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 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*. C/EBP α or C/EBP β strongly repressed *CSp.CAT* activity but stimulated *CSp.CAT.CS-Benh* activity. In contrast, the equivalent *CS-A* enhancer sequences were unable to relieve promoter repression. Elk-1 overexpression also resulted in differential effects on the *CS-Aenh* versus *CS-Benh*. Finally, we provide evidence for the association of C/EBP β with the *CS-A* and *CS-B* genes in human placental chromatin, including differential involvement of C/EBP β with the *CS-Aenh* versus the *CS-Benh*, and therefore consistent with the notion that these are regions of regulatory significance *in vivo*. We conclude that members of the C/EBP and Ets families can differentially modulate *CS-Benh* and *CS-Aenh* activity.

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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)

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1      14      33      57
GTCTACATTTCAGCTCATCAACTTGGTGTGGACGCAATTTCtGCTGCAAATTGAGATG
          RF-1      AF-1
61      75      118
CCTAGGaTGTTTCTAAACGATGCATaCGTGAGCCCTCACTCCCTGAGATTCTGATATAA
          DF-1
121      140
TTAGACTGGAATGTGGTCCAGGCAAGAGTAgTTTGAAAACTTCCCGAGGTGATTCTAA
          TEF-1
181      240
ATGTAACAAGGTTGAGAACCACtGTGTGTAGGGACCGCAAAGATGAGACCCATGTGTT

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Figure 1 The nucleotide sequence of the human *CS-B* enhancer region. The positions of previously identified TEF-1 (Walker *et al.* 1990), RF-1 and DF-1 (Lytras & Cattini 1994), and AF-1 (Lytras *et al.* 1996) regions are indicated by underlining or shading. Differences between the *CS-B* and *CS-A* enhancers are indicated by lower case characters.

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 *CS-B* 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 *CS-B* 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 *CS-B* 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 *CS-A* (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 *CS-A* 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/EBP β associates with *CS-A* and *CS-B* sequences in human term placental chromatin *in situ*, including differential interaction of C/EBP β with the *CS-A*enh versus the *CS-B*enh. These results support the notion that factors other than TEFs, specifically members of the C/EBP and

Ets families, can differentially modulate *CS-A* 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, Mississauga, 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 *CS-A* 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 *CS-A* enhancer.

A hybrid *CAT* gene *SV40p35* containing the 22 bp high affinity TEF site inserted directionally at the BamHI/SalI 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-1 were provided by Dr Kovar; RcCMV-C/EBP α (N-terminus FLAG-tagged), RcCMV-C/EBP β (N-terminus FLAG-tagged), CMV-C/EBP α , and CMV-C/EBP β 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 $1\text{--}2 \times 10^6$ (20–40% confluence) and transiently transfected by calcium phosphate–DNA precipitation after 20–26 h as described (Cattini & Eberhardt 1987). Each plate was transfected with an individual DNA precipitation, unless otherwise stated. The culture medium containing the DNA precipitate was replaced by fresh medium 20–24 h later and cells were harvested 22–24 h after the addition of the fresh medium. Pellets were resuspended in 0.1% v/v Triton X-100/100 mM Tris–HCl pH 7.8, transferred to 1.5 ml microfuge tubes and incubated for 20 min on ice. After centrifugation for 15 min at 4 °C the supernatant (cell extracts) was assayed for luciferase and/or CAT activity or placed at –70 °C until assayed. Protein concentrations were determined by the Bradford microassay (Bradford 1976) with BSA as a standard, by measuring absorption at 595 nm (A₅₉₅). CAT activity was measured using a modification of the two-phase fluor diffusion assay as described previously (Nickel *et al.* 1990). Quantitative values for CAT activity were determined by regression analysis and were corrected for the amount of protein per sample (c.p.m./mg of cell extract protein). CAT activity was also expressed as fold increase over the basal SV40 or CSp activity. Correction for transfection efficiency and DNA uptake was obtained by co-transfection with a CMV-1/luciferase gene hybrid construct (0.2–1.0 µg/plate) and assessment of the luciferase activity of the cell extracts. For transcription factor overexpression experiments, 5 µg of each expression vector or control vector were used. For these experiments luciferase correction was only done for determining the fold induction over basal promoter

activity only when the same vector(s) were used for the CSp and CSpC*Senh* constructs as different transcription factors had differential effects on CMV promoter activity (A Lytras & PA Cattini, unpublished observations).

Preparation of nuclear extracts

Nuclear extracts were prepared from cells transfected with expression vectors carrying cDNAs for transcription factors or control vectors as described previously (Andrews & Faller 1991). Extracts in buffer C (20 mM HEPES–KOH pH 7.9, 25% glycerol, 0.42 NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride) were stored at –70 °C. Protein concentrations were determined as above.

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; $2\text{--}5 \times 10^4$ 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, $40\text{--}50 \times 10^3$ 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 disruption 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 precleared 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 GeneChip 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 *CS-A* (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).

