Mammalian Genome 11, 622–627 (2000).
DOI: 10.1007/s0033500101280

Mammalian Genome
Incorporating Mouse Genome
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Identification of the promoter, genomic structure, and mouse ortholog of LGI1

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Received: 2 December 1999 / Accepted: 27 March 2000

Abstract. The human LGI1 gene is a leucine-rich, repeat-containing gene that was cloned from the t(10:19) breakpoint of the T98G glioblastoma cell line. The LGI1 gene maps to 10q24, a region of peak LOH in malignant gliomas, and is inactivated during the transition from low to high-grade brain tumors. Here we report detailed studies of the genomic structure of the LGI1 gene and its promoter. We have also cloned and characterized the mouse *lgil* gene, which is 97% homologous to the human gene at the amino acid level and 91% homologous at the nucleotide level. LGI1 contains 8 exons, where each of the four leucine-rich repeat units is contained in an individual 72-bp exon. The cysteine-rich regions flanking the LRR and the single trans-membrane domain do not occupy individual exons. Approximately 5-kb of the genomic region 5′ to LGI1 was sequenced, but conventional CAAT and TATA motifs were not present within this sequence. A 597-bp fragment of this 5′ sequence was cloned upstream of a promoterless luciferase gene and was shown to be sufficient to drive transcription. SSCP analysis of the coding region of LGI1 in 20 glioblastomas and five cell lines did not reveal any mutations. Because LGI1 expression is considerably downregulated in gliomas, we also investigated whether this was owing to changes in the methylation status of the promoter. Southern blot analysis and 5-azacytidine treatment did not show any appreciable difference in methylation status between normal brain and glioblastomas.

Introduction

Glioblastoma multiforme (GBM) is the most common malignant tumor of the adult central nervous system. The prognosis for patients suffering from GBM is poor, with a median post-treatment survival time of less than 2 years. GBMs are thought either to arise from a number of benign precursor tumors or via a de novo route where, on first presentation, the patient is histopathologically diagnosed as suffering with GBM. Despite a considerable degree of heterogeneity in tumor origin, some of the genetic lesions associated with GBM development are consistent. The most common genetic abnormality involves the loss of markers from Chromosome (Chr) 10. Remarkably, in 70% of GBMs, one entire copy of Chr 10 is lost, and in up to 90% of GBMs at least part of Chr 10 is deleted (Fujimoto et al. 1989; Watanabe et al. 1998; von Deimling et al. 1992; Fukuyama et al. 1996; Maier et al. 1997, 1998; Chernova and Cowell 1998; Fults et al. 1998; Mao et al. 1999). Analysis of tumors lacking part of Chr 10 reveals a minimally deleted region that spans 10q24-10q26, suggesting that one or more tumor suppressor genes reside at this locale. Despite the fact that a number of genes from this region have been cloned recently, including PTEN/MMAC/TEP (Li et al. 1997; Steck et al. 1997; Li and Sun 1997), DMBT (Mollenhauer et al. 1997), and NEURALIZED (Nakamura et al., 1998), the mutation rates in these genes do not match the observed loss of heterozygosity in this area, indicating that at least one other tumor suppressor gene must reside in this region (Bostrom et al. 1998; Chiariello et al. 1998; Davies et al. 1999; Duerr et al. 1998; Lin et al. 1998; Liu et al. 1997; Ng and Lam 1998; Peraud et al. 1999; Rasheed et al. 1997; Somerville et al. 1998; Tohma et al. 1998; Tong et al. 1999; Wang et al. 1997; Watanabe et al. 1998).

Recently, we identified a gene from 10q24 that is disrupted by a translocation in the T98G GBM cell line and is also rearranged in over a quarter (26%) of primary tumors. The gene, LGI1 (Chernova et al., 1998), is a member of the leucine-rich repeat family of proteins that includes CNS-specific proteins such as TARTAN (Change et al. 1993) and SLIT (Rothberg et al., 1988, 1990; Taguchi et al. 1996). Analysis of LGI1 with Northern blots showed that, of the limited number of tissues analyzed, it is expressed in only two: the brain, where it is most highly expressed, and skeletal muscle, where the expression is considerably lower. Analysis of gliomas revealed that LGI1 expression is either abolished or greatly reduced in high-grade tumors compared with more benign ones, implying a possible role as a tumor suppressor gene. In order to understand the mechanisms of LGI1 down regulation in malignant gliomas, we have characterized the exon/intron structure, the promoter, and the methylation status of this gene. In addition, to enable the creation of both LGI1 knockout mice and transgenic animals that over-express LGI1, we have cloned and characterized the homologous mouse *lgi1* gene.

