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Genomic Organization, Chromosomal Localization, and the Complete 22 kb DNA Sequence of the Human GCMa/GCM1, a Placenta-Specific Transcription Factor Gene

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The genomic sequence of the human GCMa/GCM1 gene, a mammalian homologue of *Drosophila melanogaster* GCM, was determined. *Drosophila* GCM is a neural transcription factor that regulates glial cell fate. The mammalian homolog however, is a placenta-specific transcription factor that is necessary for placental development. The 22 kb DNA sequence spanning the GCMa gene contains six exons and five introns, encoding a 2.8 kb cDNA. Overall genomic organization is similar for the human and mouse. Several potential binding sites for transcription factors like GATA, Oct-1, and bHLH proteins were found in the 5′-flanking region of the human gene. A DNA motif for GCM protein binding exists in the 5′-flanking region that is highly homologous with that of the mouse gene. The location of this gene was mapped to chromosome 6 using fluorescence *in situ* hybridization.

Key Words: **GCMa/GCM1; transcription factor; placenta; genomic organization; Trophoblast Specific Element.**

GCMa/GCM1 is a mammalian homologue of *Drosophila melanogaster* GCM, and is a member of a small group of transcription factors that share a conserved DNA binding domain called the GCM box in the N-terminal region (1–4). *Drosophila* GCM, a transcription factor encoded by the *Drosophila* gene *glial cells missing* (*gcm*), determines the choice between glial and neuronal fates in early gliogenesis. Loss-of-function mutations in *gcm* result in a disrupted central nervous system and disorganization of the peripheral nerves whereas ectopic expression generates additional glial cells at the expense of neurons (5–7). Many vertebrate homologs of *Drosophila* transcription factors involved in neurogenesis have also been shown to be expressed in the nervous system. Mammalian homologs of dGCM have been a focus of significant interest for their possible role in neurogenesis (8). Two types of mammalian homologues GCMa/GCM1 and GCMb/GCM2 have been reported in human, mouse and rat, although none have been shown to be specific to the nervous system. The GCMa/GCM1 type is selectively expressed in the placenta (4, 9, 10). Recently genetic ablation of mGCMa was found to be embryonic lethal, with mice dying between 9.5 and 10 days *postcoitum* due to placental failure (11).

In the analysis of placenta-specific enhancer in the human aromatase gene (12), we have identified hGCMa as the binding protein for Trophoblast Specific Element 2 (TSE2) that confers trophoblast specific expression (10). The element is also found in the placenta-specific enhancers of some genes like those for the human chorionic gonadotropin α-subunit (13) and leptin (14). The available information indicates that GCMa/GCM1 is an essential transcription factor in placental development. It specifically binds to GCM recognition motifs (1, 15, 16) that have been located in some placenta-specific enhancers as *cis*-elements conferring trophoblast specific expression.

As the first step toward the molecular understanding of expression of GCMa, we isolated genomic BAC clones containing the gene and elucidated its genomic organization. In this paper we document the complete

