A splice variant acquiring an extra transcript leader region decreases the translation of glutamine synthetase gene

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The expression of glutamine synthetase (GS), catalysing the ATP-dependent conversion of glutamate and ammonia into glutamine, is transcriptionally and post-transcriptionally regulated. The genomic structure of dog GS shown in the present study is basically similar to that of other mammals in that it is composed of seven exons and six introns. Using 5′-cRACE (where cRACE stands for circular rapid amplification of cDNA ends) and reverse transcriptase–PCR, we identified an additional exon (120 bp) in the first intron, designated in the present study as exon 1′. By means of alternative splicing, the GS gene produces an altered form of GS transcript with 5′-untranslated region (UTR) containing the exon 1′. This alternative transcript is abundantly expressed in brain, whereas it is found at lower levels in other tissues. In the human and mouse GS genes, extra exons are also found at the corresponding site of the intron 1 but with different sizes. An exon-trapping experiment for the GS gene in COS-7, Madin–Darby canine kidney and SK-N-SH cells revealed that the pattern of alternative splicing is variable in different cell types. The propensity of forming a secondary structure is predicted to be considerably higher in the presence of extra 5′-UTR, suggesting the possibility of a translational effect. To test this, we performed a reporter assay for fusions with different 5′-UTRs, demonstrating that the long form with extra 5′-UTR was translated 20- and 10-fold less than the short one in SK-N-SH and Neuro-2A cells respectively. Similarly, translations of human and mouse transcripts with extra 5′-UTRs were less efficient, showing 6–8-fold reductions in SK-N-SH cells. Furthermore, when we mutated an ATG sequence contained in the exon 1′, the suppression of translation was partially relieved, suggesting that the negative regulation by an extra 5′-UTR is, to some extent, due to an abortive translation from the upstream ATG.

Key words: alternative splicing, glutamine synthetase, translational regulation, untranslated region.

INTRODUCTION

Glutamine synthetase (GS; EC 6.3.1.2; L-glutamate:ammonia ligase) catalyses the ATP-dependent conversion of glutamate and ammonia into glutamine, which is critical in nitrogen metabolism, neurotransmitter recycling and energy metabolism in animals. In mammals, it is the only enzyme capable of synthesis of glutamine *de novo* [1]. In the central nervous system, synaptically released glutamate is taken up by glial cells, where the conversion into glutamine occurs. The converted glutamine is subsequently re-used for the synthesis of the glutamate neurotransmitter as a precursor [2,3]. The diverse cellular roles of GS are achieved by spatiotemporal regulation of GS expression in a number of organs, as characterized extensively in liver cells [4]. The GS gene has been considered as a causative agent for many neurological diseases including neuronal degeneration [5], Alzheimer's disease [6–8], schizophrenia and Parkinsonism [9].

The mammalian GS gene is approx. 10 kb in length, organized into seven exons and six introns, and transcribed into mRNAs of 2.8 and 1.4 kb with variable 3′-ends [10–12]. In the human genome, there is one functional gene (GLUL located at 1q23–q25), one processed pseudogene (GLULP at 9p13) and three uncharacterized homologues (GLULL1, GLULL2 and GLULL3 at 5q33, 11p15 and 11q24 respectively) [13,14]. These genes lack introns and share 88–92 % nucleotide sequence identity in their coding sequences with GLUL. Until now, three major regions are reported to be involved in the transcriptional regulation of the rat GS as studied in hepatocytes. The first one is located at approx. 2 kb upstream of the transcriptional start site [15–18], whereas the second region is in the 3′-untranslated region (UTR) of GS mRNA [4]. The third region is within the first GS intron [15,19], in which there are three active centres positioned at approx. +350, +700 and +1150 bp from the transcriptional start [4]. The GS has also been regulated at the post-transcriptional level by decreasing the stability of its mRNA in the presence of cAMP [20]. In addition, the enzyme becomes unstable with the metal-catalysed or free radical peptide (generated by fragmentation of β-amyloid)-mediated oxidations and with growth hormone and glutamine [4]. However, none of the previous studies has focused on the translational regulation associated with splicing.

Alternative splicing is a source of creating a genomic complexity not only for protein function but also for post-transcriptional regulation. The molecular plasticity of a gene is achieved by alternative splicing, affected by combinatorial interplay of both intronic-conserved sequence and exonic splicing enhancer/silencer, which are recognized by spliceosome [21]. An extra 5′-UTR acquired by splicing has been implicated in translational regulation, e.g. proinsulin [22], GLI1 [23] and heparan sulphate/heparin GlcNAc *N*-deacetylase/*N*-sulphotransferase 2 [24]. Furthermore, an alternative promoter usage could also generate multiple mRNA transcripts differing in their 5′-UTRs [25].

