Isolation and expression analysis of a novel human homologue of the *Drosophila glial cells missing* (*gcm*) gene

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Received 26 October 1998; received in revised form 24 November 1998

Abstract A novel human homologue (GCMB) of the *Drosophila glial cells missing* gene (dGCM) was isolated using RACE. GCMB contained a gcm motif sequence and a nuclear targeting sequence similar to that of dGCM and mouse GCMb. Homology searches indicated that GCMB was located within chromosome 6p24.2. Transcripts of GCMB were detected by means of RT-PCR in fetal brain, normal adult kidney, 3/3 medulloblastomas, 1/3 gliomas and 4/8 non-neuroepithelial tumor cell lines. Our data suggest that humans have two homologues of gcm like mice and that human gcm genes form a novel family which may function not only during fetal development but also in the postnatal or pathological stage.

Key words: Human; GCMB; Nuclear targeting sequence; Chromosome 6p24.2; Tumor cell line

1. Introduction

The mammalian central nervous system (CNS) consists of two different types of cells, neuron and glia, both of which originate from common multipotential neural stem cells [1]. While several factors and mechanisms for neuronal differentiation of stem cells have been identified [2–7], the molecular mechanisms controlling gliogenesis from them has not been satisfactorily clarified [8–10].

A new gene, *glial cells missing* (*gcm*), was recently isolated from *Drosophila melanogaster* [11–13]. In *Drosophila*, the glial precursors which express gcm (dgcm, GenBank accession numbers U34039, D64040, U81164) in a specific stage of early development differentiate into glia and the dgcm encodes a 504-amino acid protein (dGCM). The N-terminal region of dGCM which has a DNA-binding activity is named the gcm motif. *Drosophila* GCM thus was thought to be a novel transcription regulatory factor which promotes gliogenesis [14,15].

Since the isolation of dgcm, several mammalian homologues of gcm have been identified in mice from not only brain (mgcmb: GenBank accession number D88611) but also non-neuroepithelial sources (mgcma/1: GenBank accession number D88612, U59876) [14,16]. All these gcm proteins (GCMs) have a common, highly conserved gcm motif in the N-terminal portion and the relevant findings strongly suggest that the gcm motif is evolutionarily well conserved and that gcms form a novel gene family of DNA-binding proteins [14,17].

In humans, a single gcm homologue has so far been isolated from placenta (human gcma; hgcma, GenBank accession number D88613) [14] but not from human brain. For the study reported here, we succeeded in isolating a novel human homologue of gcm (human gcmb; Human Gene Nomenclature Committee-approved symbol, GCMB) from human brain cDNA library by using the rapid amplification of cDNA ends (RACE) method and examined the character and expression of this homologue in human.

2. Materials and methods

2.1. Cloning of the GCMB cDNA

RACE was performed using 0.5 ng of human fetal brain Marathon-Ready cDNA (Clontech, Palo Alto, CA, USA) as a template in combination with Advantage cDNA Polymerase Mix (Clontech), according to the manufacturer's instruction. The first 3′-RACE used the adapter primer (AP1, Clontech) and gene-specific primer (GSP), and the nested 3′-RACE used the nested adapter primer (AP2, Clontech) and nested GSP (NGSP). The GSP sequences are as follows: GSP 5′-GCCATGCGCAATACCAACAACCACA-3′ (first 3′-RACE); NGSP 5′-CATCCTCAAGAAGTCCTGCCTGGGT-3′ (nested 3′-RACE).

The RACE products were subcloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced with several forward and reverse primers using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI, Foster City, CA, USA) in both directions in conjunction with an ABI 310 genetic analyzer. We generated new GSPs from the partial sequence of GCMB. We started the 5′-RACE and repeated the 3′-RACE using the GSPs to identify the full sequence of GCMB. The overlapping sequences of 5′- and 3′-RACE products were thus combined and the full-length GCMB sequence was identified. The GCMB cDNA including a complete open reading frame (ORF) was cloned from the same fetal brain cDNA by using PCR.

Searches for homologue sequences were performed with the aid of BLASTN, BLASTP and BLASTX algorithms and the protein motif search was executed by using the PROSITE algorithm.