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TABLE 1

Synthetic Oligonucleotide Primers

Name	Sequence	Position in cDNA*	Comment
pf8	AACACATCTACAGCTCGGAGG	338–353	screening
pr5	TTGCCTCAGCTTCTAACTTGG	708–688	screening
pf10	CCTGCTGGGACTTGAACCAGCAGTAAG	4–31	
pf9	AACAATCTCCTGGTCCAAGG	85–104	
pf7	TGGCCTGACCTTATCATGG	198–216	
pf8	AACACATCTACAGCTCGGAGG	337–354	
114f	AACGCTGTCCCAACTGTGAC	544–563	
pf3	AGAAGCTGAGGCAAGAAGAGCC	695–716	
pf12	GCGCAAATTGTCCAGTAGCAGAACC	1007–1031	
pf2	TACCAGCAGCTTCCATTGG	1224–1242	
B17pf4	GATAAGCAGTAGACAAGG	1633–1650	
B17pf5	GGATTACAGGCGTGAGCCATTG	1949–1970	
116f	TAGTGATGTGCATGGTCTCC	2467–2486	
134f	CGGCCATGTTTGAATGCCAG	2652–2671	
131r	CTAACTTCTTACGGAGAAGC	73–54	
125r	GGTTCCATGATAAGGTCAG	220–202	
pr6	GGAGTTGTGGTTGTTGGTATTGCGCAT	419–393	
113r	CTTGTCACAGATGGCAGGTC	521–502	
117r	TAAAGCGTCCGTCGTGCCTC	642–623	
pr7	GGTTCTGCTACTGGACAATTTGCGC	1031–1007	
pr2	AAGGAGAGAAGCTGCAAGGCC	1200–1180	
pr1	ATGAGGATGAGAGGCGTAGG	1406–1387	
pr8	TTCACACCTTAGGCAGTTCTTCCACC	2289–2264	
B17pr1	CCCCACATTGATATAGGTGCC	2586–2566	
124r	TTGTGTTTCACCTGGAAGAG	801–782	E1 and F4 assembly

* The cDNA for hGCMa referred in this study is a 2766 bp composite of two previously reported cDNAs, D88613 (1–132 bp) and AB026493 (133–2766), namely the longest reported 5′-end for the former has been joined to AB026493 that has longest 3′-untranslated region.

DNA sequence of the 22 kb human GCMa gene with 1 kb of the 5′-flanking region. The chromosomal localization of the gene has been assigned to chromosome 6 with fluorescence *in situ* hybridization method.

MATERIALS AND METHODS

Genomic DNA sequencing and analysis. A human genomic BAC library was screened for the GCMa genomic sequence with the set of primers (pf8 and pr5) listed in Table 1 by the two-step PCR based screening method described previously (17).

Direct sequencing of BAC clones. Direct sequencing of two BAC clones (1865E1 and 2002F4) was carried out with primers nested in the hGCMa cDNA (shown in Table 1) using the ABI PRISM BigDye Cycle Terminator ready reaction mix (PE Biosystems, CA). The PCR products were precipitated with ethanol, washed twice with 70% ethanol to remove excess dye-terminators, and analyzed with an ABI PRISM 310 DNA analyzer (PE Biosystems). Exon positions were identified by comparison of the genomic sequence with the hGCMa cDNA sequence using a Sequence Analysis Software Package (Genetics Computer Group, Inc.).

A shotgun strategy (18–20) was applied for sequencing of the 1865E1 clone. Five hundred thirty four shotgun clones were sequenced using the ABI PRISM BigDye Cycle Terminator ready reaction mix diluted with 5 × sequencing buffer (PE Biosystems). Excess dye-terminators were removed by gel filtration and then PCR products were automatically loaded onto an ABI PRISM 3700 DNA analyzer (PE Biosystems). Individual shotgun sequences were assembled into contigs using GENETYX-Σ/SQEX software (SDC, Tokyo, Japan) on a SUN SPARC Ultra 2 (Sun Microsystems, Mountain View, CA). Each gap was filled by the unique transposon system of GPS-1 (NEB, MA). The genomic region of hGCMa/B17 gene in the BAC 1865E1 clone reached 19,669 bp, covered by 200 sequence fragments. The average sequence read was 600 bp, resulting in a redundancy of 5.61.

Clone 1865E1 was found not to contain exon 6, while 200F4 demonstrated all exons but exon 1. When it was directly sequenced with a primer that nests in exon 6 (124r in Table 1), the 140 bp stretch identical to the 3′-end of 1865E1 was found flanking a *Hin*dIII site 216 bp upstream of exon 6. Accordingly, the 2455 bp contig from 200F4 was assembled with the contig derived from 1865E1 for the GCMa gene.

