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Identification of trophoblast-specific elements in the *HLA-C* core promoter

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Abstract

There are several aspects of *HLA-C* gene expression that distinguish it from the *HLA-A* and *HLA-B* genes. First, *HLA-C* is expressed by extravillous trophoblasts, whereas *HLA-A* and *HLA-B* are not. Second, its cell-surface expression is much lower, which has been linked to changes in transcription and efficiency of peptide loading and export. Third, *HLA-C* possesses a NK cell-specific promoter and a complex alternative splicing system that regulates expression during NK cell development. In this study, we investigate the contribution of the *HLA-C* core promoter to trophoblast-specific expression. Analysis of transcription start sites revealed the presence of a trophoblast-associated start site and additional upstream TATA and CCAAT-box elements in the *HLA-C* promoter, suggesting the presence of an overlapping trophoblast-specific promoter. A comparison of *in vitro* promoter activity demonstrated that the *HLA-C* promoter was more active in trophoblast cell lines than either the *HLA-A* or *HLA-B* promoters. Enhanced trophoblast activity was mapped to the central enhanceosome region of the promoter, and mutational analysis identified changes in the RFX-binding region that generated a trophoblast-specific enhancer.

Keywords

major histocompatibility complex; MHC class I; human leukocyte antigen; *HLA-C*; extravillous trophoblasts; promoter; transcription factors

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1 INTRODUCTION

Human leukocyte antigens (HLA) expressed by fetal extravillous trophoblasts (EVT) play a vital role in regulating the immune response at the maternal-fetal interface.¹ During pregnancy, trophoblasts invade the decidua, and despite expressing paternally-derived HLA molecules, the fetus survives without rejection.² Unlike most tissues, which express HLA-A, HLA-B, HLA-C, and HLA-E, EVT are unique in that they lack expression of HLA-A and HLA-B, but express HLA-C together with the non-classical major histocompatibility complex (MHC) class I HLA-E and HLA-G molecules.^{3–5}

HLA-C is the most recently evolved HLA gene, and it is assumed to have developed primarily as a ligand for the KIR2D family of receptors expressed by NK cells, since its appearance in higher primates correlates with the expansion of the KIR2D receptor family.⁶ All of the *HLA-C* alleles produce proteins that are recognized by at least one KIR2D, whereas *HLA-A* and *HLA-B* only have a subset of alleles that can be recognized by KIR3D receptors.⁷ In addition, HLA-A and HLA-B cell surface expression levels are 13–18 times higher than HLA-C,⁸ suggesting that the principal function of HLA-C is the regulation of NK cell responsiveness rather than antigen presentation to T cells. The recent discovery of an NK-specific promoter and a complex alternative splicing mechanism controlling HLA-C expression in developing NK cells provides further evidence for the emergence of HLA-C as a specialized ligand for the KIR family of MHC class I receptors.⁹

Reproductive fitness may represent the major driving force for HLA-C evolution in humans and great apes, since KIR:HLA-C interactions have been shown to play a role in pregnancy outcomes.¹⁰ Uterine NK cells are the dominant lymphocyte present in the decidua, consistent with NK cell recognition of fetal HLA-C by KIR being a major factor in the regulation of fetal tolerance.¹¹ Distinct combinations of maternal *KIR* and fetal *HLA-C* alleles are associated with either preeclampsia and low birth weight, or obstructed labor,^{12–14} suggesting a strong influence of KIR:HLA-C recognition on successful reproduction.

