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GCMa Regulates the Syncytin-mediated Trophoblastic Fusion*

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The human placental trophoblast cell can be classified as either a cytotrophoblast or a syncytiotrophoblast. Cytotrophoblasts can function as stem cells for the development of the syncytiotrophoblast layer via cell fusion. An *envelope* gene of the human endogenous retrovirus family W (HERV-W) called *syncytin* is specifically expressed in the syncytiotrophoblast layer. Syncytin is a fusogenic membrane protein; therefore, it can mediate the fusion of cytotrophoblasts into the syncytiotrophoblast layer, which is essential for pregnancy maintenance. GCMa is a placenta-specific transcription factor and is required for placental development. To study the placenta-specific fusion mediated by syncytin, we tested whether GCMa is involved in this process by regulating *syncytin* gene expression. In this report, we demonstrate that GCMa was able to regulate *syncytin* gene expression via two GCMa-binding sites upstream of the 5′-long terminal repeat of the *syncytin*-harboring HERV-W family member in BeWo and JEG3 cells but not in HeLa cells. Furthermore, adenovirus-directed expression of GCMa enhanced *syncytin* gene expression and syncytin-mediated cell fusion in BeWo and JEG3 cells but not in HeLa cells. Therefore, the integration site of the *syncytin*-harboring HERV-W family member in the human genome is close to the functional GCMa-binding sites by which GCMa can specifically transactivate *syncytin* gene expression in trophoblast cells. Our results may help to explain the mechanism underlying the cell fusion event specific for syncytiotrophoblast formation.

The human placenta contains a specialized cell type called a trophoblast, which is the first lineage to differentiate in embryo development and plays key roles during implantation and placentation. The human trophoblast cell can be further classified as cytotrophoblasts and syncytiotrophoblasts. In the early gestation stage, cytotrophoblast stem cells facing the maternal decidua proliferate and fuse to form a syncytium, *i.e.* the syncytiotrophoblast. Later on, vascular spaces called trophoblastic lacunae appear in the syncytium around day 8–9. The cytotrophoblast layer under the syncytium can rapidly proliferate into these spaces, which results in the formation of the primary chorionic villi. Subsequently, proliferation of the cytotrophoblasts, growth of chorionic mesoderm (under the cytotrophoblast layer), and blood vessel development transform the primary villi into secondary and tertiary villi, which are composed of a core of mesenchyme cells surrounded by an inner layer of cytotrophoblasts and an outer layer of multinucleate syncytiotrophoblasts (1, 2). The syncytiotrophoblast layer (syncytium) transports nutrients and gases and produces hormones such as placental lactogen and chorionic gonadotrophin, which are indispensable for the further progression of pregnancy (1).

Recently, a membrane protein termed syncytin has been demonstrated to mediate cell fusion of the human BeWo trophoblastic cell line (3). Syncytin is an envelope protein of the newly identified human endogenous retrovirus family W (HERV-W)[1] and is a polypeptide of 538 amino acids (3, 4). After synthesis, a post-translational cleavage is predicted to separate the syncytin polypeptide into surface protein and transmembrane protein. The latter contains the membrane-spanning segment and a hydrophobic fusion domain. *In situ* hybridization has demonstrated that *syncytin* is specifically expressed in the syncytiotrophoblast layer (3–5). These studies suggest that syncytin can mediate fusion of cytotrophoblasts into the syncytiotrophoblast layer, and the expression of *syncytin* is tightly regulated in a temporal and spatial manner to maintain an integral and functional syncytiotrophoblast layer.

GCM (glial cell missing) was originally isolated from a *Drosophila melanogaster* mutant line that produces additional neurons at the expense of glial cells (6, 7). Currently, two *GCM*-like genes (*GCMa* and *GCMb*) have been reported in mouse, rat, and human (8, 9). Altogether these GCM homologues form a novel family of transcription factors, which all share sequence homology in the amino-terminal region that constitutes the DNA-binding domain called the GCM motif. Although sequence homology is less preserved outside the GCM motif, a transactivation domain (TAD) has been identified in the extreme carboxyl terminus of GCM proteins (10, 11). The optimal recognition sequence for the GCM motif is 5′-(A/G)CCC(T/G)CAT-3′ or its 5′-ATG(A/C)GGG(T/C)-3′ complement (10, 12). *Drosophila* GCM mRNA is transiently detected in glial precursors and immature glial cells, except for mesectodermal midline glia during a short period of gliogenesis within the central nervous system (6, 7). In contrast, mouse GCMa mRNA is highly expressed in the labyrinthine trophoblast cells (13). GCMa is required for placental development because genetic ablation of mouse *GCMa* leads to a failure of labyrinth layer formation and the fusion of trophoblasts to syncytiotrophoblasts (14, 15).

To study the placenta-specific fusion mediated by syncytin, we tested whether GCMa can activate the promoter activity of

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[1] The abbreviations used are: HERV-W, human endogenous retrovirus family W, LTR, long terminal repeat; GBS, GCMa-binding site; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; nt, nucleotide(s); EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; Ad, adenovirus.

the long terminal repeats (LTRs) of the *syncytin*-harboring HERV-W family member to specifically drive *syncytin* expression in trophoblast cells. In this study, we demonstrated that GCMa recognizes two GCMa-binding sites (GBSs) in the upstream region of the 5′-LTR of the *syncytin*-harboring HERV-W family member, activating *syncytin* gene expression and consequently enhancing the syncytin-mediated cell fusion. Our data help to explain the regulatory mechanism underlying the placenta-specific trophoblastic fusion mediated by syncytin.