Chromosomal assignment of GCMa/GCM1 by FISH analysis. The chromosomal assignment of GCMa/GCM1 was performed by fluorescence *in situ* hybridization (FISH) using BAC clone 2002F4 and a set of biotinylated chromosome painting probes. FISH was performed as previously described (21), with prophase chromosome spreads prepared from the human HT1080 cell line. The specific probe for GCMa/GCM1 was prepared by nick-translation of the 2002F4 clone with digoxigenin-11-dUTP (Boehringer Mannheim). Biotinylated chromosome painting probes (CAMBIO) and digoxigenin-GCMa were detected with FITC-conjugated avidin (ENZO Biochem.) and RITC-conjugated anti-digoxigenin (Boehringer Mannheim), respectively. Chromosomes were counter-stained with DAPI. The hybridization was performed with 100 ng of GCMa probe and 1 μg of human Cot 1 DNA (Boehringer Mannheim) in 20.5 μl mixture, followed by the second hybridization to the same chromosomes with painting probes. Digital images of successive FISH results were taken by using a Zeiss Axoplan microscope equipped with a cooled CCD camera (Princeton Instruments). Psuedocoloring and merging of two images were performed using IpLab software.

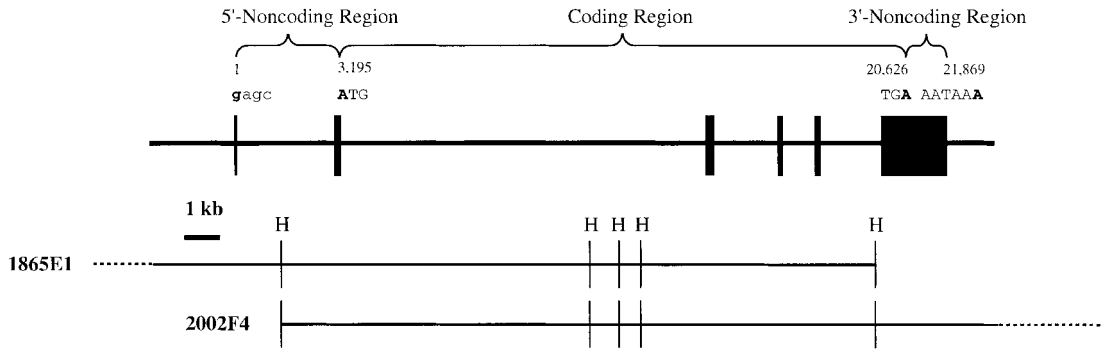

FIG. 1. Genomic structure of the human GCMa/GCM1/TSE2-BP gene. Exons are denoted as boxes. The locations of the first ATG, the TGA, and the potential polyadenylation signal AATAAA are shown with their relative numbers from the 5′-end of the longest cDNA (1). The relative positions of the two BAC clones containing the gene are shown at the bottom.

RESULTS AND DISCUSSION

Determination of Genomic Structure

Two human BAC clones 1865E1 and 2002F4 from an array of the human genomic BAC library were found to contain the GCMa/GCM1 gene by a PCR-based screening method (17). Southern blot analysis with probes corresponding to the 5′-end and the 3′-end of the hGCMa cDNA sequence (AB026493), indicated 1863E1 to contain most of the cDNA but the 3′-end, and 2002F4 most but the 5′-end (data not shown).

All the exons and exon–intron boundaries were sequenced directly using GCMa/GCM1 cDNA-specific primers (shown in Table 1) with both 1865E1 and 2002F4 clones. Sequence analysis of entire 1865E1 was achieved to an average redundancy of 5.61-fold using shotgun sequencing and finishing strategies as described above. The contig from the 1865E1 clone and the 2455 bp contig including exon 6 derived from 2002F4 clone were assembled for the GCMa gene as described in the methods section. The 23,124 bp contig has been submitted to the GenBank with Accession No. AB047819.