With regard to the unique HLA expression profile observed on trophoblast cells, the mechanisms by which HLA-A and HLA-B are inhibited, and HLA-C is preferentially expressed, have not been elucidated. The *HLA-G* gene is uniquely expressed in the placenta, and a recent study has identified an enhancer 12 kb upstream of *HLA-G* that is required for HLA-G expression in trophoblasts.¹⁵ The binding of C/EBP β and GATA2/3 transcription factors (TF) is required for *HLA-G* enhancer function, and these TFs are highly expressed in human placenta. The mechanisms responsible for the specific expression of HLA-C in trophoblasts are likely to be distinct from HLA-G, since *HLA-B* and *HLA-C* are adjacent genes located more than 1 mb from the *HLA-G* gene. The close proximity of *HLA-B* and *HLA-C* suggests that there may be trophoblast-specific elements in *HLA-C*, even though the *HLA-B* and *HLA-C* promoter regions possess greater than 90% homology. NLRP2 has been shown to suppress HLA-C expression in trophoblasts, however there is no indication that this molecule is involved in tissue-specific expression.¹⁶

The detailed analysis of the *HLA-C* promoter conducted in the current study has identified a trophoblast-specific transcription start site (TSS) ~30 bp upstream of the TSS observed in

other cell types, indicating that trophoblast-specific elements are embedded in the core promoter region. Comparison of the *HLA-A*, *HLA-B*, and *HLA-C* core promoters revealed the presence of *HLA-C* specific polymorphisms that generated novel *HLA-C*-specific transcription factor binding sites. *In vitro* analysis of promoter activity identified *HLA-C*-specific TF-binding sites in the central enhancosome region that are required for trophoblast-specific transcriptional activity.

2 MATERIALS AND METHODS

2.1 Cell Lines

Human Embryonic Kidney (HEK) 293T cells were cultured in DMEM media (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (FBS), 100 U/ml each of penicillin and streptomycin, plus L-glutamine (PSG), and sodium pyruvate. The choriocarcinoma cell lines JAR and BeWo were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained according to the supplier's instructions. JAR cells were cultured in RPMI 1640 media with 10% FBS and PSG. BeWo cells were cultured in Ham's F-12K with L-glutamine media (ATCC) with 10% FBS and PSG. All cells were maintained at 37° C in a 5% CO₂ atmosphere.

2.2 5' Rapid Amplification of cDNA Ends (5' RACE) Analysis of HLA-C transcripts in trophoblast cell lines

5' RACE was performed on RNA isolated from 1×10⁷ BeWo cells using the RNeasy Plus Mini Kit with an additional on-column DNase I digest using an RNase-Free DNase Set (Qiagen, Valencia, CA, USA). Human placental RNA was obtained from Clontech (Mountain View, CA, USA). 10 ug of isolated RNA was used to perform 5' RACE using the FirstChoice RLM-RACE kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturers' directions. *HLA* gene-specific cDNA was made using an exon 2 antisense primer (5'-GAAATACCTCATGGAGTGGGAG; IDT, Newark, NJ, USA). The first round of nested PCR was performed using the 5' RACE outer adapter provided and a *HLA-C* exon 1 gene-specific outer primer (5'-CAGGTCTCGGTCAGGGCCAGG) at an annealing temperature of 62° C for 35 cycles using ZymoTaq PCR Premix (Zymo Research, Irvine, CA, USA). Excess primers from the first round of PCR reactions were removed with the Charge-Switch PCR Clean-up Kit (Invitrogen, Carlsbad, CA, USA). Nested PCR was subsequently performed with the 5' RACE inner primer provided and *HLA-C* exon 1 gene-specific inner primer (5'-CAGGGCTCCCGAGAGCAGCAGG) at an annealing temperature of 64° C for 35 cycles. PCR products were cloned into the Topo PCR2.1 vector (Invitrogen) and sequences were analyzed to map the 5' transcription start sites.

2.3 Generation of promoter constructs

HLA core promoter fragments were generated by PCR using the following primers: HLAA-Fwd (5'-GAAGGCGGTGTATGGATTGG); HLAA-Rev (5'-GTCTGGGGAGAATCTGAGTCC); HLAB-Fwd (5'-GGCTCCGAGGGCCGCTCTGCAATG); HLAB-Rev (5'-GTCTGAGGAGACTCTGAGTCC); HLAC-Fwd (5'-GGCTCCAAGGGCCGCTGTCTGCAC); HLAC-Rev (5'-