2.2. Southern blot analysis

Normal human genomic DNA was extracted from blood lymphocytes according to standard protocols [18]. For Southern blot analysis, 5 μg aliquots of DNA digested with either *Eco*RI, *Bam*HI, *Hin*dIII or *Kpn*I restriction enzymes, fractionated by electrophoresis in 0.8% agarose gel (Seakem GTG, FMC, Rockland, ME, USA) and transferred onto a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech, Buckinghamshire, UK). A cDNA probe consisting of 417 nucleotides (nt) of the GCMB (nt 1428–1844) was generated by PCR amplification and labeled with [α-^{32}P]dCTP using a random primer labeling method (Megaprime, Amersham Pharmacia Biotech). The filter was prehybridized for 1 h in ExpressHyb Hybridization Solution (Clontech) at 60°C and hybridized for 2 h with the GCMB cDNA probe. Post-hybridization washes consisted of 3×15 min at

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PII: S0014-5793(98)01650-0

room temperature with 2×SSC/0.05% SDS and 2×20 min at 50°C with 0.1×SSC/0.1% SDS. The filter was exposed to X-ray film (Kodak XAR-5, Rochester, NY, USA) overnight.

2.3. Cell lines and RNA extraction

Seven human neuroepithelial tumor cell lines were examined; T98G, U251 and U-373MG originating from glioblastomas, Daoy, D283 Med, and D341 Med originating from medulloblastomas and KP-N-SILA-2B4 originating from a neuroblastoma [19]. Eight non-neuroepithelial tumor cell lines were also investigated as controls; HuH-6 [20] and Hep G2 from hepatocellular carcinomas, PANC-1 and BxPC-3 from pancreatic adenocarcinomas, Jurkat and K-562 from leukemias, NTera2/cl.D1 from a teratocarcinoma and 293T from transformed embryonic kidney epithelial cells [21]. U251 was purchased from Riken Cell Bank (Tsukuba, Japan), 293T was a gift from Dr. M. Yamadori (Department of Medicine III, Osaka University Medical School, Osaka, Japan) and KP-N-SILA-2B4 was gifts from Dr. T. Matsumura (Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan). HuH-6 was a gift from Dr. S. Kishimoto (Department of Surgery II, Osaka University Medical School, Osaka, Japan). The other cell lines were purchased from American Type Culture Collection (Rockville, MD, USA).

Total RNA was extracted from the cultured cells in the logarithmic phase with the acid guanidinium thiocyanate-phenol-chloroform extraction procedure using TRIZOL reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions.

2.4. Northern blot analysis

The filters containing 2 μg of poly(A)$^+$ RNA from a variety of human fetal and adult normal tissues were purchased from Clontech (Human Multiple Tissue Northern Blot). For tumor cell lines, 10 μg of total RNA was denatured, fractionated by electrophoresis in 1.2% agarose/formaldehyde gels and blotted onto nylon membrane filters. These filters were prehybridized for 4 h in ExpressHyb at 68°C and hybridized for 24 h with the radiolabeled GCMB cDNA probe above described. Post-hybridization washes consisted of 3×15 min at room temperature with 2×SSC/0.05% SDS and 2×20 min at 50°C with 0.1×SSC/0.1% SDS. The filters were exposed to an X-ray film (Kodak BioMax-MS) with double intensifying screens at −80°C for 3 days, 7 days and 14 days. To control loading of the RNA extracts, the filters were rehybridized with a 220-bp cDNA probe of GAPDH after removal of the GCMB probe used, and exposed overnight.