EXPERIMENTAL PROCEDURES

Library Screening and Plasmid Constructs—The human syncytin and GCMa cDNAs were cloned by PCR using a human placental cDNA library as template. The syncytin cDNA fragment was radiolabeled and used to screen a λDASH II human genomic library (Stratagene, La Jolla, CA). A genomic clone, L13, covering the entire proviral genome of the *syncytin*-harboring HERV-W family member was isolated and used to build promoter constructs (Fig. 1*A*). The human BAC clone, 083M05 (GenBank™ accession no. AC000064), was used to isolate more distal genomic regions upstream and downstream of the proviral genome of the *syncytin*-harboring HERV-W family member (Fig. 1*A*).

A GCMa cDNA fragment containing an amino-terminal HA epitope sequence was subcloned into the pRcCMV plasmid (Invitrogen) to generate pCMVHAGCMa. Genomic fragments were subcloned into the pE1bCAT reporter plasmid, which is derived from pCAT3-Basic (Promega, Madison, WI) by insertion of the adenovirus *E1B* TATA box in front of the bacterial *CAT* (chloramphenicol acetyltransferase) gene. For simplicity, the range of genomic fragments used for these constructs was based on the numbering of nucleotide (nt) residues in the 083M05 BAC clone. Genomic fragments of nt 25468–30953 with deletions of GBS-(25538–25545), GBS-(28026–28033), or both were subcloned into pCAT3-Basic to generate deletion constructs pCATΔd-(25468–30953), pCATΔp-(25468–30953), or pCATΔdp-(25468–30953), respectively.

Cell Culture, Transfection, and Reporter Gene Assays—The mammalian cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA). BeWo cells were grown at 37 °C in F-12K, 15% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. JEG3 and HeLa cells were grown at 37 °C in minimum Eagle's medium, 10% fetal bovine serum, and the same antibiotics as mentioned above. BeWo or HeLa cells were transfected by using the Geneporter system (GTS, San Diego, CA). CAT assays were performed as described (16). The Student's *t* test was performed to determine statistical significance for differences between means of relative CAT activities. A *p* value of less than 0.05 was considered statistically significant. HAGCMa proteins in transfected cells were subject to immunoblotting with an horseradish peroxidase-conjugated rat monoclonal anti-HA antibody (Roche Molecular Biochemicals). The membranes were stripped in 62.5 mM Tris-HCl (pH6.7), 100 mM 2-mercaptoethaniol, and 2% SDS at 50 °C for 30 min and reprobed with a rabbit polyclonal anti-actin antibody (Sigma).

Preparation of Recombinant GCMa Protein—Sf9 cells (Invitrogen) were maintained as suspension cultures at 28 °C in Sf-900II SFM (Invitrogen), 0.125 μg/ml amphotericin B, 50 μg/ml streptomycin, and 50 units/ml penicillin. A GCMa cDNA fragment with a carboxyl-terminal FLAG epitope sequence was subcloned into the pVL1392 transfer plasmid (BD Biosciences). The resultant construct was cotransfected with *Bsu*36I-digested baculoviral genomic DNA (Novagen, Madison, WI) into Sf-9 cells to generate recombinant GCMa-FLAG baculoviruses, which were used to express GCMa-FLAG proteins. GCMa-FLAG proteins were purified by the FLAG M2 monoclonal antibody affinity column (Sigma).

Electrophoretic Mobility Shift Assay and DNase I Footprinting Analysis—The electrophoretic mobility shift assay (EMSA) was performed as described (16) with minor modifications. End-labeled DNA fragments or oligonucleotide probes were incubated with 20 ng of GCMa-FLAG proteins in a binding reaction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 4 mM spermidine, 0.05% Nonidet P-40, 5 mM dithiothreitol, 10% glycerol, 0.25 μg poly(dI-dC) and 7.5 μg of bovine serum albumin. After incubation, the reaction mixtures were analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels in running buffer (25 mM Tris-HCl, pH 8.5, 190 mM glycine, 1 mM EDTA) at 4 °C. Two oligonucleotides, dGCMa (5′-ACTTCTGTCCCTCATGGCCAGT-3′) and pGCMa (5′-TTCTGGGATGAGGGCAAAACG-3′), were synthesized. A mutant pGCMa oligonucleotide, Mut (5′-TTCTGGGATGATAGCAAAACG-3′), was also synthesized as a negative control. Antiserum against human GCMa was induced in guinea pigs using a His-tagged GCMa recombinant protein expressed in BL21(DE3). 1 μl of antiserum or normal serum was used for supershift experiments. DNase I footprinting analysis was performed essentially as described by Chen *et al.* (17).

Chromatin Immunoprecipitation (ChIP) Assay—Approximately 3×10^7 BeWo cells transfected with 20 μg of pCMVHAGCMa were subject to a ChIP assay as described by Boyd and Farnham (18). HAGCMa-DNA complexes were immunoprecipitated by a rat monoclonal anti-HA antibody (Roche Molecular Biochemicals) and Protein A-agarose beads (Oncogene, Boston, MA). Specific sequences of regions upstream of the 5′-LTR of HERV-W in the immunoprecipitates were detected by PCR with specific primers. PCR products were analyzed on 2% MetaPhor agarose gels (FMC, Rockland, ME). Sequences of primers are 5′-CTCAGTCCGGCTTACAGTTTCGTTC-3′ and 5′-GAATAAGACGGCCTTCTGACCCTTC-3′ for region 22473–22371; 5′-GGCGTCAGATCCCATTACTCTAGG-3′ and 5′-AATAGAATGGGCCTGTGAGGCTGG-3′ for region 25461–25686; 5′-GCCCATTTCGATTGTAACATCTGCCAC-3′ and 5′-GCAAGATAATTGCTGTATCTCCAGGC-3′ for region 27800–28064.