GTCTGGGGAGAATCTGAGTCC). The vector targeting sequence (5'-CTATCGATAGGTACCGAGCTC) was added to the 5' end of all forward primers, and (5'-CAGTACCGGAATGCCAAGCTT) was added to the 5' end of all reverse primers, to insert the PCR fragments into the *SacI* and *HindIII* sites of the pGL3-Basic (Promega, Madison, WI, USA) luciferase reporter vector using the CloneEZ® PCR Cloning Kit (GeneScript, Piscataway, NJ, USA). Chimeric *HLA-A/HLA-C* constructs and site-directed mutations were generated using a pair of Ultramer (IDT) oligonucleotides of up to 150 bp in length to synthesize a complete 220 bp core promoter region containing specific sequence elements of either *HLA-A* or *HLA-C*. The fidelity of all constructs was verified through sequencing.

2.4 Cell transfection and luciferase assays

The JAR, BeWo, and HEK293T cells were used due to their ability to be efficiently transfected using lipid-based transfection, thereby producing high levels of luciferase activity. JAR and BeWo cells were plated at 5×10^4 cells per well in a 24-well plate. In order to compensate for their more rapid growth, HEK293T cells were plated at 2.5×10^4 cells per well in a 24 well plate. Cells were incubated for 24 hours at 37°C in 5% CO₂ prior to transfection. All cells were co-transfected with control pRL-SV40 *Renilla* Reporter Vector (Promega) to normalize luciferase readings. JAR, BeWo, and HEK293T cells were transfected using the HilyMax transfection reagent (Dojindo, Rockville, MD, USA) with DNA and HilyMax concentrations that produced optimal transfection of each cell type. For each well, 3–10 µL of HilyMax (10 µL for BeWo cells, 3 µL for JAR and HEK293T cells) was combined with 30 µL of OptiMEM and incubated at room temperature for 5 minutes. A DNA mixture containing 500 ng–2.5 µg of the HLA promoter construct (500 ng for HEK293T, 1 µg for JAR, and 2.5 µg for BeWo cells) and 20–500 ng of pRL-SV40 *Renilla* luciferase control DNA was then added to each and incubated at room temperature for 30 minutes. After 48 hours of incubation, cells were lysed and analyzed for promoter activity using the Dual-Luciferase Reporter Assay System (Promega) according to the supplier's instructions. Measurement of the firefly luciferase activity of the HLA promoter constructs was normalized relative to the *Renilla* luciferase activity produced by the pRL-SV40 control vector. Each construct was tested in triplicate in at least four independent experiments.

2.5 Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from cell lines using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich). Protein concentration was measured with a Bio-Rad protein assay, and samples were stored at –70°C until use. Double-stranded DNA oligonucleotide probes were synthesized (IDT) containing either the upstream CREB element (UP-CREB: 5'-GCAACCTACGTAGGGTCC-*HLA-A**01; 5'-GCAACCTATGTAGGGTCC-*HLA-A**02; GCAACTTGTGTCGGGTCC-*HLA-B*; GCAACCTGCGTCGGGTCC-*HLA-C*), the enhancer site (ETS/RFX: 5'-GGTCCTTCATCCTGGATACTCAC-*HLA-A*; 5'-GGTCCTTCTTCCAGGATACTCAC-*HLA-B*; 5'-GGTCCTTCTCCTGAATACTCA-*HLA-C*), or the previously characterized CREB site^{17–19} (CREB: 5'-GGACTCACGACGCGGA-*HLA-A*; 5'-GGACTCATGACGCGTC-*HLA-B/C*). Probes were labeled with α-[32P]deoxycytidine triphosphate (3000 Ci/mmol; PerkinElmer, Waltham, MA, USA) by fill-in using the Klenow fragment of DNA polymerase I (Invitrogen). [32P]-labeled double-stranded oligonucleotides were purified using mini Quick Spin Oligo

Columns (Roche Diagnostics, Indianapolis, IN, USA). DNA–protein binding reactions were performed in a 10- μ l mixture containing 5 μ g nuclear protein and 1 μ g poly[dI-dC] (Sigma-Aldrich) in 4% glycerol, 1 mM MgCl₂, 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5). Nuclear extracts were incubated with 1 μ l 32P-labeled oligonucleotide probe (10,000 cpm) either alone, in the presence of specific antibodies (Santa Cruz Biotechnology, Dallas, TX, USA), or with unlabeled consensus TF-binding oligos (Santa Cruz), incubated at room temperature for 20 min, and then loaded on a 5% polyacrylamide gel (37:5:1). Electrophoresis was performed in 0.5 $\times$ TBE for 2 h at 130 V, and the gel was visualized by autoradiography.