2.5. Detection of GCMB transcripts by RT-PCR

For RT-PCR analysis in normal human tissues, human first-strand cDNA generated from poly(A)$^+$ RNA was purchased from Clontech (Multiple Tissue cDNA Panels). For tumor cells, cDNA was generated from 1 μg of the total RNA using MMLV reverse transcriptase (Gibco-BRL) and random hexamers [22,23]. Next, PCR was performed using Takara Ex Taq polymerase (Takara, Otsu, Shiga, Japan) and GCMB-specific primers (PF and PR) [22,23]. The primer sequences were as follows: PF (forward) 5′-CACCTGGCGCACCT-

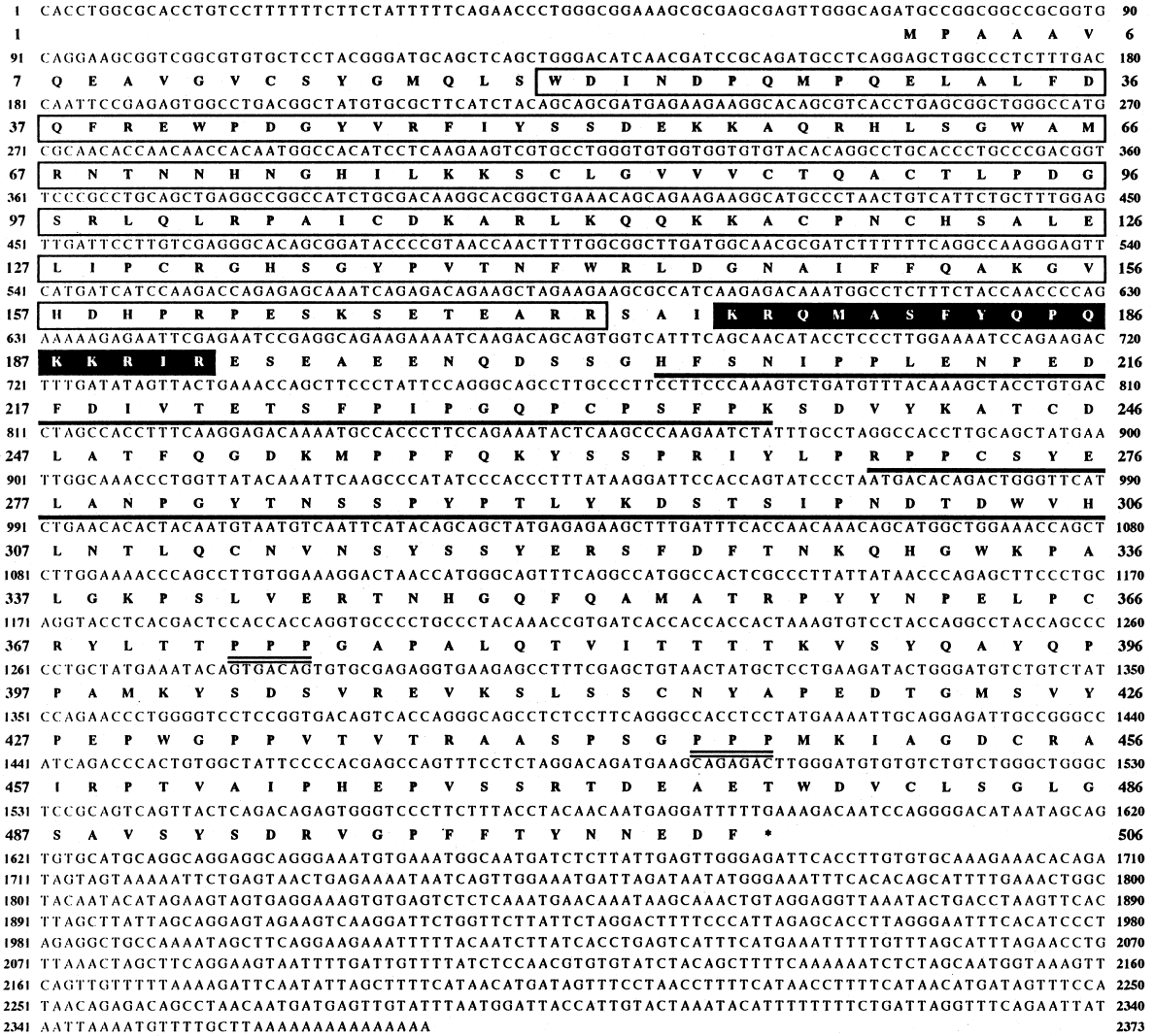

Fig. 1. Nucleotide and deduced amino acid sequence of the GCMB. The highly conserved N-terminal region including the gcm motif is boxed. The proposed nuclear targeting sequence is highlighted in black, PEST-like sequences are underlined and two homopolymeric stretches of proline residues are denoted with a double underline.