Construction of Ad-HAGCMa—Recombinant HAGCMa adenoviruses (Ad-HAGCMa) were generated in CRE8 cells by cotransfection of the linearized transfer vector (pAdlox-HAGCMa) and the ψ5 genomic DNA (19). For a control, an empty recombinant adenovirus (Adlox) was generated using a linearized pAdlox and ψ5 genomic DNA. Ad-HAGCMa and Adlox were grown and amplified in CRE8 cells for two consecutive cycles. Cells in culture plates were transduced with Ad-HAGCMa or Adlox at an multiplicity of infection of 100 or 200 at 37 °C for 90 min. After that, the virus was removed, and fresh culture media were added and incubated for an additional time until analysis.

RNA was isolated using RNeasy reagents (Qiagen, Hilden, Germany). RNA (20 μg) were assayed for Northern analysis using human GCMa or β-actin cDNA probes. To detect syncytin transcripts, 15 μg of RNA from Ad-HAGCMa-transduced cells were analyzed by ribonuclease protection assays using the RPA III kit (Ambion, Austin, TX). The syncytin riboprobe contains nucleotides 417–821 relative to the translation start site. A kit-provided β-actin riboprobe was used as an internal control. The protected syncytin bands were quantified by BAS-1500. HAGCMa and syncytin proteins in transduced cells were subject to immunoblotting with an anti-HA antibody and a syncytin antibody, respectively. Antiserum against syncytin was induced in guinea pigs using a His-tagged surface protein (amino acids 21–215) expressed in BL21(DE3).

Fusion Assay and Fluorescence Microscopy—293 cells were transfected with the red fluorescent protein plasmid pDsRed1-N1 (Clontech) 24 h before cell fusion assay. HeLa, JEG3, or BeWo Cells were transduced with Adlox or Ad-HAGCMa. Four hour post-infection, cells were trypsinized, and 8×10^5 infected cells and 1×10^6 transfected 293 cells were cocultured onto a 60-mm culture dish. After another 30 h at 37 °C, cell fusions were examined under an Olympus microscope (Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP50). Images were prepared for presentation using Adobe Photoshop® 6.0.

RESULTS

Promoter Analysis of the syncytin Gene—Syncytin is encoded by the *envelope* (*env*) gene of an HERV-W family member with a genomic configuration of 5′-LTR-*gag-pro-pol-env*-LTR-3′ (Fig. 1*A*) (4). To investigate the placenta-specific expression of *syncytin*, promoter analysis was performed to identify potential elements and transcription factors that could enhance the LTR promoter activity of the *syncytin*-harboring HERV-W family member. For simplicity, we refer to the *syncytin*-harboring HERV-W family member as HERV-W for the rest of this report. A λDASH II genomic clone (L13) containing the HERV-W genome was isolated using the syncytin cDNA probe (Fig. 1*A*). A human BAC clone, 083M05, which encompasses the L13 clone, was used together with L13 to build a series of promoter constructs covering genomic regions up to 14.8-kb upstream of the 5′-LTR and 5.1-kb downstream of the 3′-LTR of HERV-W (Fig. 1, *A* and *B*).

The role of the placenta-specific transcription factor GCMa in the expression of *syncytin* gene was investigated, because GCMa is known to play an important role in murine placental development (14, 15). Transient expression experiments of the promoter constructs were performed in BeWo cells. As shown