Materials and Methods

Sequencing of BAC 306010. BAC DNA was purified by using the Qiagen low copy protocol. The genomic copy of LGI1 was sequenced using the Big Dye Terminator cycle sequencing kit from Applied Biosystems Incorporated (Foster City, Calif.). The sequencing reactions were carried out in accordance with the manufacturer's instructions with the following minor modifications. BAC DNA used in the sequencing reactions was pre-digested with *Not* 1 in order to relax supercoiling. DMSO was also added to a concentration of 5% in the final reaction, and 39 rather than the recommended 29 cycles were performed. Sequences obtained were aligned with the cDNA sequence by using the ALIGN program in the DNASTAR (Lasergene) sequence analysis package. The point at which the cDNA and the BAC derived sequences diverged was analyzed for sp1ice acceptor and sp1ice donor sequences by using the sp1ice site prediction program.

SSCP analysis. Genomic DNA was isolated from tumor samples as described by Sambrook et al. (1982). Each exon of LGI1 was amplified from 250 ng of genomic DNA, with 2 mM of exon-specific primers (Table 1), in the presence of 2 mM of dATP, dGTP, and dTTP and 0.1 ml of a ^{32}P dCTP (3000Ci mmol^{-1}). The products of the PCR reactions were analyzed on agarose gels to ensure only a single product of the correct size had been

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Table 1. Summary of the DNA sequences at the splice acceptor and splice donor sites for each exon of LGI1.

Exon number	Acceptor sequence		Donor sequence		Exon phasing
	Intron	Exon	Exon	Intron	
1	N/A		TCTCATT**gt**aaggcc		2
2	ctctttttttgttttctttc**ag**ATCCTTTGTGAGATCTGGTT		AGCTCTT**gt**gagaaa		2
3	ttgtgtacttttttctgggc**ag**GTTATTCACATCGAACTCCT		AGTATTT**gt**aagtaa		2
4	atatattataacttattgc**ag**ATTCATAGAATACAACAACA		TTCACTT**gt**aagtat		2
5	ctttttttttttttttttcc**ag**GAGCCTTGCAAACAACAATC		CAAATGT**gt**aagagg		2
6	cagctgaagtttgtctttc**ag**GGACCTGAGGGGTAATTCAT		ATTACAG**gt**aatgta		1
7	tattatttcctatttttgc**ag**AATTTGCAAAGTCTCAAGAC		CATTCAG**gt**atgaaa		1
8	gtntcnaccttcttctccc**ag**GCACTTCCACTGTAGTACGC		N/A		N/A

amplified. The PCR products corresponding to the larger exons were digested to ensure they were of optimal length for SSCP. The PCR products were denatured by boiling for 10 min, chilled on ice, and then loaded onto a 1 × MDE gel (FMC Bioproducts). Electrophoresis was carried out for 14 h at 6 watts. Gels were dried and exposed to film for 5 h.

Southern blotting protocol. Tumor and cell line DNA was prepared using standard methods (Sambrook et al., 1982). Of each DNA sample, 10 μg was digested with either the *Hpa*II or *Msp*I restriction endonucleases. The DNA samples were then electrophoresed through 0.7% TBE agarose gels until the desired separation had been achieved. Gels were depurinated in 0.2 *N* HC1 for 15 min before being soaked twice in denaturation solution for 30 min. The DNA was then transferred to Hybond N+ (Amersham) in denaturation solution overnight. The membrane was then neutralized for 20 min, and the DNA was fixed by UV cross-linking. ^{32}P-labeled probes were prepared by random priming by using the Megaprime system (Amersham). Hybridization was carried out overnight in 0.5 M sodium phosphate and 7% SDS at 65°C. Filters were washed with 0.1 × SSC and 0.1% SDS at 65°C, twice for 30 min. Filters were exposed to film at −70°C.

Treatment of cells with 5-azacytidine. U373 and U87 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in the presence of 10% CO_2. Cultures of these human glioma cells were then treated with 1 μm or 2 μm 5-azacytidine for 72 h. The plates were then washed twice with ice-cold PBS, and RNA was extracted by the method of Chomczynski and Sacchi (1987). The RNA was then reverse transcribed with Superscript II (Gibco) and random hexamers (Pharmacia) in accordance with the manufacturer's instructions. PCR reactions were then carried out with a series of primer pairs that were complementary to the LGI1 cDNA sequence but mapped to different exons, allowing us to distinguish between PCR products that were derived from the cDNA or from contaminating genomic DNA in the RNA preparation.