Sequence analysis of the clones revealed that the cDNA for GCMa/GCM1 contains six exons separated by five introns and spans 21.9 kb (Fig. 1). The lengths of introns and the sequences at the exon–intron boundaries of the human GCMa gene are shown in Table 2. All of the exon–intron splice signals were found to be consistent with the GT-AG rule (22). The coding region starts in exon 2. The largest exon (exon 6) contains the 3′-coding region as well as 3′-untranslated region and spans 1984 bp. The largest intron (11,237 bp) lies between exon 2 and 3. AATAAA elements directing cleavage and polyadenylation signals reside at 21,845 and 21,864 from the 5′-end of the cDNA. The amino-terminal region of the GCMa protein consists a DNA binding domain that is conserved through evolution. Interestingly, the domain composed of 134 amino acid residues is encoded in four exons and spans 14 kb, whereas 245 amino acid residues at the C-terminal are encoded in a single exon.

Comparison with the Mouse Gene

The overall gene structure of the human GCMa gene is similar to that of the mouse gene (9). The sites at which the coding sequence is interrupted in the genome are exactly the same as those for the mouse gene except with the first intron. Overall human and mouse cDNAs demonstrate approximately 59% identity (Geneworks), that result in 73% identity in amino acid sequence. The level of homology is not uniform throughout the cDNA. The nucleotide identity in the

TABLE 2

Exon–Intron Junctions of hGCMa Gene

Exon	3′-Exon junction	Donor site sequence	Intron	Size (bp)	Acceptor site sequence	5′-Exon junction	Exon	Size (bp)
						GAGCCT	1	76
1	TAGAAG	**gt**aaga	1	2,982	ttt**ag**	CCCTAG	2	211
2	CCACAG	**gt**atgt	2	11,237	tatc**ag**	AACGTG	3	253
3	AGCAGC	**gt**gagt	3	1,954	ttgt**ag**	GGAAAC	4	113
4	TTCCAG	**gt**tgga	4	1,075	ttta**ag**	TCAAAG	5	129
5	ACCAGG	**gt**aatc	5	1,855	ttcc**ag**	TCTCTT	6	1,984
6	AATAAA							