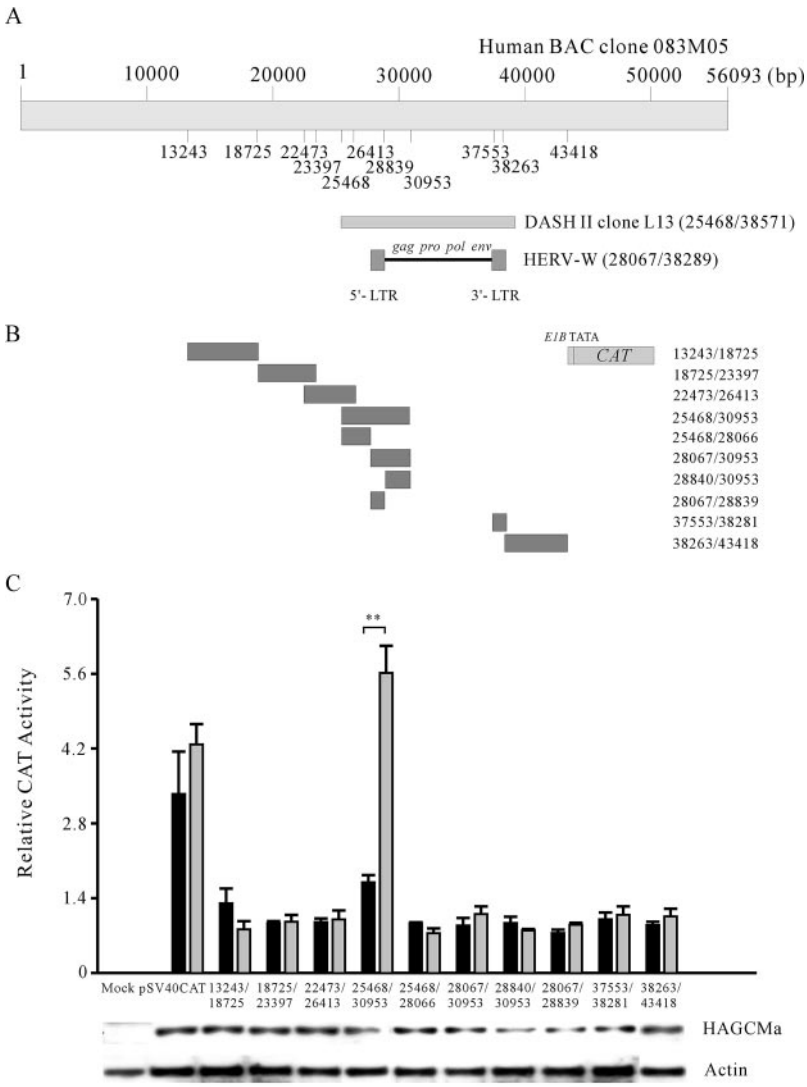

FIG. 1. **Promoter analysis of the *syncytin* gene.** *A*, schematic representation of the human BAC clone 083M05, the λDASH II clone L13, and HERV-W. The numbers above and underneath 083M05 denote the positions of the numbered nucleotides. *B*, schematic representation of promoter constructs used in this study. The genomic fragment inserted in pE1bCAT is indicated on the *left* by a *horizontal filled bar*, denoting its location in the BAC clone 083M05 as listed in *panel A*. The *numbers* on the *right* indicate the ranges of the genomic fragments in the BAC clone 083M05. *C*, promoter analysis for the *syncytin* gene. BeWo cells were transfected with 0.5 μg of the indicated promoter construct in the absence (*black bar*) or presence (*gray bar*) of 0.5 μg of pCMVHAGCMa. Mean values and S.E. obtained from six independent transfection experiments are provided. *Asterisks* denote statistically significant differences (**, $p < 0.01$) between mock- and expression plasmid-transfected groups. HAGCMa proteins in the total cell lysate of mock- and pCMVHAGCMa-transfected groups were analyzed by Western analysis with a horseradish peroxidase-conjugated rat monoclonal anti-HA antibody. As a loading control, actin proteins in the lysate were detected with a rabbit polyclonal anti-actin antibody.

in Fig. 1*C*, the CAT activity directed by pE1bCAT-(25468–30953) is higher than those of the other constructs examined. In addition, a statistically significant 3.3-fold transactivation by GCMa on pE1bCAT-(25468–30953) was observed when it was cotransfected with pCMVHAGCMa. This up-regulation was not due to a differential expression of HAGCMa proteins in transfected cells because comparable amounts of HAGCMa proteins were detected (Fig. 1*C*). When the pE1bCAT-(25468–30953) was divided into pE1bCAT-(25468–28066) and pE1bCAT-(28067–30953), transcriptional activation by HAGCMa was not observed with either construct. pE1bCAT-(28067–30953) contains the 5′-LTR; therefore, these results suggest that potential GBSs are present in nucleotides 25468–30953, which, in conjugation with 5′-LTR, can be up-regulated by HAGCMa. The positive effect of pCMVHAGCMa on pE1bCAT-(25468–30953) was also observed in another human trophoblastic cell line, JEG3 (data not shown). The optimal recognition sequence for the DNA binding domain of *Drosophila* GCM is 5′-(A/G)CCC(T/G)CAT-3′ or its 5′-ATG(A/C)GGG(T/C)-3′ complement (10, 12). Indeed, close scrutiny of GBS(s) in nucleotides 25468–30953 revealed two potential GBSs (25538–25545 and 28026–28033) upstream of the 5′-LTR of HERV-W. The sequences for GBS-(25538–25545) and GBS-(28026–28033) are TCCCTCAT and ATGAGGGC, respectively.

Analysis of the Binding Elements of GCMa in the syncytin Promoter—To test whether GCMa directly binds the two potential GBSs, we performed EMSA with radiolabeled (25488–25587) and (27978–28077) DNA fragments and a recombinant GCMa-FLAG protein (Fig. 2*A*). GCMa-FLAG specifically bound to radiolabeled (25488–25587) and (27978–28077) DNA fragments (Fig. 2*B*, *lanes 2* and *8*) because the unlabeled oligonucleotide pGCMa, consisting of the GBS-(28026–28033) at a 100-fold excess, competed with complex formation (Fig. 2*B*, *lanes 3* and *9*). However, a pGCMa mutant oligonucleotide, Mut, containing mutated nucleotide residues in GBS-(28026–28033) could not compete with complex formation (Fig. 2*B*, *lanes 4* and *10*). Furthermore, the GCMa antiserum, but not the control serum, was able to supershift the DNA-protein complex (Fig. 2*B*, *lanes 5*, *6*, *11*, and *12*). These results suggest that GBSs are existent in (25488–25587) and (27978–28077) DNA fragments.

To localize the binding sites of GCMa in (25488–25587) and (27978–28077) DNA fragments, DNase I footprinting analyses were performed using GCMa-FLAG and the radiolabeled probes of the two fragments. As shown in Fig. 2*C*, the regions protected by GCMa-FLAG in both fragments encompass the GBS core sequence and some immediate 5′-flanking nucleotides (Fig. 2*C*). We further verified the footprinting results by EMSA using GCMa-FLAG and labeled dGCMa and pGCMa oligonucleotides spanning the GCMa-FLAG-protected regions GBS-(25538–25545) and GBS-(28026–28033), respectively (Fig. 2*D*). Specific complexes were observed in lanes without