Promoter analysis of LGI1. A 597-bp fragment of the LGI1 promoter was PCR-amplified from BAC 306010 by using the primers, GLKpn-1 (5′-taggatccattagccaggtag-3′) and GLXho-1 (5′-gcactcgagaaaatatcccc-3′). The PCR amplification product was then cloned in to the TA vector pCR2.1 (Invitrogen); the insert was then sequenced to check for any proofreading errors. The insert was released by digestion with *Xho*1 and *Kpn*1, gel purified, and cloned into the promoterless luciferase vector pGL3-Basic (Promega). DNA was prepared for transfection with the Qiagen midiprep kit. Three cell lines (NIH3T3, C3H10T1/2, and Cos7) were used initially in the luciferase activity assays. Luciferase activity for this gene could not be detected at any appreciable level in Cos7; all subsequent experiments were undertaken with the NIH3T3 and C3H10T1/2 cell lines. Five μg of DNA was transfected into 4×10^5 3T3 or 10T1/2 cells by using lipofectamine in accordance with the manufacturer's instructions. Prior to transfection, cells were washed twice with optimem (Gibco). Twenty-four hours post transfection, cells were washed with PBS and lysed by using passive cell lysis buffer (Promega). Luciferase activity was measured with the dual luciferase assay kit from Promega. All results were normalized against the Renilla luciferase activity.

Cloning of the mouse cDNA. A mouse λgt 10 skeletal muscle cDNA library (Clontech) was screened to obtain the mouse *lgi1* clone. Briefly, 300,000 plaques were screened with a human LGI1 coding region probe. The human LGI1 probe was labeled as described above by using the Megaprime kit from Amersham. Library filters were hybridized overnight in 0.5 M sodium phosphate 7% SDS at 65°C. Unbound probe was removed by washing two times in 0.2 × SSC and 0.1% SDS at 65°C. Autoradiography was carried out by standard procedures. Positive plaques were subjected to two more rounds of screening to ensure they were plaque pure. The DNA was then isolated by using the Qiagen 1 midi preparation kit and sequenced.

Results

To determine the intron/exon structure of LGI1, we designed a series of primers covering the entire cDNA. Combinations of primers that gave identical sized bands using the cDNA and genomic DNA as templates indicated a lack of an intervening intron between the two primers. The presence of an intron was indicated by a discrepancy in the size of PCR product produced by using genomic and cDNA templates or by the absence of a band in the reaction using a genomic DNA template. Primer combinations that indicated the presence of an intron were then used to sequence directly from BAC 306010, which had previously been shown to contain the entire LGI1 gene (Chernova et al. 1998) in order to identify the sp1ice donor and acceptor sites.

LGI1 spans ~25 kb of genomic sequence (GDB Accession No. AF055636) as judged by Southern blotting and is composed of eight exons that range in size from 72 bp to 1197 bp (Fig.1). The LGI1 protein contains four and a half leucine-rich repeats. The first leucine-rich repeat is the least conserved, as is often noted in proteins containing this motif, and the amino acid substitutions are consistent with those described in other leucine-rich, repeat-containing proteins. Each repeat is broken by an intron at the 5th amino acid (either a leucine or a valine) and at the 2nd base of this codon. The first exon contains the start methionine, signal peptide, N terminal-cysteine-rich flank and amino acids 1–5 of the first leucine-rich repeat (LRR). The four small, 72-bp exons (exon 2–5) each contain an entire LRR, running from the 5th amino acid of one repeat to the 5th amino acid of the next. The phasing of the sp1ice sites of each LRR-containing exon is identical. This phasing has allowed this gene family to evolve by duplication or deletion of these exons, since it does not change the open reading frame. Additionally, this exon phasing gives these genes the ability to produce a number of protein products by differential sp1icing of the mRNA. All other exons have a different exon phasing. Exon 6 contains the final half leucine-rich repeat and the C terminal cysteine rich flank (Fig. 1). There are no features of note in exon 7, while exon 8 contains the putative transmembrane domain and the stop codon. All exon/intron boundaries conform with the GA/GT rule (Table 1) described by Padgett et al. (1986).