GTCCTTTTTTC-3′ (nt 1–25 of GCMB), PR (reverse) 5′-GGCCATTGTGGTTGTTGGTGTTGCG-3′ (nt 271–295 of GCMB).

The PCR amplification program consisted of 30 or 40 cycles of a denaturing step at 94°C for 30 s and an annealing/extending step at 68°C for 2 min.

The RT-PCR products were fractionated by electrophoresis in 2% agarose gel (3:1 NuSieve GTG, FMC) and stained with ethidium bromide. For the specific detection of the amplified mRNA transcript by RT-PCR, the fractionated products were transferred onto a nylon membrane and hybridized with a γ-^{32}P-end-labeled oligoprobe which contained the 25-nt sequence of GCMB (nt 140–164 of GCMB) for 2 h in ExpressHyb at 60°C. Post-hybridization washes consisted of 3×15 min at room temperature with 2×SSC/0.05% SDS and 2×20 min at room temperature with 0.1×SSC/0.1% SDS. The filter was exposed to X-ray film (Kodak XAR-5) overnight. To identify any non-specific amplification from genomic DNA, which could have been included in the RNA extract, separate PCRs under the same conditions were performed by using either the same RNA samples without reverse transcription or genomic DNA as a template. For the control of RNA loading and RT reaction, a 220-bp transcript of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in parallel for all reactions as an internal standard for RNA.

3. Results

3.1. Cloning of the GCMB cDNA

Since the cDNA sequence of the mouse gcm motif is highly conserved between mgcma/1 and mgcmb, we assumed that a novel human gcm homologue (GCMB) should contain a gcm motif sequence very similar to or completely matching hgcma. We first generated two GSPs (GSP and NGSP) based on the gcm motif sequence of hgcma for the 3′-RACE and nested 3′-RACE of GCMB cDNA. An 841-bp fragment which contained partial sequences of GCMB was isolated from the nested 3′-RACE and used to generate eight more GSPs for several 5′- and 3′-RACE procedures. Three independent products were isolated from 5′-RACE and three from 3′-RACE. Sequence analysis of these RACE products with overlapping regions revealed the full-length of the GCMB cDNA sequence.

We then generated GCMB-specific primers and cloned a 1639-bp product (nt 1–1639 of GCMB) including a complete ORF by means of PCR from the same fetal cDNA. One of the aforementioned 3′-RACE products was 1922 bp (nt 452–2373 of GCMB). Since a 1188-nt sequence (nt 452–1639 of GCMB) was common in these two products, full-length sequence of GCMB was judged to be derived from a single mRNA.