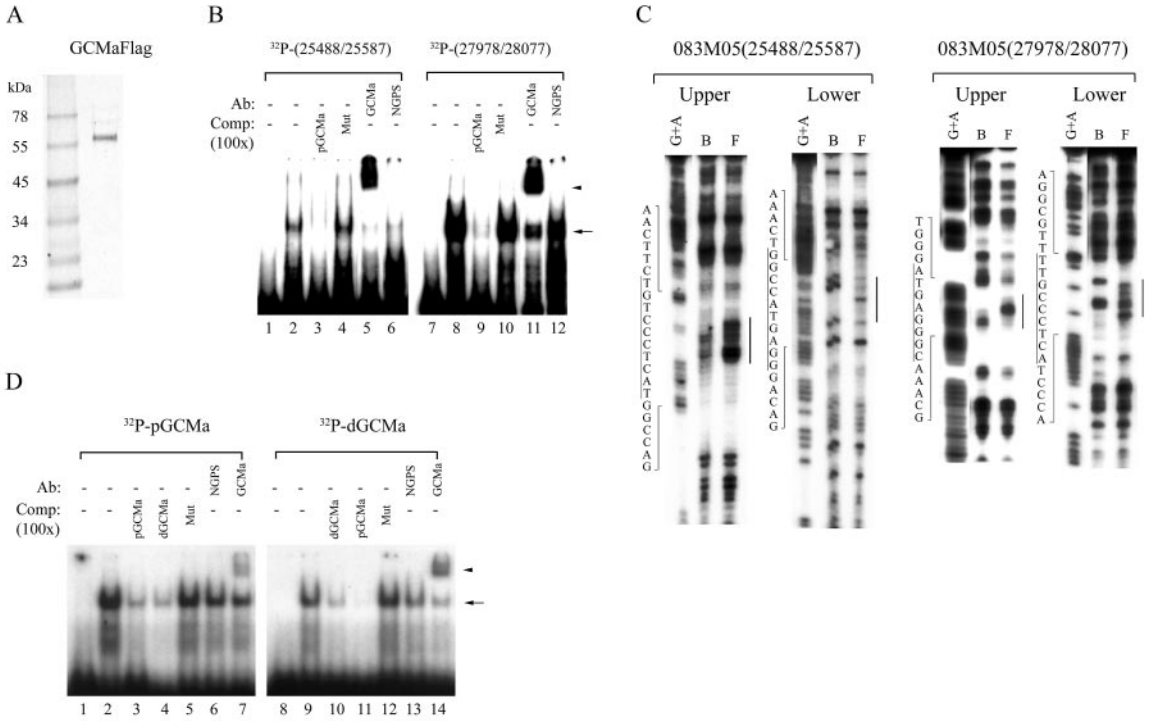

FIG. 2. **Analysis of GCMa binding to GBSs in the 5′-flanking region of HERV-W 5′-LTR.** *A*, GCMa-FLAG proteins were affinity purified from Sf-9 cells infected by a recombinant GCMa-FLAG baculovirus. Approximately 0.2 μg of purified GCMa-FLAG was analyzed by SDS-PAGE and detected by Coomassie Blue R-250 staining. *B*, EMSA of GCMa-FLAG and radiolabeled genomic fragments containing potential GBSs. *Ab*, antibody. *Comp*, the presence or absence (−) of unlabeled pGCMa or Mut oligonucleotide in a 100-fold (100×) excess. *NPGS*, normal guinea pig serum. The *arrow* and *arrowhead* indicate the GCMa-FLAG-DNA complex and its supershifted complex, respectively. *C*, DNase I footprinting analysis of GBSs in the 5′-flanking region of HERV-W 5′-LTR. Two genomic fragments (nt 25488–25587 and nt 27978–28077) in the HERV-W 5′-flanking region were asymmetrically radiolabeled. The labeled DNA probe was incubated with GCMa-FLAG protein, digested with DNase I, and analyzed on 8% polyacrylamide-urea gels. The *vertical lines* on the *right* denote the protected regions of which sequences are listed and underlined on the left. *G+A*, Maxam and Gilbert G plus A sequencing reaction of the probe. *B*, bound or protected probe. *F*, free probe. *D*, oligonucleotides pGCMa and dGCMa, derived from the footprinting analysis, were radiolabeled as probes for GCMa-FLAG in EMSA.

pGCMa and dGCMa competitor oligonucleotides and in lanes with the Mut negative control oligonucleotide (Fig. 2*D*, *lanes 2*, *5*, *9*, and *12*). A supershifted complex was only observed using the GCMa antiserum (Fig. 2*D*, *lanes 7* and *14*). Taken together, the EMSA and footprinting results suggest that GCMa specifically recognizes GBS-(25538–25545) and GBS-(28026–28033) in the 5′-flanking region of HERV-W 5′-LTR.

Transactivation of HERV-W 5′-LTR by GCMa Depends on the Two GBSs and Is Cell Type-dependent—Transient expression experiments were performed in BeWo cells, using GBS-deletion promoter constructs, to test whether transactivation of HERV-W 5′-LTR by GCMa depends on GBS-(25538–25545) and GBS-(28026–28033) (Fig. 3*A*). As shown in Fig. 3*B*, transcriptional activation by HAGCMa of both pCATΔd-(25468–30953) and pCATΔp-(25468–30953) was observed; however, this activation was lower than that observed with pCAT-(25468–30953). Moreover, transactivation was abolished in pCATΔdp-(25468–30953). In contrast, no transcriptional activation by HAGCMa of these constructs was observed in HeLa cells. These results suggest that transactivation of HERV-W 5′-LTR by GCMa depends on the two GBSs and is cell type-dependent.

In Vivo Interaction between GCMa and GBSs Upstream of the HERV-W 5′-LTR—Because GBS-(25538–25545) and GBS-(28026–28033) could be functionally transactivated by GCMa, we further tested whether GCMa interacts with both sites *in vivo* by means of a ChIP assay. BeWo cells were transfected with pCMVHAGCMa, and the DNA-HAGCMa complexes were immunoprecipitated by anti-HA antibody for PCR analysis (Fig. 4*A*). Positive signals were detected when regions 25461–25686 and 27800–28064 were amplified by PCR (Fig. 4*B*). These two regions encompass GBS-(25538–25545) and GBS-(28026–28033), respectively. No signal was detected when a more distal upstream region, 22473–22731, was amplified. These results suggest that GCMa associates with the two GBSs in the 5′-flanking region of HERV-W 5′-LTR in the nuclei of BeWo cells.