It was previously observed that, in malignant gliomas, the LGI1 gene is rearranged in 20–30% of tumors and shows reduced levels of mRNA expression when compared with either normal brain or the lower-grade brain tumors (Chernova et al. 1998). Since mutations are one way that tumor suppressor genes can be inactivated, we analyzed the mutational status of the LGI1 gene in GBM. Primers were designed from within the flanking intron sequences which amplified all of the exons individually (Table 2)

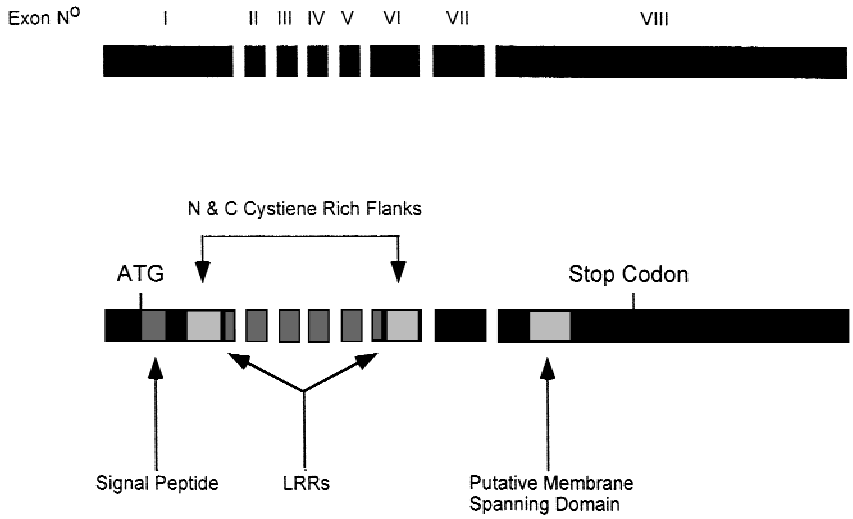

Fig. 1. Diagrammatic representation of (**a**) the exon-intron organization of the LGI1 gene and (**b**) how the structural motifs within this gene are distributed.

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tacagacaggagaatagattcagcaagttcacag
cagacctgcgttctcaaagccaggtgtctggttc
tcagtccagcatttcccccaccatgtcacagcct
cggggctgagccaggccttttcaaagccccagg
aagctagagctggccgcctcctgctggcagggag
ttcctttccaagcttctgggtgctacttgccaaa
gaagggagggtgggagaaaagagggaaggtggat
tatacacagagagggtcccgcacttctgtttccc
ttccttgtcatGGAAGGAAAGAGAATGAATTACG
TGGGGGACCCAGGGGGATAGGAAAATCAATCATT
CATGACAGTTAATAAGTGTCAGAAAAGTATGTAC
TCACATTCATCCCCTGAAAGTCACATTCTTCATC
TGTTCTGATTTGTCTTTCACTGATGGCATTAGCC
AGGTAGGTCAGCAAGAAACATCAAAAGGTTTTAG
CCCAATATTGTGGAGAGAGTTTCATTTAGCTTCA
TTTTTTCCAGCCGGGGCTGGCTCCAAAGCTAGTT
TTTCTTACTAGGTTGTTTGCTACATGAAAAGCAG
ACTCCCAGAGGTTTCTGAGATTGCCGACATCCTA
TGCAAGATGACTGCTCCCACCCACCCGCCCACCC
ACTCACTAATAAGACATAATGCCGTCCTATTTTT
GGTAACAATTAAAGGGCCTCCCGGTGAAAGCAGA
AAGCTGTTTATTTTCTGTGACATCACTTCACTTT
GCCTTCGAAGGCTGGTCTGTGCTCAGTGTTTTCG
TGGTGATGCAAGTCGGCTCTCTCCTCCAGCAGTT
GGATCCCTCCCATCTCACAGTACCTCACAGGTCT
CTTCCCCCGAGCAGTGCATTGCTGGAGCGA
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Fig. 2. DNA sequence of the minimal promoter region for LGI1. The sequence in upper case represents the 597 bp cloned into pLUC. The lower case sequence is part of the upstream sequence obtained (see Results). The 5′ and 3′ orientation is shown.

and SSCP was then performed on 20 GBM tumors and five cell lines. No alteration in banding patterns was observed in the coding region of LGI1 in any of the tumors analyzed. Since SSCP may not detect all mutations, or specific recurrent mutations, we completely sequenced the entire coding region from two tumor DNA samples. Thus, the individual exons from GBM3 and cell line U373 were analyzed, and no mutations were detected with this approach either. It appears, therefore that mutation of LGI1 is not a common event in glioma tumorigenesis.