2.6 Statistical Analysis

One-way ANOVA, the Kruskal-Wallis test, was performed using GraphPad Prism version 7 for Mac OSX, GraphPad Software (La Jolla, CA, USA), and a p value less than 0.05 was considered significant.

3 RESULTS

3.1 Identification of a trophoblast-specific transcription start site in the *HLA-C* core promoter

In order to gain insight into the mechanisms responsible for the selective expression of *HLA-C* by trophoblast cells, we analyzed *HLA-C* transcription start sites (TSS) in trophoblasts to determine if a separate trophoblast-specific promoter was present. 5' RACE (Rapid Amplification of cDNA Ends) was performed with primers located in exon 1 of the *HLA-C* gene. Two novel TSS in the *HLA-C* core promoter were identified using RNA from BeWo cells and placental RNA. (Figure 1A). These start sites are 32 and 34 nucleotides upstream from the principal *HLA-C* proximal promoter TSS used in most tissues that have been studied. The presence of trophoblast-specific TSS in this region was supported by a search of the GenBank EST database which identified two clones from a placenta cDNA library that had TSS within 7 nucleotides of the trophoblast TSS identified by 5' RACE (GenBank #s DA847926 and DA831787). In light of our recent discovery of an NK-specific promoter 1.3 kb upstream of the core *HLA-C* promoter,⁹ we used a series of primers upstream of all known TSS to determine if an additional upstream promoter was active in trophoblasts. No TSS upstream of the core promoter region were detected in any of the trophoblast cell line or placental RNA samples tested. Therefore, the trophoblast-specific TSS that we have observed indicate that there is a trophoblast-specific promoter overlapping the proximal core promoter region of the *HLA-C* gene.

3.2 Comparison of the *HLA-A*, *HLA-B*, and *HLA-C* promoters reveals a duplication of core elements in the *HLA-C* promoter

A close examination of the promoter region of the *HLA-A*, *HLA-B*, and *HLA-C* genes reveals a remarkable duplication of the CREB, NF-Y (CCAAT box), and TBP (TATA box) binding sites that is specific to the *HLA-C* promoter, with nearly identical spacing and position relative to the TSS of the core promoter versus the trophoblast TSS (Figure 1B). The additional predicted CREB-binding site present in the *HLA-C* gene has a single non-consensus nucleotide, however it is identical to the CREB site of the *HLA-A* gene, and this

variation did not affect the CIITA-mediated transactivation of the *HLA-A* promoter.¹⁷ Other than the single nucleotide difference in the *HLA-A* CREB site, the CREB, NF-Y, and TBP - binding sites associated with core promoter activity are conserved among all three genes. However, the additional CREB and NF-Y sites present in the *HLA-C* gene are disrupted by non-consensus SNPs in the *HLA-A* and *HLA-B* genes (Figure 1A).

3.3 Increased *HLA-C* promoter activity compared to *HLA-A* or *HLA-B* in trophoblast cells

Luciferase reporter assays were performed to assess differences in core promoter activity between the *HLA-C*, *HLA-A*, or *HLA-B* genes in trophoblast cell lines as compared to HEK293T. A 220 bp core promoter fragment¹⁸ containing the region immediately upstream of the ATG codon of each gene (Figure 1A) was cloned into the pGL3 reporter vector. The promoter activity of the construct was assessed in the HEK293T, and compared with the activity observed in BeWo and JAR trophoblast cell lines (Figure 2). In HEK293T cells, *HLA-A* and *HLA-B* promoter activity was similar to *HLA-C* promoter activity. However, in the two trophoblast cell lines, *HLA-C* promoter activity was approximately 2 to 3-fold higher than *HLA-A* and *HLA-B* promoter activity (Figure 2). This indicates the presence of trophoblast-specific elements in the *HLA-C* core promoter region.