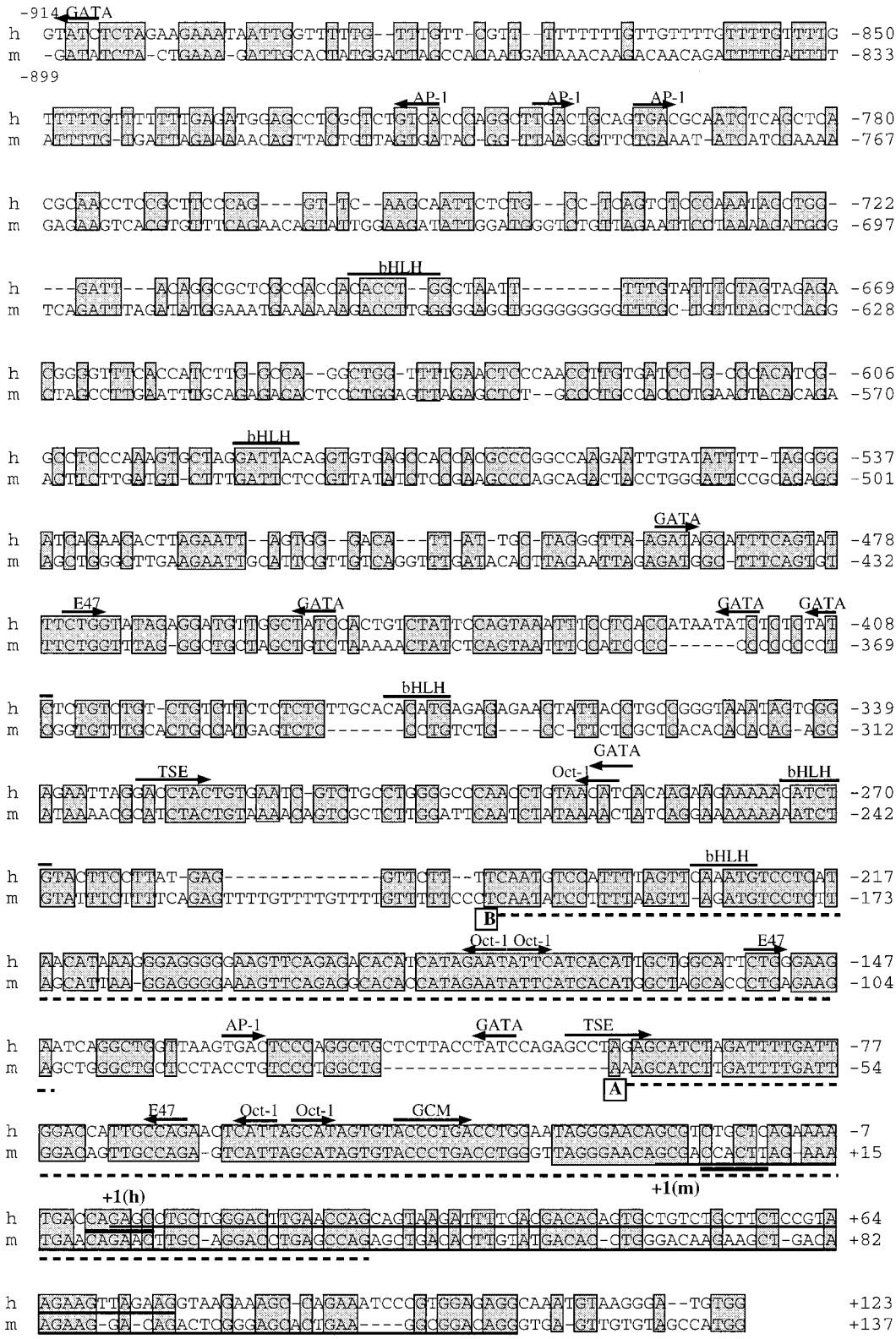

FIG. 2. Comparison of the 5′-untranslated regions of human and mouse GCMa/GCM1 gene. The underlined letters represent exonic sequences. Bold underlining shows initiator sequence. Identical sequences are shown in shaded boxes and the two regions with higher homology (>83%), A and B are illustrated by broken underlines. The 5′-end of cDNA is indicated by +1. Potential binding motives for AP-1, Oct-1, and E47 are searched with MatInspector (30) on TRANSFAC databases.

coding region is 79%. Homology is less conserved in the 3′-untranslated region (39%) and the 5′-untranslated exon 1 bears least homology (26%), where the insertion position of the first intron differs.

A DNA sequence of 1046 bp has been documented as the 5′-flanking region including exon 1 for mouse GCMa gene. Corresponding 1037 bp sequence of the human gene is compared with the mouse sequence in Fig. 2. Overall homology of the region is 48%. Although direct comparison of untranslated exons 1 of both genes showed relatively low homology (26%), highly homologous (83%) stretches revealed in this alignment in the proximal 5′-flanking region extending to exon 1: a 119 bp stretch (A) and a 102 bp (B) are shown with broken underlines in Fig. 2. There are other shorter homologous stretches upstream, but the homology gradually declines in distal upstream region. The homologous regions are likely to be important in regulation of gene expression although the precise initiation start sites have not been identified for either gene. The highly conserved putative promoter regions in both cases lack typical TATA and CCAAT boxes. In the mouse gene, an "initiator" sequence (23) resides at the 5′-end of the longest cDNA reported (bold-underlined in Fig. 2). A distinct "initiator" sequence is similarly present at the 5′-end of the human cDNA.