The transcription of certain tumor suppressor genes can be silenced by methylation of the promoter, or other regulatory elements, responsible for controlling gene expression. It was possible, therefore, that the reduction in LGI1 mRNA levels was caused by methylation. Prior to determining the methylation status of the promoter, however, the promoter sequence itself needed to be defined. A continuous sequence of 4995 bp was obtained from BAC306010 by a single pass sequencing effort. No identifiable promoter could be detected. We therefore verified the approximately 1800 bp 5′ to the initiation of transcription site (GDB accession No. AF246992). Analysis of the 699 bp immediately upstream of the transcription start site promoter with the MATinspector program indicated the presence of a number of potential transcription factor-binding sites, although computer prediction programs failed to identify this sequence as a promoter.

In order to determine whether the DNA sequence 5′ of the start of transcription acts as a true promoter, we cloned the 597 bp immediately 5′ of the first transcribed base upstream of a promoterless luciferase gene. This construct was cotransfected into cells with a plasmid that expressed renilla luciferase under the control of the SV40 promoter. The firefly luciferase activity measured in these cells was normalized with respect to the renilla luciferase activity. Transfection of the LGI-Luc construct into both NIH 3T3 and C3H10T1/2 cells produced high luciferase activities (80,069 and 60,100 respectively), whereas the pGL3-basic vector gave undetectable amounts of activity (Fig. 3). These data confirmed that the 597 bp immediately 5′ of the transcription start site does indeed contain the basic elements that are required to drive transcription.

Southern blot analysis was then used to determine the methylation status of the genomic DNA surrounding the LGI1 gene. Genomic DNA from both GBM tumors and normal brain was digested with either *Hpa*II or *Msp*I, two restriction enzyme isoschizomers that differ in their susceptibility to methylation of their recognition sequence. The results shown in Fig. 4 demonstrate that the genomic DNA surrounding the LGI1 locus is indeed methylated in those GBM tumors which have very low or absent expression of LGI1. The LGI1 locus, however, is equally methylated in normal brain tissue, where the gene is normally most highly expressed. This observation suggests that differential methylation of the LGI1 locus is not responsible for the observed reduction in its expression in GBM. To confirm this result, the U87 and U373 GBM cell lines, which do not express LGI1, were treated with 1 μm or 2 μm 5 azacytidine, which has previously been shown to reverse methylation-induced repression of gene expression in malignant gliomas (Furnari et al. 1997). As shown in Fig. 4, the methylation status of the LGI1 promoter is clearly altered by 5-azaC treatment, resulting in the elimination of the larger (methylated) bands and the presence of the same profile seen in the MSP1-digested DNA. This treatment of cells did not result in a restoration of the expression of the LGI1 gene, indicating that a change in methylation status of the LGI1 promoter is probably not responsible for the loss of gene expression in tumors.

Many LRR-containing genes are highly conserved throughout evolution; thus, to compare the mouse and human orthologs, we screened a mouse skeletal muscle cDNA library with a human LGI1 probe. From the library screen a number of cDNA clones were identified. The sequence established from these clones, when compared with the Genbank batabase, showed high levels of homology to a number of EST sequences. However, the highest

Table 2. Details of the primer sequences used to amplify the individual exons of LGI1.

Exon number	Forward primer	Reverse primer	Product size[a]	Mouse[b]
1	gaggcagaggaccagggtggact	tgcttacgggccttacaatgagt	253	+
2	taacttattgcagattcat	atagcaacattcatacttacaa	103	−
3	aatttatcactacagttacatca	atttctaaggtcctcttacacatt	152	+
4	gcaacgccgggtaaggtc	ttttgaggtggaatgatgatgagt	317	+
5	ttagaaccctgattttt	tcccagatgaagtaagaga	234	+
6	ctagccaaccaaagaggtat	gggggaattatggtttagag	347	+
7	agaggatggccacacaac	gaagaagtcatcatggcattg	156	+
8a	tcagacactcagaacgcctcatcc	cttcactgcgtacacatcctccat	123	+
8b	ggtgttccagcctcttca	tacgcttattaatggacaca	187	+

[a] The size of the expected PCR product is shown. For exon 8, primers that produce two overlapping PCR products were designed to keep their size within that ideal for SSCP analysis.
[b] Those exons that could be amplified in the mouse by using the human primer pair are indicated (+).