3.4 The central region of the *HLA-C* promoter is associated with trophoblast-specific expression

The core promoter of the *HLA-C* gene can be divided into three functional regions, all of which contain potentially disruptive polymorphisms in key transcription factor binding sites relative to the *HLA-A* and *HLA-B* promoters (Figure 1A): the 5' region contains NF- κ B and IRF transcription factor binding sites; the central region corresponds to the well-characterized enhanceosome¹⁹ which contains RFX-binding, CREB-binding, and NF-Y binding sites, as well as the additional *HLA-C*-specific CREB, and NF-Y transcription factor binding sites (Figure 1A); the 3' region contains the NF-Y and TBP-binding sites associated with the core promoter, and both TSS regions. In order to determine which region(s) of the core promoter region are important for trophoblast-specific transcription of *HLA-C*, chimeric promoter constructs were generated. Long PCR primers (100–150 bp) were used to generate all possible combinations of *HLA-A* and *HLA-C* sequences within each of these three areas in the core promoter. The 5' domain includes sequence up to and including the IRF site. The central region contains both CREB sites, the additional *HLA-C*-specific NF-Y site, ETS/RFX sites, and ends at the STAT6/TBP site. The 3' region contains the conserved NF-Y, TBP, and TSS sites associated with the principal TSS site used by *HLA-A*, *-B* and *-C* (Figure 1A). As shown in Figure 3, six different chimeras of the *HLA-A* and *HLA-C* promoters were generated (A/A/C, A/C/C, A/C/A, C/C/A, C/A/A, and C/A/C). In BeWo and JAR cells, constructs that contained the *HLA-A* sequence from the central region had significantly lower promoter activity as compared to constructs with *HLA-C* sequence in the central region (Figure 3). In contrast, the 5' and 3' regions of the core promoter had little impact on promoter activity, with the exception of the A/C/A construct that had 50% greater activity than the native *HLA-C* promoter in BeWo cells. This may be due to the presence of a functional NF- κ B site in the 5' region of the *HLA-A* promoter. There was no significant difference in promoter activity between any of the constructs in HEK293T cells, indicating

that variation within the central promoter region is important for the preferential expression of HLA-C by trophoblast cells.

3.5 Identification of the ETS/RFX region of the enhanceosome as the primary trophoblast-specific element

In order to identify the contribution of each *HLA-C*-specific promoter element in the central region to trophoblast-specific transcriptional activity, site-directed mutation of the upstream CREB, NF-Y, and putative ETS/RFX binding sites was performed. The insertion of the *HLA-C* CREB and NF-Y binding sites into the *HLA-A* promoter either alone or in combination, did not have a significant effect on promoter activity in either the BeWo or JAR trophoblast lines (Figure 4), however these changes did result in decreased activity in the HEK293T cell line. Conversely, mutation of these sites in the *HLA-C* promoter to the *HLA-A* sequence did not decrease promoter activity in any of the cell lines tested. However, insertion of the ETS/RFX element of *HLA-C* into the *HLA-A* promoter resulted in promoter activity in trophoblast cell lines that was comparable to that of *HLA-C* and produced a two-fold increase in activity in 293T cells. Conversely, mutation of the *HLA-C* ETS/RFX element to the *HLA-A* sequence, decreased promoter activity to the level observed with the wild-type *HLA-A* promoter in the trophoblast lines and resulted in higher activity in HEK293T cells. These results implicate the ETS/RFX site of the *HLA-C* promoter in trophoblast-specific activity. This element overlaps with the RFX-binding site characterized in the *HLA-B* gene,¹⁹ and is also referred to as the X1 site.¹⁷ The RFX binding consensus contains a central G nucleotide that has been replaced by an A residue in *HLA-C* (Figure 1A). RFX cooperates with CREB and NF-Y to recruit CIITA, as shown for the class II genes.¹⁹ Since CIITA is not expressed in trophoblasts, and it cannot be induced by IFN- γ ,²⁰ the altered RFX site of the *HLA-C* gene may recruit the CBP/p300 coactivators via ETS molecules in trophoblasts.^{21–22}