Placenta-specific enhancers have been functionally analyzed in several genes. Like many other tissue specific enhancers, they are composed of various sets of ubiquitous elements and at least one tissue-specific element. Up to date, the master gene for the trophoblast lineage has not been known. Accordingly *cis*-elements that bind to placenta-specific *trans*-factors differs significantly. As summarized by Wang and Melmed (24), at least five distinct *cis*-elements have been claimed for placental specificity. Trophoblast Specific Element (TSE) (25) (core:RNCCTNNRG) (12, 13), that recognized by AP-2γ (26), is the most often found element in the placenta-specific enhancers. CSEF-1 binding site (core:TGGAATGTG) that is recognized by placenta-enriched TEF-5 (27), is extensively characterized in hCS-B gene. GCM recognition motif (core:RCCCTAK) (10) is found in some placenta-specific enhancers as the core element and described as PLE1 (14), URE (13, 25) or TSE2 (10). Two other elements, PLE3 and PSE are less characterized but bound by distinct yet unknown proteins (14, 24). These placenta specific elements were searched in the upstream region. Exact matches for PLE3, PSE, or CSEF-1 site are not found. There are two TSEs in the human gene. A GCM binding motif is found in the conserved stretch A as shown in Fig. 2.

Among ubiquitous transcription factors, GATA-2,3, TEF and Oct-1 are often involved in the placenta-specific enhancers (24, 26, 27). Binding motif for bHLH protein (CANNTG) might be important, since two bHLH proteins, Mash-2 and Hand 1 are involved in the placental development. The *cis*-elements for these factors are shown on the human sequence. Several of them are found in the region but they are not necessarily associated to conserved regions. There are rather fewer known elements in the homologous stretches. The potential function of the conserved stretch is hardly to predict from the mere combination of known elements. In this respect, the presence of a GCM recognition motif in the conserved stretch A may be important, since an auto regulatory mode of expression has been suggested for the gene (11).

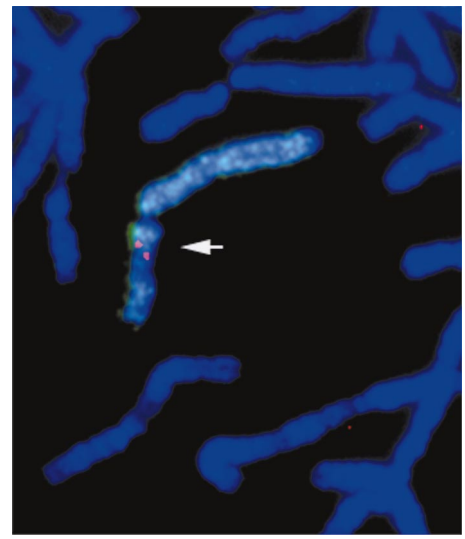

FIG. 3. Chromosomal mapping of hGCMa to chromosome 6 by fluorescence *in situ* hybridization. Representative picture of a FISH. The twin-spot FISH signal for GCMa probe is shown in red and an arrow, counter-stained with DAPI (dark blue). The same preparation was stained with a painting probe for chromosome 6 (light blue).

GCMa/GCM1 could be assigned here to the short arm of chromosome 6 by FISH analysis using the 2002F4 BAC clone as a probe (Fig. 3). The GCMb/GCM2 gene is also reported to reside on chromosome 6 (6p24.2) (3).

The trophoblast is the first lineage to differentiate in mammalian development and this has been well described morphologically. The molecular processes controlling the event, however, are relatively poorly understood and so-called "master gene" or cascade of transcription factors for this lineage have yet to be identified. Recently increasing numbers of transcription factors have been revealed to be essential for placental development by gene targeting strategies. GCMa/GCM1 is among them, and mGCMa is expressed selectively in labyrinthine trophoblasts. In GCMa knock out mice, mutant placentas form the chorion and allantois normally but they fail to develop a functional labyrinth layer, which is necessary for nutrient and gas exchange between maternal and fetal blood. However, GCMa does not seem to function in the earliest step of cascade of transcription factors in-

volved in placental development (11). The obvious next question is which gene(s) controls the expression of GCMa. Functional analysis of the promoter region will help clarify the molecular sequence of events that takes place in differentiation of the trophoblast lineage.

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