3.2. Sequence analysis of the GCMB cDNA and deduced protein

The isolated GCMB transcript is 2373 nt in length and has

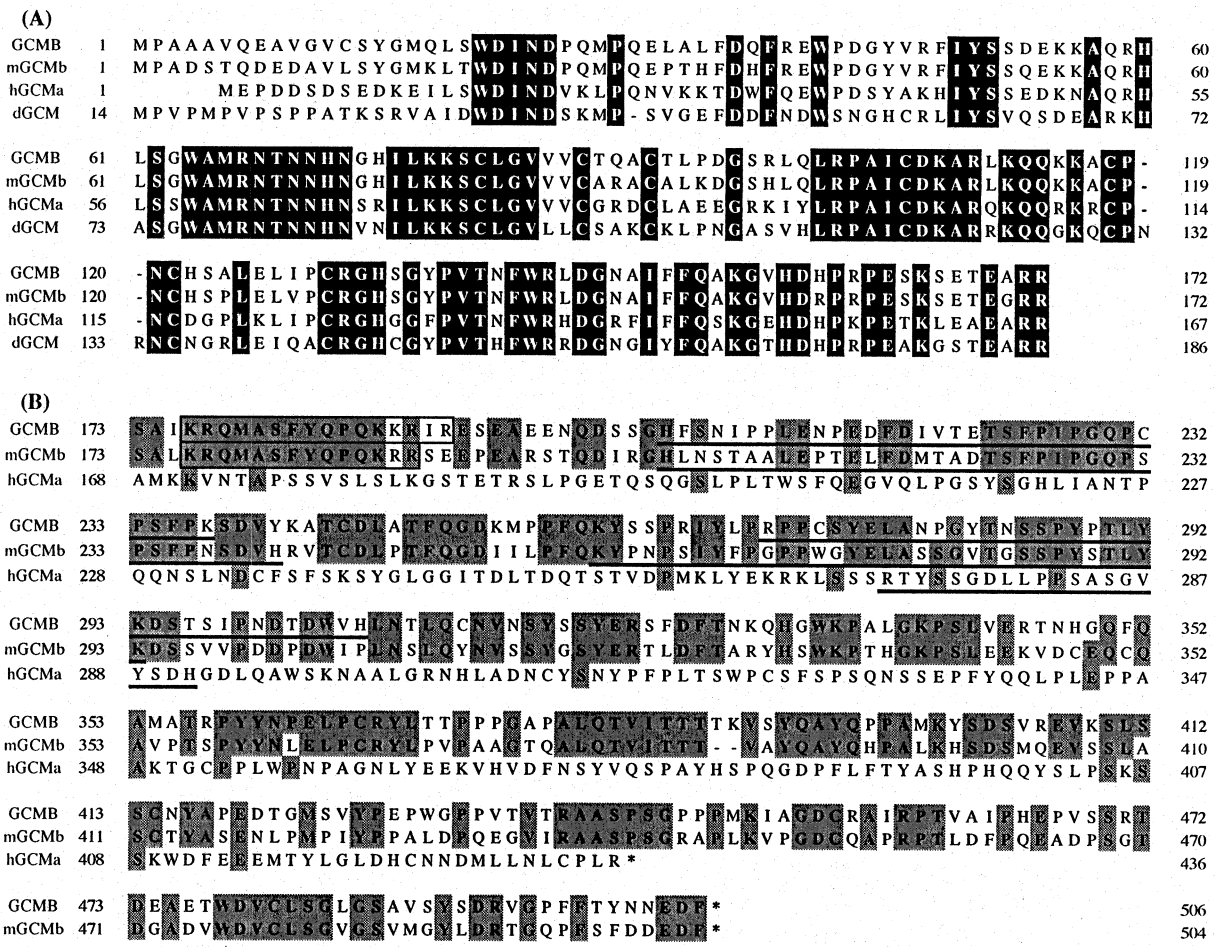

Fig. 2. A: Comparison of GCMB, mouse GCMb (mGCMb), human GCMa (hGCMa) and *Drosophila* GCM (dGCM) sequences in the N-terminal region of the proteins. The amino acid residues conserved through the four proteins are highlighted in black. Gaps are indicated by dashes. B: Comparison of GCMB, mGCMb and hGCMa sequences in regions other than that of the gcm motif. Amino acid residues shared by at least two products are highlighted in gray, the proposed nuclear targeting sequence is boxed and PEST-like sequences are underlined.

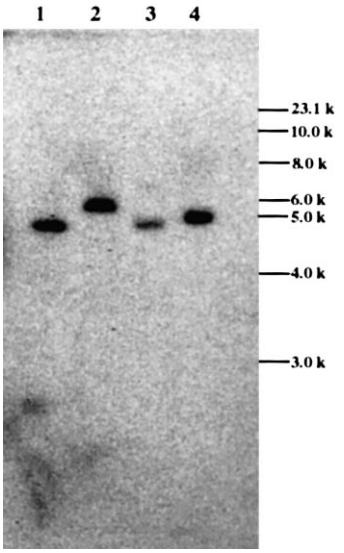

Fig. 3. Southern blot analysis of GCMB gene using a 417-nucleotide radiolabeled cDNA probe. Human genomic DNA digested with either *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3) or *Kpn*I (lane 4) exhibited a single signal band. The size markers are indicated on the right.

an ORF which encodes a 506-amino acid protein. The AUG codon at nt 73–75 which is likely to be the initiator methionine is embedded in a poor Kozak consensus sequence (CAG AUG C) similar to mgcmb (CAG AUG C), in contrast to other gcms, which have a good Kozak consensus sequence, such as dgcm (AAA AUG G), mgcm1 (ATC AUG G), mgcma (ATC AUG G) and hgcma (ATC AUG G) [24]. The 3′-untranslated region (UTR) contained an AU-rich sequence (67.9%) which carries two mRNA instability motifs, AUUUA [25], as does mgcmb (Fig. 1).