GCMa Transactivates syncytin Gene Expression—We next tested whether the expression of GCMa increases the synthesis of syncytin proteins. A recombinant HAGCMa adenovirus, Ad-HAGCMa, and an empty recombinant adenovirus, Adlox, were generated. Expression of HAGCMa in BeWo, JEG3, or HeLa cells transduced with Ad-HAGCMa was analyzed by Northern and Western analyses at different time points. As shown in Fig. 5*A*, Northern analyses revealed that HAGCMa transcripts in HeLa and BeWo cells were detected from 16 h to at least 72 h post-transduction. In JEG3 cells, HAGCMa transcripts were detected from 24 to 72 h post-transduction. Correspondingly, increasing levels of HAGCMa protein were detected in the transduced cells (Fig. 5*A*, *lower panel*). To investigate the effect of HAGCMa on *syncytin* expression, ribonuclease protection assays were performed to specifically detect the *syncytin* transcripts in Ad-HAGCMaC-transduced cells at 24 or 48 h post-transduction. In comparison with untransduced cells, the level of *syncytin* transcripts in transduced JEG3 and BeWo cells at 48 h post-transduction increased ~4.2- and 3.4-fold, respectively (Fig. 5*B*). Interestingly, no *syncytin* transcript was detected in transduced HeLa cells in the presence of a higher level of the HAGCMa protein. Western analyses on the syncytin proteins in BeWo and HeLa cells, transduced with Ad-HAGCMa or Adlox, were performed at 40 h post-transduction. As shown in Fig. 5*C*, HeLa cells transduced with the control

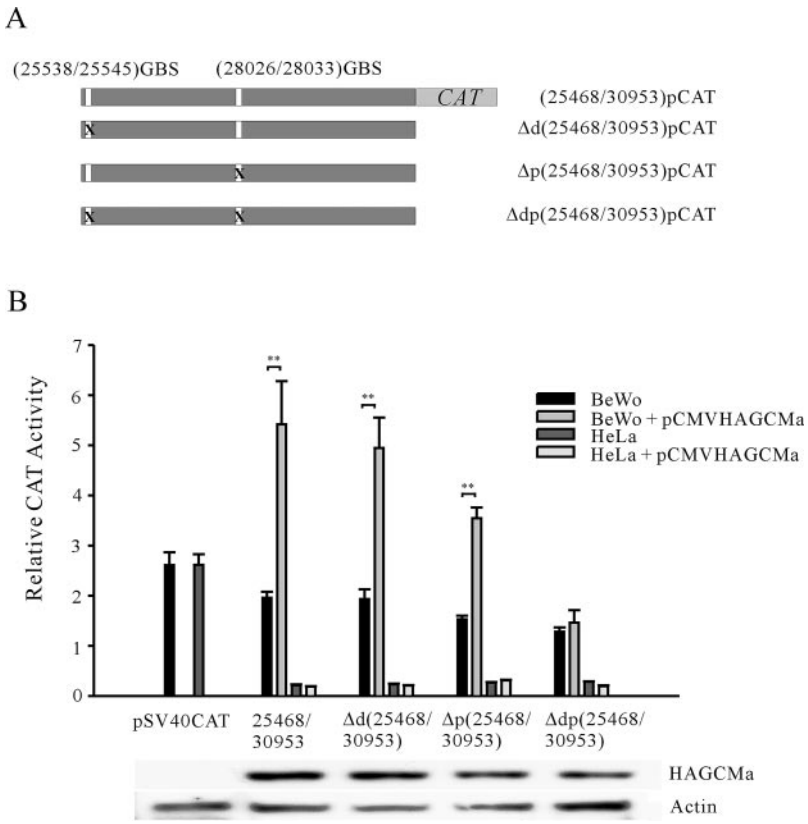

FIG. 3. **Transactivation of GCMa depends on the presence of GBSs in the 5′-flanking region of HERV-W 5′-LTR.** *A*, schematic representation of the mutant promoter constructs. The deleted site is indicated by *X*. *B*, BeWo or HeLa cells were transfected with 0.5 μg of the indicated promoter construct in the absence or presence of 0.5 μg of pCMVHAGCMa. Mean values and S.E. were obtained from seven and three independent transfection experiments for BeWo and HeLa cells, respectively. *Asterisks* denote statistically significant differences (**, $p < 0.01$) between mock- and expression plasmid-transfected groups. HAGCMa and actin proteins in the transfected cells were analyzed as in Fig. 1*C*.

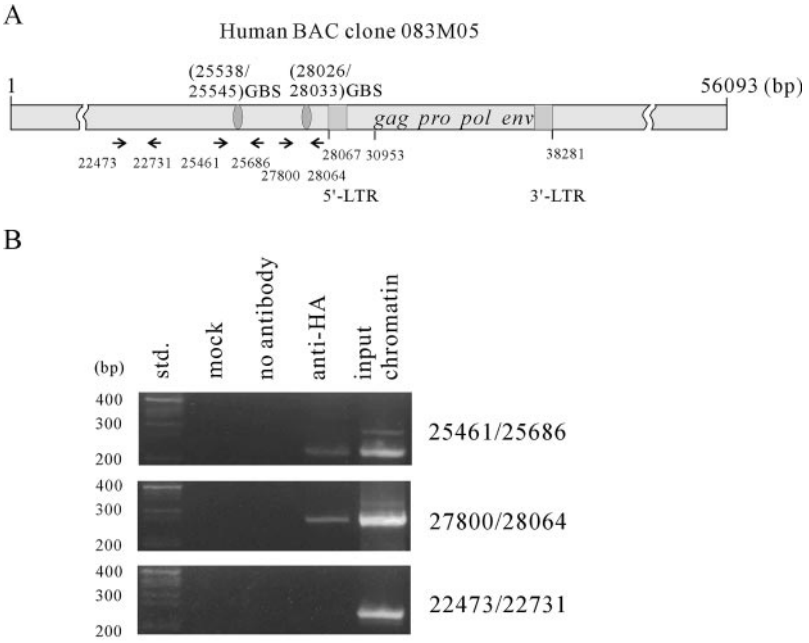

FIG. 4. ***In vivo* interaction between GCMa and the GBSs in the 5′-flanking region of HERV-W 5′-LTR.** *A*, schematic representation of the GBSs, HERV-W, and primer sets used in a ChIP assay. *B*, HAGCMa interacts with GBS-(25538–25545) and GBS-(28026–28033) *in vivo*. ChIP assays of BeWo cells transfected with pCMVHAGCMa were performed for genomic fragments covering nt 25461–25686 (containing GBS-(25538–25545)), nt 27800–28064 (containing GBS-(28026–28033)), and nt 22473–22731. Immunoprecipitates without input chromatin (*mock*) or in the absence of antibody were used as controls. Input chromatin represents a portion of the sonicated chromatin prior to immunoprecipitation.