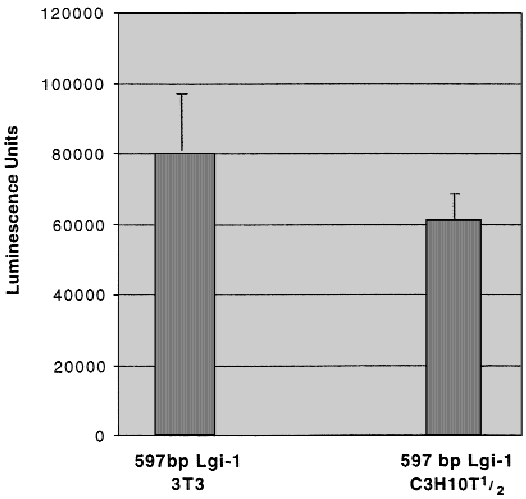

Fig. 3. Luciferase activities from cells transiently transfected with different promoter-deletion constructs. Two different mammalian cell lines, C3H10T1/2 and NIH3T3, were used for transfection. For each individual transfection, 30 ng of SV40 renilla luciferase was used as an internal control. The firefly luciferase and renilla luciferase activities were measured and the promoter activity determined after normalization. Results are expressed as an average of three independent transfections with duplicates per transfection. In both NIH3T3 and C3H10T1/2 cells, strong promoter activity was detected for the 597 bp used in this assay.

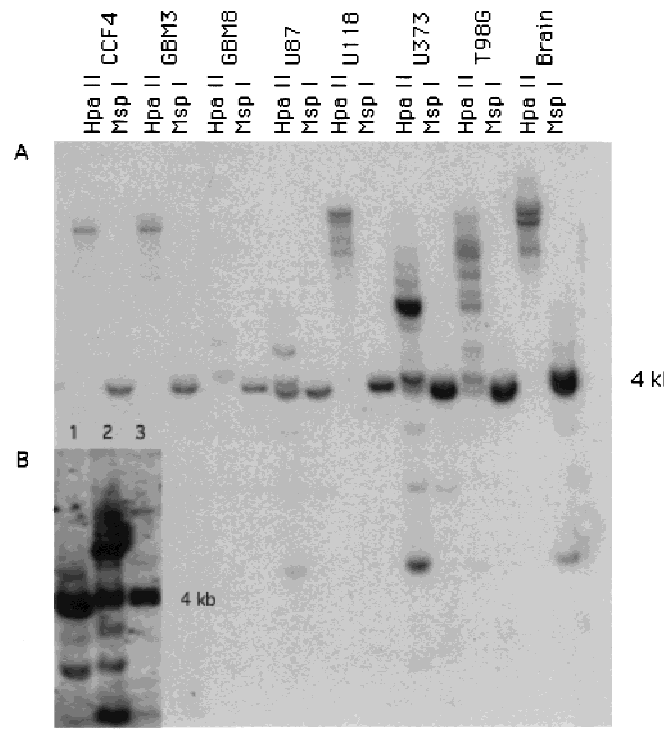

Fig. 4. (A) Southern blot analysis comparing methylation status between various GBM tumors and normal brain by using methylation-sensitive (*HPa*II) and -insensitive (*Msp*I) restriction enzymes. CCF4 represents a primary culture of a GBM tumor, whereas GBM3 and GBM8 represent DNA isolated directly from surgically resected specimens. U87, U118, U373, and T989 are well established cell lines derived from GBM tumors. In (**B**) the effects of 5 azacytidine treatment of U373 cells is shown. Cells were harvested after three days treatment and subjected to *Hpa*II digestion in treated (lane 1) and untreated cells (lane 2). The hybridization pattern in the *Hpa*II-digested cells is compared with untreated cells digested with *Msp*I (lane 3). The expected size of the *Msp*I DNA fragment is 4 kb. In this gel it can clearly be seen that the large DNA fragments in lane 2, which are produced as result of methylation in the promoter region, are lost following 5 Aza-C treatment (lane 1).

identity was with human LGI1, with an identity of 91% at the nucleotide level and 97% at the amino acid level (Fig 5). In contrast to human LGI1, which is 2.2 kb long, mouse *lgi1* (GDB) accession No. AF246818) is 4.2 kb long. The difference in size of the two mRNAs is accounted for entirely by an extra 2 kb in the mouse 3′ untranslated region. With mouse genomic DNA, PCR amplification was performed across the regions where the exon/intron boundaries reside in the human genomic sequence. We did not undertake extensive sequencing to define the precise position of the exon borders, but our PCR analysis confirmed that the position of the exon/intron boundaries is broadly conserved between the two species. The only exon that could not be amplified in this way was exon 2. The high degree of identity between the mouse and human nucleotide sequence also extends into the untranscribed regions, all but one (exon 2) of the primer pairs that were designed to amplify individual human LGI1 exons also produced PCR products from mouse DNA (Table 2).

