS-A enhancer was unable to compensate for the repression of the CS promoter by C/EBPα (n=2) and C/EBPβ (n=2), resulting in a net ~75% reduction in CSp.CAT.CS-Aenh construct activity (P<0.001).
Regulation through interaction of Ets/Elk-1 and C/EBPs, and specifically with C/EBPα and β, have been reported, and these data suggest multiple mechanisms for affecting promoter activity. These include acting through independent DNA elements, acting synergistically, participating through protein–protein interaction, and contributing to efficient expression as well as inducing hormone (insulin) responsiveness (Hanlon et al. 2000, Jacob & Stanley 2001, Lin et al. 2005, Hung et al. 2010). Our functional data give insight into the possible interactions of C/EBP and Elk-1 with their overlapping sites. Interestingly, Elk-1 overexpression represses CSp.CAT activity. Importantly, it affects the CS-A and CS-B enhancers differently as the CS-A, but not the CS-B, enhancer responded negatively to Elk-1 overexpression (Fig. 10A). Like Elk-1, C/EBPα or β overexpression also represses CSp.CAT activity in JEG-3 cells. Unlike the case with Elk-1, however, the presence of the CS-B enhancer region reverses the effect of C/EBP overexpression and in fact results in enhanced CSp.CAT: CS-Benh activity. In contrast, the CS-A enhancer region as well as promoter (p) and coding regions are shown under a higher resolution map of the intervals for each of the CS-A and CS-B genes, to allow better alignment of intervals and regulatory sequences. See text in Results and Table 1 for further details regarding exact numbering of the interval sequences.
likely modify C/EBP interactions with the basal transcription machinery. This is supported by the fact that the activity of the CSp.CAT.CS-Benh construct is increased by C/EBPs suggesting that they exert a direct enhancing effect on the combined promoter–enhancer function.

Additional interactions of the AF-1 and TEF elements with other sequences in the CS enhancers are likely required for the orchestration of TEF-C/EBP cooperation as suggested by the repression of the enhancer function of the TEF element by both AF-1A and AF-1B (Fig. 2). Thus, although these studies confirm that AF-1 interferes with the interactions of the TEF element with the promoter region, they also suggest that an AF-1/TEF interaction is still not sufficient for enhancer function. However, considering the competition of AF-1 complexes, which are associated, directly or indirectly, with C/EBP factors, by TEF oligonucleotides (Fig. 4), our results link the TEF with the C/EBP family of factors known to interact with chromatin components and to be involved in transcription factor coactivator recruitment (Mink et al. 1997, Kowenz-Leutz & Leutz 1999).

The identification of an Ets binding site in the CS-A enhancer (AF-1A) might be significant given that Ets-signaling has already been shown (through an Ets-2 null mutation) to be crucial for placental development in mice (Yamamoto et al. 1998). Ets factors appear to play an important role in the development of organs that require extensive tissue remodeling, which includes the placenta, a role that is possibly associated with their effects on the expression of tissue matrix metalloproteinases (Westermarck & Kahari 1999).

Enhancer sequences have been linked with efficient in vivo expression through interactions with locus control region (LCR) sequences (Anagnou et al. 1995, Stamatoyannopoulos et al. 1997, Jin et al. 1999, 2004). During placential development the CS-A to CS-B mRNA ratio increases, and this pattern is preserved when the human GH/CS locus, including CS-A and CS-B, is introduced into transgenic mice (Jin et al. 2009). This suggests that the CS-A gene is more transcriptionally active than its CS-B counterpart at term compared with early placentas. Assuming the functional significance of the CS-B and/or the CS-A enhancer region for efficient expression of the CS genes in vivo, possible explanations for this phenomenon are a) that the CS-B enhancer is the only efficient enhancer in the human GH locus and a switch in promoter selection occurs during the course of placential development in a manner similar to the example of the globin locus (Foley et al. 1994, Langdon & Kaufman 1998) and b) that the CS-A enhancer is in fact active in vivo in spite of the low CS-A enhancer activity in choriocarcinoma cell lines. In partial support of the latter is the fact that stronger CS-A enhancing activity has been observed in primary trophoblast cultures from term placentas (Jacquemin et al. 1996) suggesting that the CS-A enhancer may be more active in mature syncytiotrophoblasts. In addition, the CS-A enhancer region has been reported to be hypersensitive specifically in placental cells (Jimenez et al. 1993). Furthermore, specific expression of CS was observed in the placental labyrinth of transgenic mice containing the human GH LCR and CS-A but lacking the CS-B gene (Jones et al. 1995) and, as indicated, the CS-A promoter appears to be more active than the CS-B promoter, based on relative RNA levels, when both genes and enhancer regions are contained in the transgene (Jin et al. 2009). Thus, crosstalk of enhancer (and related factors) with other regulatory sequences in vivo might result in CS-A enhancer function greater than that of the CS-B enhancer.