virus Adlox or Ad-HAGCMa did not reveal any signals for syncytin proteins (*lanes 3* and *4*). However, two bands of 75 and 200 kDa were observed in Ad-HAGCMa-transduced BeWo cells (*lane 2*). The band of 75 kDa may represent the syncytin precursor, whereas the band of 200 kDa may represent trimeric syncytin (5). The endogenous syncytin proteins in the Adlox-transduced BeWo cells were detected after a longer exposure (data not shown). The results of ribonuclease protection assays and Western analyses also suggest that GCMa transactivates *syncytin* gene expression in a cell type-dependent manner.

Cell fusion assays were performed to investigate the effect of GCMa-activated *syncytin* expression on cell fusion. 293 cells expressing red fluorescent protein were cocultured with Adlox- or Ad-HAGCMa-transduced cells and examined under fluorescence microscopy 30 h after coculture. In comparison with Adlox-transduced cells (Fig. 6, *A–C*), fusion events were significantly increased in Ad-HAGCMa-transduced BeWo and JEG3 cells (Fig. 6, *E* and *F*). No fusion events were observed in Ad-HAGCMa-transduced HeLa cells (Fig. 6*D*). Taken together, our study indicates that GCMa up-regulates *syncytin* gene expression via two GBSs upstream of the HERV-W 5′-LTR and consequently enhances syncytin-mediated cell fusion.

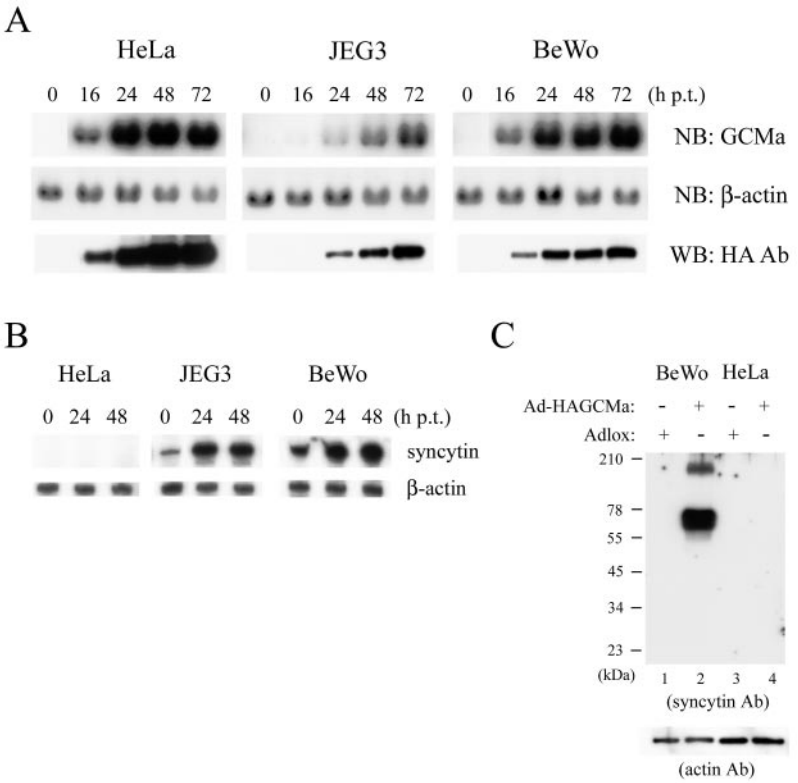

FIG. 5. **Adenovirus transduction of GCMa induces syncytin expression.** *A*, Northern (*NB*) and Western (*WB*) analyses of HAGCMa expression in Ad-HAGCMa-transduced cells at the indicated time post-transduction (*p.t.*). *B*, HA-GCMa transactivates *syncytin* expression in trophoblastic cells. The syncytin transcripts in Ad-HAGCMa-transduced cells were detected by ribonuclease protection assays using a syncytin-specific riboprobe. A β-actin riboprobe was used as an internal control. *C*, Western analyses of HAGCMa and syncytin proteins in BeWo or HeLa cells transduced with Adlox or Ad-HAGCMa 40 h post-transduction. As a loading control, the blot was reprobed with an actin antibody.