Discussion

LGI1 lies within the most common region of LOH in GBM and is inactivated in high-grade tumors and cell lines but not in benign tumors, suggesting it may be a tumor suppressor gene. To determine whether mutations in the coding region were responsible for inactivation, we established the exon/intron structure of LGI1 which contains 8 exons. Each of the leucine-rich repeats is encoded by a small, 72-bp exon. The final exon contains a putative transmembrane domain and the 3′ untranslated region. The 2nd exon, which contains the first leucine-rich repeat, is the most divergent from the consensus LRR amino acid sequence. The observation that it is the outer leucine-rich repeats that usually diverge from the consensus sequence is probably because these represent the oldest of the repeats, and therefore have had the most

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1   MESERSKRMGNACIPLKRIAYFLCLLSALLLTEGKKPAKP HUMAN
1   ....S.R.................F.VV.......... MOUSE

41  KCPAVCTCTKDNALCENARSIPRTVPPDVISLSFVRSGFT HUMAN
41  ........S.............................. MOUSE

81  EISEGSFLFTPSLQLLLFTSNSFDVISDDAFIGLPHLEYL HUMAN
81  ..................................... MOUSE

121 FIENNNIKSISRHTFRGLKSLIHLSLANNNLQTLPKDIFK HUMAN
121 ..................................... MOUSE

161 GLDSLTNVDLRGNSFNCDCKLKWLVEWLGHTNATVEDIYC HUMAN
161 ............A.......................... MOUSE

201 EGPPEYKKRKINSLSSKDFDCIITEFAKSQDLPYQSLSID HUMAN
201 .............P..................... MOUSE

241 TFSYLNDEYVVIAQPFTGKCIFLEWDHVEKTFRNYDNITG HUMAN
241 ..................................... MOUSE

281 TSTVVCKPIVIETQLYVIVAQLFGGSHIYKRDSFANKFIK HUMAN
281 ..........D.................G..... MOUSE

321 IQDIEILKIRKPNDIETFKIENNWYFVVADSSKAGFTTIY HUMAN
321 .....V.............D.............. MOUSE

361 KWNGNGFYSHQSLHAWYRDTDVEYLEIVRTPQTLRTPHLI HUMAN
361 .........................A.P.LA..... MOUSE

401 LSSSSQRPVIYQWNKATQLFTNQTDIPNMEDVYAVKHFSV HUMAN
401 ............S.......................... MOUSE

441 KGDVYICLTRFIGDSKVMKWGGSSFQDIQRMPSRGSMVFQ HUMAN
441 ..................................... MOUSE

481 PLQINNYQYAILGSDYSFTQVYNWDAEKAKFVKFQELNVQ HUMAN
481 ..................................... MOUSE

521 APRSFTHVSINKRNFLFASSFKGNTQIYKHVIVDLSA    HUMAN
521 ................................... MOUSE
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Fig. 5. Comparison of the amino acid sequence of the human (above) and mouse (below) LGI1 genes. Only the positions where the amino acids were different in the mouse are shown in this comparison.

time to evolve divergently. Primers were then designed that amplified individual exons, and SSCP was performed. Despite the consistent loss of activity in brain tumors, no mutations were detected in the coding region of LGI1. Whether mutations occur in the intronic or extended promoter regions that affect transcription by causing aberrant processing or expression of the LGI1 transcript requires more extensive analysis.