3.6 EMSA analysis of the enhanceosome region demonstrates the presence of distinct complexes in the *HLA-A*, *HLA-B*, and *HLA-C* genes

An electromobility shift assay (EMSA) was performed using oligonucleotide probes containing the upstream CREB element (UP-CREB), ETS/RFX element, and previously characterized CREB-binding site of the *HLA-A*, *HLA-B*, and *HLA-C* genes (Figure 5A). Since the *HLA-A* gene possesses a predicted CREB-binding site that differs from the consensus by a single nucleotide, and this sequence is duplicated upstream in the *HLA-C* gene, we tested the transcription factor binding properties of these sites. Surprisingly, the *HLA-A* CREB site (Figure 1A) and the *HLA-C* upstream CREB site only showed very weak CREB protein complexes, indicating that the single 3' nucleotide change had a significant effect. However, the upstream *HLA-A**01 CREB site bound to CREB strongly, even though it differed from the CREB-binding consensus at two 5' nucleotides. Supershift analysis confirmed that the observed complexes contained CREB (data not shown). Surprisingly, the upstream *HLA-A**02 CREB site did not show any CREB binding. The upstream *HLA-B* CREB site did not produce any complexes, indicating that only the *HLA-A**01 upstream CREB site is functional. Therefore, each gene has a single strong CREB-binding site, the upstream site in some *HLA-A* alleles and the downstream CREB site in the *HLA-B* and *HLA-C* genes. The distinct CREB-binding properties of *HLA-A* alleles suggests that there

may be differences in the transactivation of alleles that differ in the CREB-binding ability of the core promoter enhanceosome.

3.7 *HLA-C*-specific ETS/RFX-region complexes contain ETS family members

The core GGAA sequence of the predicted ETS-binding element that overlaps with the RFX-binding element is conserved in all three genes (Figure 1A). A shared complex observed with all probes and in all cell lines tested, was found to contain the ETS family member ELF1 by supershift analysis (Figure 5A-C). However, distinct protein complexes were observed with each of the ETS/RFX probes. The *HLA-C* specific complex observed was competed with a consensus ETS-binding site (Figure 5B), indicating that an ETS family member was binding to the trophoblast-specific *HLA-C* element. The weak inhibition of the ELF1 complex observed with the STAT consensus oligonucleotides is likely due to the presence of the core GGAA sequence in all of the STAT consensus sequences. A recent study of enhancers activated during trophoblast invasion of the placenta implicated AP1, ETS, and Tcfap2-binding sites with invasion,²³ and RNA-seq demonstrated that ETS2 and ELF3 expression is highly induced during invasion. Therefore, we tested the effect of ETS2 and ELF3 antibodies on the *HLA-C* complexes. ELF3 antibody produced a supershifted complex, implicating this transcription factor in trophoblast-specific enhancer activity. The principal difference between the *HLA-C* and *HLA-A/HLA-B* enhanceosome region is the loss of the RFX-binding site in the *HLA-C* gene, suggesting that RFX binding prevents binding of ELF3 to this region. However, the G to A change that disrupts the RFX site also generates a potential C/EBP-binding site in *HLA-C* (TTCCTGAA; Figure 1A) that differs from a consensus C/EBP site (TTGCGCAA) in the central region. In light of the identification of C/EBP β as a key factor in the *HLA-G* enhancer,¹⁵ we tested the ability of the *HLA-C* region to bind C/EBP. A P32-labeled consensus C/EBP-binding oligonucleotide produced strong complexes in the JAR cell line (data not shown), however, the unlabeled C/EBP consensus did not compete with any of the *HLA-C* complexes observed, excluding C/EBP as a component of the *HLA-C* core enhancer-binding complex (Figure 5B).