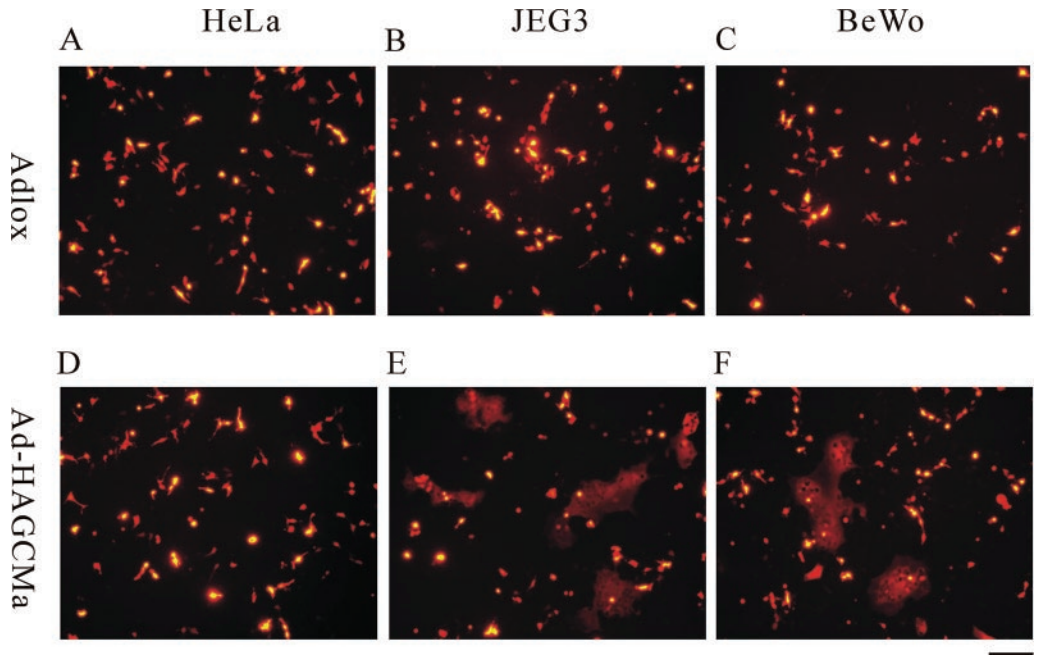

FIG. 6. **Expression of GCMa increases syncytin-mediated cell fusion.** 293 cells transiently expressing red fluorescent protein were cocultured with cells transduced with Adlox (*A–C*) or Ad-HAGCMa (*D–F*). After 30 h, cell fusions were examined under a fluorescence microscope. *Bar*, 120 μm.

DISCUSSION

The fusogenic activity of the syncytin protein has been demonstrated in a variety of primate cell lines including BeWo, COS, HeLa, and 293 (3, 5). *In situ* hybridization has revealed that the expression of the *syncytin* gene is restricted to the syncytiotrophoblast layer in human placenta (3). These observations suggest that syncytin can mediate fusion of cytotrophoblasts into the syncytiotrophoblast layer. Strict regulation of *syncytin* gene expression is important in maintaining an integral syncytiotrophoblast layer, because the overexpression of syncytin in cultured cells causes extensive cell fusion and leads to cell death.[2]

In this study, we identified GCMa as a transactivator for the trophoblast-specific expression of the *syncytin* gene. Several lines of evidence support this conclusion. First, GCMa associated with GBS-(25538–25545) and GBS-(28026–28033) in the 5′-flanking region of HERV-W 5′-LTR *in vivo* based on ChIP

[2] P. Chen and H. Chen, unpublished data.

analysis. Second, the expression of GCMa specifically increased the levels of syncytin transcripts and proteins in trophoblastic cells. Third, syncytin-mediated cell fusion was increased after GCMa expression. Interestingly, expression of the *syncytin* gene was not detected in HeLa cells expressing a high level of GCMa protein. This suggests that regulation of *syncytin* expression by GCMa is cell type-dependent or that other placenta-specific factors may be involved in the trophoblast-specific expression of *syncytin* gene.

Mutation analysis revealed that nucleotide residues 2, 3, 6, 7, and 8 in the optimal GCM recognition sequence (5′-ATG(A/C)GGG(T/C)-3′) are important for interaction with *Drosophila* GCM and mouse GCMa (12). We demonstrated that two GCMa binding sites in the 5′-flanking region of HERV-W 5′-LTR were responsive to GCMa. The proximal site, GBS-(28026–28033), is 34 bp upstream of the 5′-LTR, and its sequence matches the optimal binding sequence perfectly. The distal site, GBS-(25538–25545), is 2522-bp upstream of the 5′-LTR, and its sequence has a mismatch in position 8 in comparison to the optimal binding sequence. Correspondingly, GBS-(25538–25545) has a lower binding efficiency with GCMa in EMSA (Fig. 2*D*, *lanes 9–11*). Deletion of GBS-(25538–25545) had less of an effect than deletion of GBS-(28026–28033) on the transcriptional activation by GCMa, suggesting that the two sites may contribute differentially to the promoter activity (Fig. 3*B*). In fact, it has been shown that there are at least five GCM-binding sites in the 5′-flanking region of *Drosophila GCM* gene, each contributing differentially to the promoter activity of the *GCM* gene (20).

Our Western analyses detected the syncytin precursor proteins and their trimers in Ad-HAGCMa-transduced BeWo cells (Fig. 5*C*). Blond *et al.* (5), using a mouse monoclonal anti-syncytin antibody, have also detected syncytin precursor proteins and their trimers in transient expression experiments. It is possible that the efficiency of post-translational cleavage of syncytin protein is too low to produce a detectable level of surface protein for our Western analyses. Further investigations into the biosynthesis of syncytin protein and syncytin-mediated cell fusion may help to clarify this possibility.

GCMa is a placenta-specific transcription factor required for placental development (14, 15). In addition, GCMa proteins have been immunolocalized in human syncytioblasts and cytotrophoblasts (21). In this study, two functional GBSs were identified upstream of the HERV-W 5′-LTR due to the integration of HERV-W in the human genome. We found that GCMa recognizes these two functional GBSs, induces the trophoblast-specific expression of the *syncytin* gene, and consequently enhances syncytin-mediated trophoblastic fusion. These events could ensure the formation of an integral syncytiotrophoblast layer only in the placenta. A recent clinical survey on the expression of syncytin in human placentas has revealed a lower syncytin mRNA level in patients with placental dysfunction, including preeclampsia and hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome (22). Because *syncytin* is a target gene of GCM, this warrants an investigation into the role played by GCMa in the etiology of preeclampsia and HELLP syndrome.

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