### Table 1: Functional C/EBP and Ets sites in hCS enhancers

<table>
<thead>
<tr>
<th>Start</th>
<th>Length</th>
<th>Probes</th>
<th>Peaks</th>
<th>Peak center</th>
<th>Gene list</th>
<th>Distance to start</th>
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<tr>
<td>59299261</td>
<td>59299929</td>
<td>668</td>
<td>14</td>
<td>3</td>
<td>CS-B</td>
<td>5512</td>
</tr>
<tr>
<td>59300535</td>
<td>59303012</td>
<td>2477</td>
<td>53</td>
<td>1</td>
<td>CS-B, GH-V</td>
<td>3311, 11445</td>
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<tr>
<td>59300824</td>
<td>59304164</td>
<td>340</td>
<td>10</td>
<td>1</td>
<td>CS-B, GH-V</td>
<td>877, 9011</td>
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<tr>
<td>59313656</td>
<td>59313933</td>
<td>277</td>
<td>8</td>
<td>4</td>
<td>CS-B, GH-V</td>
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</tr>
<tr>
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<td>59317627</td>
<td>237</td>
<td>7</td>
<td>1</td>
<td>GH-V, CS-A</td>
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<tr>
<td>59322131</td>
<td>59322799</td>
<td>668</td>
<td>15</td>
<td>1</td>
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<tr>
<td>59323740</td>
<td>59325870</td>
<td>2130</td>
<td>49</td>
<td>1</td>
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<tr>
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<td>59327098</td>
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<td>1</td>
<td>CS-A</td>
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<tr>
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<td>59332541</td>
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<td>18</td>
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<td>1927, 9507</td>
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We have now identified two candidate members of the Ets and the C/EBP families of transcription factors, Elk-1 and C/EBPβ/β, which can participate in a functional interplay and affect the CS-A and CS-B enhancers differently. The presence of overlapping Ets and C/EBP sites in AF-1A, suggests that a state of equilibrium may normally exist between these factors and the AF-1B element. This notion is also reflected in the reduction in intensity of the C/EBP-related complex on AF-1A, when Elk-1 is over-expressed (Fig. 6B). Furthermore, our array analysis of chromosome 17 sequences in human placental chromatin provides evidence for the existence of a differential C/EBP equilibrium on the AF-1A versus AF-1B regions. C/EBPβ is the primary C/EBP species in the placenta and specifically the villous syncytiotrophoblasts versus cytotrophoblasts (Bamberger et al. 2004), and was, thus, targeted for assessment. C/EBPβ ‘intervals’, indicating transcription factor binding in situ, were detected in both the CS-A and CS-B genes. These intervals appear to originate at the CS-A and CS-B enhancer regions and extend out to ~3312 and 3311 nts downstream of their transcription initiation sites respectively. Importantly, the observed difference in the spread of the C/EBPβ intervals between the CS-A and CS-B enhancer regions is consistent with a differential impact on CS-A versus CS-B expression. The spread of the C/EBPβ interval over the full 241 bp CS-Benh region including the main TEF-1 site (nts 117–119) may conceivably interfere with effective binding of TEF-1/5 on this enhancer in term placenta. In contrast, in the case of the CS-Aenh, the C/EBPβ interval starts at the core of the enhancer modulatory domain just upstream of the overlapping Elk-1/C/EBP binding site and expands upstream in the opposite direction relative to the main TEF-1 site, which lies downstream of the modulatory domain (Fig. 11 and Table 1).

The identification of Ets and C/EBP binding sites in the CS enhancer regions raises several possibilities for further investigations. Ets and C/EBP proteins are expressed in placenta and have been shown to interact in a variety of tissues and gene contexts, including placental and pituitary gene regulation (Antonson & Xanthopoulou 1995, Hanlon et al. 2000, Jacob & Stanley 2001, Bamberger et al. 2004, Lin et al. 2005, Chakrabarty & Roberts 2007, Hung et al. 2010). We have shown that a site in the CS-A enhancer region can bind at least one member (Elk-1) of the Ets family. Thus, it is possible that this site is a target for additional family members that bind with higher or lower affinity, tissue specificity and a differential ability to interact with C/EBP or other regulatory GH/CS sequences. Histone modifications within the GH/CS locus have been ‘mapped’, and the CS enhancer regions are contained in hyperacetylated and hypermethylated domains in placental cells (Kimura et al. 2007). In addition Elk-1 overexpression was reported to induce endogenous GH/CS expression in heterologous human (non-pituitary and non-placental) cells (Yang et al. 2010). Furthermore, earlier work has implicated the CS enhancers in the pituitary repression of the CS genes (Jiang & Eberhardt 1997). In combination, these observations raise the possibility that members of the Ets family, possibly in association with C/EBPs, contribute to the lack of CS expression in the pituitary by participating in promoter/enhancer complexes and/or promoting chromatin modifications. The latter includes the potential involvement of chromatin loops; the generation of two loops required for specificity and efficient expression during activation of the pituitary GH gene has been described (Ho et al. 2008). In this context, preferential commitment of the CS-A, CS-B, or GH-V promoter in a placental cell might be dependent on the relative dynamics of additional regulatory sequences. Further study will be required to address the importance of the relative binding efficiency of C/EBPβ versus C/EBPβ, and the possible role of Elk-1 for tissue specificity and efficient placenta expression. Determining whether the CS-A and CS-B enhancers ‘compete’ for association with the LCR and/or other regulatory sequences, including additional Ets and C/EBP binding sites in the GH/CS locus, will also require further examination.

In summary, C/EBP and Ets binding sites have been identified in the AF-1 regions that differ between the CS-A and CS-B enhancer regions, and have been shown to account, at least in part, for the substantial difference in the activity of the two enhancers in vitro. Our observations are consistent with the participation of factors other than TEF to differentially modulate CS-A and CS-B enhancer activity, presumably either by competing with TEFs for binding or by directly or indirectly interacting with TEFs.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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