4 DISCUSSION

There has been considerable interest in the mechanisms responsible for the unique expression pattern of class I MHC proteins observed in extravillous trophoblasts. *HLA-G* expression is confined to trophoblasts, and it lacks the elements required for NF- κ B, IRF, and CIITA-mediated induction found in other MHC class I and class II genes.²⁴ The lack of CIITA-responsiveness of the *HLA-G* gene is consistent with the absence of CIITA expression in trophoblasts²⁰, and it has recently been reported that *HLA-G* expression is controlled by a distant trophoblast-specific enhancer¹⁵. In contrast, *HLA-C* is widely expressed, but is distinct from *HLA-A* and *HLA-B* in its ability to be expressed in trophoblasts³⁻⁵. In order to be expressed in trophoblasts, the *HLA-C* gene would require a CIITA-independent mechanism to recruit the CBP/p300 transcriptional coactivators required for chromatin remodeling at active promoters. Numerous transcription factors are able to directly recruit CBP/p300,^{21,22} including C/EBP β ,²⁵ which was found to bind to the *HLA-G* distal enhancer element.¹⁵ The core promoter region of the *HLA-B* gene alone is sufficient to mediate tissue-specific expression in transgenic mice,¹⁸ suggesting that the tissue-

specificity of *HLA-C* could also be controlled by this region. In the current study, we report the discovery of trophoblast-specific elements within the *HLA-C* core promoter region. The enhanced transcriptional activity of *HLA-C* promoter constructs relative to *HLA-A* and *HLA-B* promoters in trophoblast cell lines was mapped to the central enhanceosome region of the core promoter. Although an initial comparison of this region suggested that an additional CREB-binding site was present in the *HLA-C* gene (Figure 1B), EMSA analysis revealed that neither this site or the identical downstream CREB element in the *HLA-A* gene were able to bind CREB. This result is consistent with site-specific mutation studies indicating that the upstream CREB site is not associated with trophoblast-specific promoter activity (Figure 4). However, mutation of the RFX-binding element was sufficient to switch the trophoblast specificity of *HLA-A* and *HLA-C*. Oligonucleotides containing the ETS/RFX region of the enhancer produced unique protein complexes for each of the *HLA* genes when tested using trophoblast nuclear extracts, consistent with this region playing a role in the distinct tissue specificities observed. The predicted RFX-binding site found in this region of the *HLA-A* and *HLA-B* genes contains a non-consensus nucleotide in the *HLA-C* gene. The core ETS-binding element is conserved in all three genes; however, the 3'-flanking nucleotides are distinct. ELF-1 was found to bind to all three genes, however ELF3 was found to bind to the *HLA-C* element, suggesting that it may be a component of a trophoblast-specific enhancer complex. This is consistent with the recent observation of ELF3 upregulation during trophoblast invasion. Furthermore, ELF3 has been shown to interact with p300 and CBP,²⁶ thus providing a CIITA-independent mechanism to recruit the CBP/p300 transcriptional coactivators required for chromatin remodeling. Although C/EBP β is highly expressed in placenta and the trophoblast cell lines used in this study, we did not detect any binding of this factor to the *HLA-C* enhanceosome, distinguishing this trophoblast-specific enhancer from the distal *HLA-G* enhancer.¹⁵ The observed upregulation of Fos and Jun in invading trophoblasts²³ may also enhance expression of *HLA-C* in trophoblasts, since a consensus AP1 element is present in the upstream NK-specific promoter,⁹ and it may also function as enhancer element in trophoblasts. In conclusion, we have demonstrated that the *HLA-C* promoter possesses trophoblast-specific activity that is associated with distinct TF-binding properties of the enhanceosome region of the core promoter as compared to *HLA-A* and *HLA-B*.

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(A)

(B)

Core promoter elements: TGACGC — 43bp — CCAAT — 20bp — TTCTAAA — 25bp — CTCAGATTCT
HLA-C upstream elements: CGACGC — 32bp — CCAAT — 23bp — TTCTAGA — 26bp — TCCCGTTCT

Figure 1.