Sequence analysis of the approximately 600 bp upstream of the transcriptional start site failed to identify TATA or CAAT boxes. Luciferase assays, however, demonstrated that this region has promotor activity. This observation is not unique to LGI1, since other members of the LRR family such as PRELP (Grover and Roughly 1998) and the human and mouse biglycan genes (Fisher et al. 1991; Wegrowski et al. 1995) do not have TATA or CAAT boxes either. These genes, however, are able to use SP1 sites which have been shown to be able to drive transcription. In other genes, such as the mouse lumican gene, Sp1 sites facilitate the recognition of a weak TATA box (Ying et al. 1997). LGI1, however, does not have an Sp1 site in the promoter region either, and so it appears that there are other critical elements in the promoter region that drive transcription. In the normal brain, these are presumably strong promoter elements, since expression levels are high. A more detailed study of individual elements of the promoter, however, was beyond the scope of this study. The LGI1 gene is expressed almost exclusively in the brain, and so the characterization of this promoter may provide a useful tool in directing transgene expression to the brain during development.

Most GBM contain only one genomic copy of LGI1, and this one is almost invariably not expressed (Chernova et al. 1998). Since mutations do not appear to be the cause of this inactivation, we investigated whether transcription was regulated by methylation as has been shown for tumor suppressor genes such as the RBI gene in retinoblastoma (Greger et al. 1994), the elongin gene in VHL-related tumors (Graff et al. 1997), and the p16 gene in melanoma (Lo et al. 1996). In gliomas and GBMs, expression of the GFAP (Fukuyama et al. 1996) and PTEN (Furnari et al. 1997) genes has also shown to be blocked by methylation.

Addition of 5-azacytidine to the cultures of GBM-derived cell lines that did not express LGI1 failed to reactivate it, suggesting that methylation is not responsible. This observation was complemented by the fact that methylation-sensitive enzymes failed to show differences in banding patterns on Southern blots containing normal brain and gliomas. A comparison between tumor cells and whole brain, which is a complex tissue, however, would not identify more subtle differences in methylation status that might be present in individual cell types. Exactly how this gene is inactivated, therefore, is not clear, although one possibility is that chromosome or gene rearrangements, which occur in 20–25% of tumors (Chernova et al. 1998) as determined by the relatively crude approach of Southern blotting, cause inactivation as a result of a position effect. We previously described a cell line, CCF4 (Chernova et al., 1998), which contained a single cope of Chr 10 where the region containing LGI1 was translocated into the pericentromeric 11q region. No mutations in LGI1 were found in this cell line, but mRNA expression was suppressed. It appears, in this cell line at least, that the translocation event is related to the inactivation of LGI1. FISH analysis of several other cell lines (Chernova et al. in preparation) also demonstrates rearrangements involving the single remaining copy of Chr 10, suggesting that this may be a more common mechanism than expected. The other alternative is that LGI1 is part of a highly regulated pathway where inactivation of other key members or high specific transcription factors results in either inactivation of all genes in the pathway or a failure to initiate transcription. A better understanding of whether this is the case will depend on a more complete understanding of the function of LGI1.

Since functional inactivation of LGI1 occurs during the transition of low-grade to high-grade brain tumors, the creation of either knockout or transgenic mice that overexpress LGI1 may provide insights into the role of this gene in tumorigenesis. It was necessary, therefore, to establish the mouse cDNA sequence and investigate the genomic structure. Human and mouse LGI1 are highly conserved, showing 91% identity at the nucleotide level and 97% similarity at the amino acid level, with most of the amino acid substitutions being conservative. The mouse *lgi1* gene is 4.2 kb in length, while human LGI1 is 2.2 kb in length. This difference in size between the human and mouse gene is owing to the inclusion of a 2-kb sequence in the 3′untranslated region in the mouse gene. Whether this additional sequence affects gene expression is not clear. The conservation between these genes is not restricted to the coding sequence, since an analysis of the genomic sequence demonstrates that both the position and number of exon/intron boundaries is also similar in humans and mice. In addition, several of the intronic primer pairs that were designed to amplify the individual human exons, also amplify mouse-specific exons, indicating that the intronic sequences in the proximity of the splice sites are also conserved. The high degree of LGI1 conservation between mice and humans implies that this gene has experienced a strong selection pressure. It is intriguing to speculate that any major deviations in the primary protein sequence may result in a loss of function of this gene product. Total or partial loss of the LGI1 gene function could, therefore, be lethal, which in turn implies that LGI1 plays an important role in normal brain development as well as in tumor formation, making further analysis of this gene a high priority.

Acknowledgments. We thank Karen Toil for her help in the preparation of this manuscript and Drs. D. Miller, G. Barnett, and J. Hahn for providing tumors. This work was supported by grant R01 NS35791 from NIH and in part by the Rose-Ella Burkhardt endowment fund.

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