The 5′-UTR is known to be involved in the regulation of translation by varying its sequence, length and secondary structure [26–30]. The formation of an extensive secondary structure

Abbreviations used: GS, glutamine synthetase; MDCK, Madin–Darby canine kidney; ORF, open reading frame; cRACE, circular rapid amplification of cDNA ends; RPA, RNase protection assay; RT, reverse transcriptase; SV40, simian virus 40; uATG, upstream ATG; UTR, untranslated region.

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[2] The mRNA and partial genomic sequence containing introns 1 and 2 of the dog GS gene have been deposited in the GenBank® Nucleotide Sequence Database under the accession numbers AF544242 and AF544243 respectively.

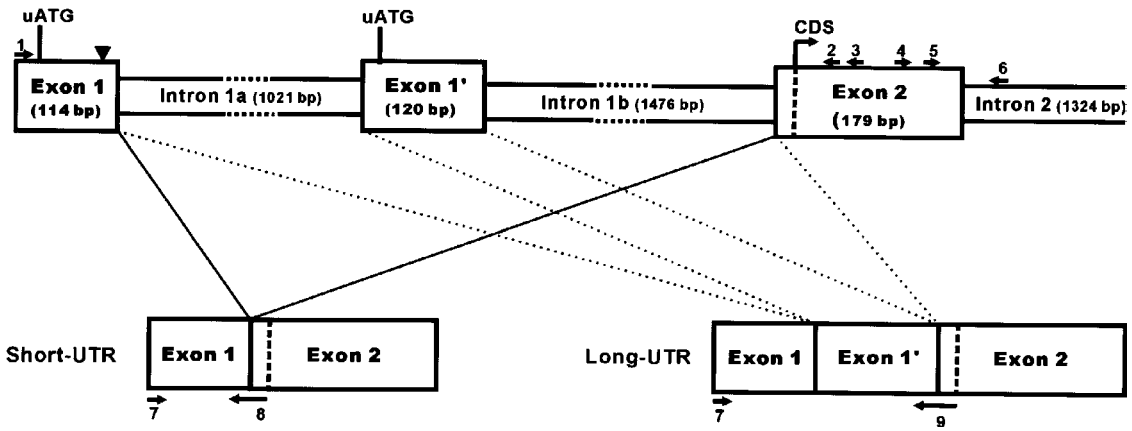

Figure 1 Schematic drawing of the partial genomic structure of dog GS generating an alternative 5′-UTR

The partial genomic structure of dog GS, around exons 1 and 2, are shown with its alternative transcripts, designated here as the short and long UTRs. Relevant exons and introns are presented. ——, Splicing of the short UTR; · · · · · ·, splicing of the long UTR. Arrows with numbers represent primers used. Positions of uATGs and authentic coding sequences (CDS) are indicated. The GCCC tetranucleotide repeat (▼) is located at the distal end of exon 1.

involving 5′-UTR could lower the chance of entry or of finding the initiation codon of mRNA for the ribosome [28]. In the c-Jun of chicken as well as in thymidine kinase of human, it was found that the degrees of secondary structure formation involving their 5′-UTRs correlated inversely with the translational efficiency [31,32]. An upstream ATG (uATG) within the leader sequence, associated with an upstream open reading frame (ORF), may also influence the translation of the downstream cistron [30]. A small peptide encoded by an upstream ORF was reported to suppress the translations of the β_2-adrenergic receptor, the type-1A angiotensin II receptor and the corticotropin-releasing hormone type-1 receptor [33–35]. In some cases, the region contains an internal ribosome entry site or its homologue, which may facilitate association of the 40 S ribosome with UTR, thereby enhancing the scanning efficiency of the start codon [36].

During the analysis of the GS gene in dog brain, we found an alternative transcript containing an extra 5′-UTR. A similar splicing event of GS also occurs in human and mouse brains. The degree of alternative splicing is variable in different species and cell types. The extra 5′-UTR shows a negative effect on protein translation in transfected cells, providing an additional mechanism for the regulation of GS expression.

EXPERIMENTAL

Primers and DNA sequencing

All primers used in the present study were synthesized by Genotech Co. (Taejon, Korea), unless otherwise stated. All PCR products were separated by agarose or PAGE and visualized with ethidium bromide. The separated fragments were purified using QIAquick Gel Extraction kits (Qiagen) and sequenced directly using the Big Dye termination kits for the ABI-Prism 3100 DNA sequencer (PerkinElmer).