In the 172 amino acids of the N-terminal region of deduced GCMB protein, the gcm motif has mostly preserved (Fig. 1) and the N-terminal region revealed 86.0% homology to mGCMb, 68.2% homology to hGCMa and 62.0% to dGCM (Fig. 2A). In regions other than that of the gcm motif, GCMB deduced protein was 59.3% similar to mGCMb but quite different from hGCMa (Fig. 2B). GCMB deduced protein has a PEST-like sequences [26,27] like other GCMs and contains a nuclear targeting sequence [28] and two homopolymeric stretches of proline residues [29], both of which were absent in hGCMa (Figs. 1 and 2B). The overall GCMB deduced protein showed 68.4% similarity to mGCMb but only 46.5% to hGCMa.

3.3. Analysis of the GCMB gene structure, deduced chromosomal localization and homology searches

Southern blot analysis showed a single hybridization signal for each human genomic DNA digested with a restriction enzyme (Fig. 3). The size of the signal band was 4.6 kbp for *Eco*RI, 6.2 kbp for *Bam*HI, 4.8 kbp for *Hin*dIII and 5 kbp for *Kpn*I. From the results of homology searches of GenBank, we determined that the P1 artificial chromosome (PAC: PAC No. dJ417M14, GenBank accession number AL024498) contained 1703 nucleotides corresponding to nt 655–2357 of the GCMB with 99.8% homology. This PAC contains the 146-kbp human genomic DNA sequence of chromosome 6p24.2 (Sanger Center, http://www.sanger.ac.uk./HGP/Chr6/). The size of the

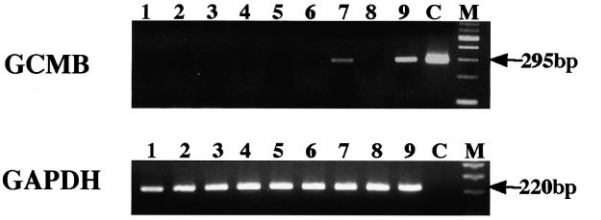

Fig. 4. Detection of GCMB transcripts in normal human tissues by RT-PCR. Specific products for GCMB (295 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (220 bp) used as controls in agarose gel stained with ethidium bromide are shown. The size marker (bp) is indicated by an arrow. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, fetal brain; lane C, positive control and lane M, marker.

Southern hybridization signals for *Hin*dIII or *Kpn*I coincided with the restriction map of the PAC. These findings suggest that the GCMB is most likely a unique gene and that GCMB gene is located within the regions of 6p24.2.

We also identified 14 expressed sequence tags (ESTs) that were almost completely consistent (over 98%) with the GCMB sequence. All these ESTs were identified from human sporadic parathyroid adenomas (GenBank accession numbers AA724352, AA772631, AA782779, AA845533, AA845538, AA860827, AA890229, AI022866, AI028145, AI034434, AI051443, W16423, W19939 and W39214) which did not contain any neuroepithelial tissues.