Trophoblast cells have a unique transcription start site associated with an additional array of *HLA-C*-specific TF-binding sites. A. The sequence of the core promoter region of *HLA-C*01* is shown, and nucleotides that are polymorphic in other *HLA-C* alleles are shown in orange type. Only nucleotide differences that are present in all alleles are shown for the *HLA-B* and *HLA-A* genes. Predicted TF-binding sites and their consensus sequences are shown in bold above the sequence, and nucleotides that match the consensus are underlined in the *HLA-C* sequence. The conserved TBP and NF-Y binding sites are indicated by black boxes, whereas sites that vary between genes are shown in blue boxes. Nucleotide differences found in all *HLA-A* and *HLA-B* alleles are shown in green type, as is the RFX consensus binding sequence. *HLA-C* transcription start sites (TSS) mapped by 5' RACE in BeWo cells are marked by black asterisks. The core TSS found in most tissues is marked by a black asterisk. Previously defined functional elements, Enhancer A, ISRE, X1, X2 and Y sites are labeled. The vertical red lines indicate junctions between the 5', central, and 3' regions. B. The sequence of promoter elements and their relative positions is shown for the core and trophoblast-associated TSS. The relative position of the overlapping TF-binding arrays is shown below, with putative trophoblast elements indicated by blue boxes, and core elements shown as green boxes.

** $p < 0.01$, ANOVA (Kruskal-Wallis test)

Figure 2.

Comparison of *HLA-A*, *HLA-B*, and *HLA-C* promoter activities in HEK293T cells with trophoblast cell lines. Luciferase activity *HLA-A*, *HLA-B*, and *HLA-C* 220 bp core promoter constructs. The average activity of each construct shown is relative to the activity of the empty pGL3 vector in at least 4 independent experiments. Error bars represent $\pm$ SEM.

Figure 4.

Site-directed mutation of CREB, NF-Y, and ETS/RFX sites. Sequence elements were exchanged between the *HLA-A* and *HLA-C* genes. The CREB and NF-Y sites of *HLA-C* were inserted into the *HLA-A* gene either alone (A+CREB; A+NF-Y) or in combination (A+CREB/NF-Y). The RFX-binding site was mutated to the *HLA-C* sequence (A-RFX). Conversely, *HLA-A* sequence was used to disrupt the CREB (C-CREB) or NF-Y (C-NF-Y) sites, add the RFX (C+RFX) site, or add the RFX site and mutate the ETS site (C+RFX-ETS) of the *HLA-C* core promoter. Constructs were transfected into HEK293T (A), BeWo (B), or JAR (C) and promoter activity was normalized to *HLA-C* core promoter activity. Results are the average of four independent experiments. Error bars represent $\pm$ SEM.

Figure 5.

TF binding to the HLA enhanceosome. A. EMSA of HLA probes from the upstream CREB site (UP-CREB), ETS/RFX site, and CREB site. Nuclear lysate from JAR, BeWo, or HEK293 cells was used, as indicated below each panel. Probe regions are indicated at the top of each panel, with brackets encompassing the individual *HLA* gene probes used. Arrows indicate CREB-binding protein complex (CREB), ELF1 complex (ELF1), HLA-C-specific (C-sp), and HLA-A-specific (A-sp) complexes. B. Competition of *HLA-C* ETS/RFX complexes formed in JAR cell nuclear lysate with unlabeled TF consensus oligonucleotides. The left panel shows results with decreasing amount of a consensus ETS oligonucleotide from 50-fold excess (50x) to equimolar (1x). Arrows indicate inhibited complexes. The right panel shows competition with a 50-fold excess of C/EBP, ETS, and STAT consensus oligonucleotides. C. Supershift analysis with anti-ETS family antibodies. JAR nuclear lysate was pre-incubated with the indicated antibodies prior to adding the labeled *HLA-C* ETS/RFX probe. The positions of the ELF1 complex and the supershifted antibody bands are indicated by arrows.