Figure 3 Electrophoresis mobility shift patterns of the AF-1A and AF-1B regions. Nuclear extracts from placental choriocarcinoma JEG-3 cells were used to compare the EMSA patterns obtained with radiolabeled AF-1A and AF-1B regions, as indicated. EMSA experiments were carried out as described in the section Materials and methods, and the binding reactions resolved on a non-denaturing 4% acrylamide gel. Complexes of various mobilities observed with either or both oligonucleotide probes are indicated (C1–C6).

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 (Lytras *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 *CS-A* 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

Figure 4 Electrophoresis mobility shift patterns of the AF-1 regions combined with competition assays with AF-1A, AF-1B, and TEF oligonucleotides. Competition assays using JEG-3 nuclear extracts and increasing amounts of unlabeled competitor oligonucleotides. The identity of the radiolabeled probe is indicated underneath the figure; amount (ng) and the competitors used are shown at the top. Apart from the specific complexes, an apparently non-specific/low affinity complex is observed migrating slightly faster than C2 with both AF-1A and AF-1B probes. In general, minor differences such as observed in the binding patterns in Fig. 4 compared with Fig. 3 can be attributed to minor modifications of the experimental conditions, including use of different batches and amounts of nuclear extracts or incubation (room) temperature. Higher amounts of nuclear extracts tend to increase the relative intensity of lower mobility complexes (rather including more associated factors); in Fig. 3, the AF-1A probe seems to be associated with more pronounced lower mobility complexes (mainly C6), while in Fig. 4, the AF-1A probe seems to be associated with relatively higher intensity of C2 and C3/4.

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/EBP α 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/EBP α or C/EBP β (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/EBP α 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/EBP α might physically participate in the formation of the novel C5 complex on AF-1B. To directly

determine the presence of C/EBP α 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/EBP α 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

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.

(*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 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.

C/EBP α and C/EBP β 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 α or C/EBP β 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 α and C/EBP β resulted in increased *CSp.CAT.CS-Benh* construct activity (by 64.3 and 71.7%, respectively, $n=4$,

Figure 6 Effect of Elk-1 overexpression on DNA-protein interactions in the AF-1 regions. (A) Nuclear extracts (5 μ g) were prepared from JEG-3 cells transfected with expression vectors carrying the cDNAs for Ets family members (Ets-1, Ets-2, Elf-R, Elf-1, and Elk-1) and were examined in EMSAs using AF-1A and AF-1B radiolabeled oligonucleotides (indicated below the figure). A high mobility complex (HMC) and a lower mobility complex either identical to or 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. (B) Competition assays using radiolabeled AF-1A, and AF-1A or AF-1B competitor (ng) as indicated. Nuclear extracts were prepared from JEG-3 cells transfected with the Elk-1 expression vector (Elk-1) or with pcDNA (Control).

Figure 7 Electrophoresis mobility ‘supershift’ assays for the detection of Elk-1-related complexes with AF-1A. Nuclear extracts (6 µg) from JEG-3 cells transfected with the same Elk-1 expression vector used in Fig. 6 (Elk-1 +), or with pcDNA as a control (Elk-1 –), were preincubated with monoclonal antibodies (M2, Sigma) against the FLAG epitope (anti-FLAG) or against FGFR-1 (anti-FGFR, as a negative control). Following binding to radiolabeled AF-1A, samples were resolved on a non-denaturing 5% polyacrylamide gel. Complexes are labeled to correspond to previous figures.

$P<0.005$) in spite of a strong negative effect on independent CSp activity (approximately eightfold or $>87\%$ repression of control activity, $n=4$, $P<0.001$). This is reflected in the activity of the CS-B enhancer (expressed as the fold difference of the activity of the CSp.CAT.CS-Benh over the activity of the CSp.CAT) that increased from ~ 12 - to ~ 330 -fold. In contrast, the CS-A enhancer was unable to fully compensate for the repression of the CSp by either C/EBP α or C/EBP β . This resulted in a net $\sim 75\%$ reduction in the activity of CSp.CAT.CS-Aenh ($n=2$, $P<0.01$). It has to be noted, however, that the activity of the CS-A enhancer, (expressed as the fold difference of the activity of the CSp.CAT.CS-Aenh over the activity of the CSp.CAT) increased from ~ 1.5 -fold under control conditions to ~ 4.4 -fold in the presence of C/EBPs. Thus, C/EBP α and C/EBP β appeared to increase CS-B enhancer activity much more effectively than CS-A enhancer activity, increasing the fold-difference between the activities of these enhancer regions by over an order

of magnitude (from 7.9-fold under control conditions to 81.9- and 87-fold in the presence of C/EBP α or C/EBP β respectively).