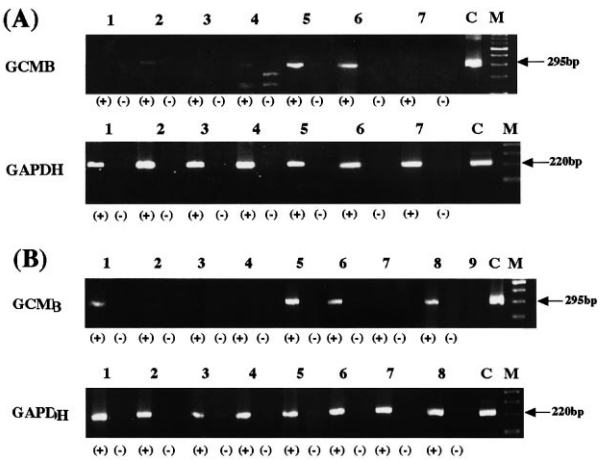

Fig. 5. Detection of GCMB transcripts in human tumor cell lines by RT-PCR. Specific products for GCMB (295 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (220 bp) used as controls in agarose gel stained with ethidium bromide are shown. The size marker (bp) is indicated by an arrow. (+) and (−): PCR with (+) and without (−) reverse transcription. A: The expression of GCMB mRNA transcript in neuroepithelial tumor cell lines. Lane 1, U251; lane 2, T98G; lane 3, U-373MG; lane 4, Daoy; lane 5, D283 Med; lane 6, D341 Med; lane 7, KP-N-SILA-2B4; lane C, positive control and lane M, marker. B: The expression of GCMB mRNA transcript in non-neuroepithelial tumor cell lines. Lane 1, NTera2/cl.D1; lane 2, HuH-6; lane 3, Hep G2; lane 4, Jurkat; lane 5, K-562; lane 6, PANC-1, lane 7, BxPC-3; lane 8, 293T; lane 9, human genomic DNA; lane C, positive control and lane M, marker.

3.4. Detection of GCMB transcripts in various normal human tissues and tumor cell lines

Despite the use of radiolabeled cDNA probes with high specific activity, Northern blot analysis failed to reveal any significant signals in either normal tissues or tumor cell lines even with a 14-day exposure of the filter (data not shown). However, we managed to detect the expression of GCMB transcripts by using RT-PCR. While no products from any sources could be detected with 30-cycle amplification, a visible 295-bp product was identified in kidney and fetal brain with 40-cycle amplification (Fig. 4). The product was also detected in one (T98G) of three gliomas, all three medulloblastomas (Daoy, D283 Med, D341 Med) (Fig. 5A) and four (NTera2/cl.D1, K-562, PANC-1 and 293T) of eight non-neuroepithelial tumor cell lines (Fig. 5B). The product proved to be specific for GCMB as a result of hybridization analysis using a radiolabeled oligonucleotide probe (data not shown). The PCR products from RNA without reverse transcription or from normal genomic DNA did not exhibit any detectable signals as a result of hybridization analysis, indicating that the signal was from the RNA extract and not from possibly contaminated genomic DNA in the extract.

4. Discussion

Drosophila gcm mRNA is transiently expressed in glial precursors and immature glial cells except for mesectodermal midline glia during a short period of gliogenesis within the CNS [11,12]. The cells which express dgcm differentiate into glia, which are marked by the expression of the glial homeodomain protein, *repo* [30,31]. In the absence of dgcm expression, presumptive glial cells differentiate into neurons. An ectopic overexpression of the dgcm forces many presumptive neurons to be transformed into glia. These findings suggest that GCM is required as a binary switch between neuronal and glial determination in *Drosophila* [11,12]. Analysis of dGCM revealed that dGCM had DNA-binding activity in the gcm motif, and several dGCM-binding sites were found in the promoter region of the *repo* gene [14,15]. Thus, dGCM is likely to be a transcriptional activator which induces the expression of *repo*, which encodes a downstream transcription factor required for the terminal differentiation of the *Drosophila* CNS glial cells [30,31]. These findings led many investigators to explore the mammalian homologues of GCM to reveal the regulatory mechanisms of gliogenesis in mammalian nervous systems.

Since the isolation of dgcm, it has been found that *Drosophila* and mice have several gcm genes. Another *Drosophila* gcm (dgcm2), whose function has not been analyzed yet, was isolated from genomic DNA [14]. Two gcm analogues have been identified in mice from adult brain (mgcmb) and placenta (mgcma/1) [14,16]. The proteins (GCMs) deduced from these gcms were highly similar in the N-terminal region and the sequence was therefore named gcm motif [14]. The amino acid sequence except for the gcm motif varied for each homologue. It is therefore thought that gcms constitute a novel gene family which contain the gcm motif as a common core sequence.

We isolated a novel human homologue of gcm, GCMB, in the present study. GCMB have several unique characteristics. GCMB had two mRNA instability motifs in the 3′-UTR like dgcm and mgcmb, suggesting that GCMB (or mgcmb) is unstable in vivo as is dgcm. In addition to the structures common to all GCMs including the gcm motif and the PEST-like sequence, the GCMB deduced protein contained a nuclear targeting sequence and two homopolymeric stretches of proline. The nuclear targeting sequence was present in dGCM and mGCMb, but not in hGCMa and mGCMa/1. GCMB exhibited a high degree of homology to mgcmb in terms of both mRNA and the deduced protein sequence and so did hgcma to mgcma/1. These findings indicate that mammals possess a novel family of gcm motif proteins which consist of at least two different gcm isoforms, one with a nuclear targeting sequence (B isoform) and the other without (A isoform).

Detection of mgcma/1 and mgcmb transcripts by means of Northern blot has not been successful and it is confined to the RT-PCR method. Transcripts of both mgcma/1 and mgcmb at very low levels were observed in fetal mouse brain (personal communication in July, 1998 from Drs. T. Hosoya and Y. Hotta at the National Institute of Genetics, Japan). Although the expression analysis of mgcma/1 was performed by means of in situ hybridization, no significant signal was detectable in both embryonal and adult brain of mice [16]. As in the case of mice, detection of GCMB transcripts was limited to use of the RT-PCR method in our study. In normal tissues, the transcripts were detected in fetal brain and adult kidney. *Drosophila* gcm expression has been reported to be indispensable for development of not only CNS glia but also larval optic lobe primordia, adult peripheral nervous system and scavenger lineage cells in post-developmental stages [11,32]. These findings suggest that GCMB may be expressed and play certain roles in various tissues in both developmental and post-developmental stages.

GCMB is likely to be located within chromosome 6p24.2. These loci have been demonstrated to be linked to a series of CNS disorders such as schizophrenia and orofacial clefting [33–35]. This finding suggests a possible correlation of the GCMB gene with such CNS diseases. Moreover, chromosome 6 has the fourth most frequent abnormalities in human cancer [36] and a correlation between abnormal chromosome 6 and some adult or pediatric CNS tumors was previously reported [37–39]. Several ESTs matching GCMB have recently been isolated from parathyroid tumor cells. These findings indicate a possible correlation between GCMB and human tumor cells. We therefore screened the expression of the GCMB transcript in various tumor cells by means of the RT-PCR method. The GCMB transcript was found to be not limited to neuroepithelial tumor cells and to be relatively specific to embryonic or pediatric tumors such as medulloblastoma, teratocarcinoma and transformed embryonic kidney epithelial cells. In brain and kidney, GCMB transcript was detectable in both normal and tumor cells. These data suggest that GCMB may have a certain significance in these tumors. Similarly, we detected hgcma transcripts by means of RT-PCR in the various lineages of human cell lines (unpublished data). These findings may provide some indications for clarification of the function of human gcm genes.

In conclusion, our study has provided a novel hypothetical idea that humans have at least two gcm homologues: one isoform with a nuclear targeting sequence similar to dgcm and the other without. Although low-level transcripts of both isoforms were detectable in both the developmental and post-developmental stages, their exact roles remain to be identified.

Acknowledgements: We thank Drs. T. Hosaya and Y. Hotta (National Institute of Genetics, Japan) for information on mouse gcm homologue analysis, and Drs. R. Fukuyama and Y. Tomozawa (Kyoto Prefectural University of Medicine) for their helpful discussions. We are grateful for Mr. T. Tanaka (Osaka University) for his outstanding technical help with DNA sequencing and Ms. W. Murata for her technical assistance. We used the Genome Information Research Center of Osaka University for the homology searches of GCMB. The sequence of GCMB has been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession number AF079550. This study was supported in part by a Grant-in-Aid for Scientific Research, (C) 10671299 from the Ministry of Education, Science and Culture, Japan.

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