C/EBP β 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/EBP β associates with CS-A and CS-B sequences in the context of human term placenta chromatin. C/EBP β was targeted for examination based on its high level of placental expression, particularly during late pregnancy (Bamberger *et al.* 2004). C/EBP β ‘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/EBP β ‘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/EBP β intervals over the enhancer regions were observed. In the case of the CS-A gene, the C/EBP β 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/EBP β interval spans from nts 59323740 to 59325870 (Table 1) extending upstream toward the CS-A gene-coding region. As the overlapping Elk-1/C/EBP site in the modulatory domain of the CS-A enhancer spans nts 59323721–59323739, the detected C/EBP 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/EBP β 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

Figure 8 Effects of C/EBP α and C/EBP β overexpression on the DNA-protein interactions on the AF-1 regions and electrophoresis mobility supershift assays for the detection of C/EBP α -related complexes on AF-1A. (A) Nuclear extracts (5 μ g) from JEG-3 cells transfected with control expression vector (RcCMV) or expression vectors for C/EBP α or C/EBP β (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/EBP α -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.

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 β 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 9 Competition assays for the novel C/EBP α -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/EBP α or C/EBP β (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.

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/EBP α complex (C5) was generated with C/EBP α 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 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/EBP α (α) or C/EBP β (β) co-transfection (5 μ g) resulted in a strong negative effect on independent *CS* promoter activity (approximately eightfold or 88.5 and 87.1% repression of control activity, by C/EBP α ($n=4$) and C/EBP β ($n=4$) 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/EBP α ($n=4$) and C/EBP β ($n=4$) respectively ($P<0.005$). In contrast, the *CS-A* enhancer was unable to compensate for the repression of the *CS* promoter by C/EBP α ($n=2$) and C/EBP β ($n=2$), resulting in a net ~75% reduction in *CSp.CAT.CS-Aenh* construct activity ($P<0.01$).

Figure 11 Association of C/EBP β with the *CS-A* and *CS-B* genes in human term placenta chromatin. Map of the peak intensities for C/EBP β binding across all five members of the human GH/CS gene family on chromosome 17 in human term placenta as detected by ChIP on chip analysis. The line running horizontally through each set of 'peaks' represents the threshold applied to peak intensities based on normalization against input DNA array data to obtain intervals for the C/EBP β ChIP DNA (Materials and methods). Thick horizontal lines indicate C/EBP β intervals. The positions and 5'–3' directions of the five GH/CS genes are indicated under the low resolution map of the GH/CS locus. Nucleotide numbering corresponds to human chromosome 17 sequences as included in the University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>). The *CS-A* and *CS-B* enhancer (enh) 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.

Regulation through interaction of Ets/Elk-1 and C/EBPs, and specifically with C/EBP α 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/EBP α 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 compensates only slightly with the net effect still being repression (Fig. 10B). It is possible that in the presence of the *CS-B* enhancer, the interaction of C/EBP proteins with the promoter is neutralized by factors other than C/EBPs; thus, a 'competition' occurs between enhancer-related complexes and C/EBP proteins. Alternatively, enhancer complexes that include C/EBP proteins interact with the C/EBP complexes in the promoter region. Certainly putative C/EBP elements can be detected by sequence analysis within the first 500 bp upstream of the *CS-A* and *CS-B* transcription initiation sites (not shown), and elements >1 kb upstream have been reported (Stephanou & Handwerger 1995). In either case, enhancer sequences

Table 1 CCAAT/enhancer-binding protein β (C/EBPβ) intervals on the GH/CS locus on chromosome 17 as detected by ChIP on chip tiling array assay. Nucleotide numbering corresponds to human chromosome 17 sequences as included in the University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>). Intervals on the CS-A (2130 nts) and CS-B (2477 nts) genes that include enhancer sequences are indicated (bold). The equivalent CS-L interval that differs in length (1045 nts) is also indicated

	End	Length	Probes	Peaks	Peak center	Gene list	Distance to start
Start							
59299261	59299929	668	14	3	59299309	CS-B	5512
59300535	59303012	2477	53	1	59301510	CS-B, GH-V	3311, 11445
59303824	59304164	340	10	1	59303944	CS-B, GH-V	877, 9011
59313656	59313933	277	8	4	59313795	CS-B, GH-V	(-)8974, (-)840
59317390	59317627	237	7	1	59317614	GH-V, CS-A	(-)4659, 10105
59322131	59322799	668	15	1	59322786	GH-V, CS-A	(-)9831, 4933
59323740	59325870	2130	49	1	59324407	CS-A	3312
59326747	59327098	351	10	1	59326903	CS-A	816
59332182	59332541	359	10	1	59332444	CS-A, CS-L	(-)4725, 9906
59337280	59337566	286	8	1	59337553	CS-A, CS-L	(-)9834, 4797
59338177	59339222	1045	18	2	59338818	CS-L, GH	3532, 11112
59339763	59340594	831	18	2	59340423	CS-L, GH	1927, 9507

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 co-operation 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 placental 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 placental 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.

We have now identified two candidate members of the Ets and the C/EBP families of transcription factors, Elk-1 and C/EBP α/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-1A but not 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/EBP β 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/EBP β '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/EBP β 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/EBP β 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/EBP β 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 & Xanthopoulos 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/EBP α versus C/EBP β , 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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