Tissue and RNA preparations

Tissues from the frontal lobe, parietal lobe, occipital lobe, medulla oblongata, thalamus, cerebellum, heart, skeletal muscle and kidney of dog (*Sapsari*, a Korean breed) [37] were obtained from an adult animal in a *Sapsari* colony at Kyungbook province (Korea), with the approval of the University Animal Care and Use Committee, Kyungbook National University of Veterinary Medicine (Taegu, Korea) and by the Committee at Korea Advanced Institute of Science and Technology (KAIST; Taejon, Korea). The mouse brain was obtained from an adult animal of the ICR strain (a well-known outbred mouse), which was purchased from Daehan Breeding Center Co. (Seoul, Korea) and housed under controlled temperature and lighting (22 °C; 12 h light–dark cycle) with free access to food and water, according to the NIH guidelines for the care and use of laboratory animals. The human brain tissue was a mixture of samples from the parietal lobe, cortex and white matter, kindly provided by Chong-Jai Kim (Seoul National University College of Medicine, Seoul, Korea), with the informed consent of a 16-year-old male who died of primitive neuroectodermal tumour in the chest wall and with the approval of the Ethical Committee of Seoul National University Hospital. Total RNAs for reverse transcriptase (RT)-PCRs were prepared from the tissues or cell lines by the single-step method [38] or by RNeasy mini kits (Qiagen). mRNAs for 5′-cRACE (where cRACE stands for circular rapid amplification of cDNA ends) were purified using the Oligotex mRNA mini kits (Qiagen).

RT-PCR

First-strand cDNA was synthesized from 5 μg of total RNA primed with oligo(dT)$_{30}$ using SuperScript™ II/RNase H$^-$ reverse transcriptase (Invitrogen) in a total volume of 20 μl, and then used as a template for subsequent PCR. Each PCR was repeated at least twice with different RNA preparations including a negative control for each set of reactions. Primers 1 (Figure 1) and GSe3-r1 (positioned in the exon 3 and directed upstream) were used for semiquantitative RT-PCR of dog GS, and the primers hGSe1-f1 (5′-GTGAGCAGTACTGCGGCCTC-3′ in exon 1) and hGSe3-r1 (5′-TCGAAATTCCACTCAGGCAACT-3′, exon 3) were used for semiquantitative RT-PCR of human GS. Similarly, the primers mGS-f (5′-GAACAGCACGCTC-ACCCATC-3′, exon 1)/mGS-r (5′-AACTTGCTGAGGTGGCC-ATG-3′, exon 2) and mGS120-f1 (5′-TTGCTTAGACCAGCG-AGAAATG-3′ in the putative exon 1′)/mGS120-r1 (5′-CATTT-CTCGCTGGTCTAAGCAA-3′ in the putative exon 1′) were used for the mouse GS. Luciferase forward primer was 5′-GATAC-GCCCTGGTTCCTGG-3′ and reverse primer was 5′-CTGAC-GCAGGCAGTTCTATG-3′.

Northern-blot hybridization

Northern-blot analyses with 10 μg of total RNAs from dog tissues were performed as described by Sambrook and Russell [39]. The probes were prepared as the [α-^{32}P]dCTP PCR products for exon 3 of dog GS (162 bp) and the dog glyceraldehyde-3-phosphate dehydrogenase (508 bp).

RNase protection assay (RPA)

A 348 bp fragment containing 120 bp of exon 1′, 179 bp of exon 2 and 49 bp of exon 3 was subcloned into pBluescript II SK(+) (Stratagene). The 432 nucleotides of the full-length [α-^{32}P]CTP antisense probe were generated using T7 RNA polymerase (Takara, Osaka, Japan). Hybridization was performed with the gel-purified probe (10^5 c.p.m.) for 24 h at 42 °C with 25 μg of total RNAs from dog tissues. The assay was performed using the RPA III™ kit (Ambion).

5′-cRACE

The 5′-cRACE experiment was performed by the method of Maruyama et al. [40]. The primers of 2, 3, 4, 5 and GSe3-r1 were designed based on the dog GS ORF sequence (GenBank® Nucleotide Sequence Database accession no. AF544242; Figure 1). The first-strand cDNA was synthesized from 0.5 μg of mRNA by the SuperScript™ II/RNase H$^-$ reverse transcriptase (Invitrogen) and the GSe3-r1 primer phosphorylated by T4 polynucleotide kinase (Takara). After the reverse transcription, NaOH was added to a final concentration of 0.5 M, and the reaction was incubated at 37 °C for 10 min to hydrolyse the template mRNA. cDNA was then precipitated with ethanol and redissolved in 10 μl of a reaction mixture containing 25 % (w/v) poly(ethylene glycol) (molecular mass 6000 Da), 0.1 % BSA, 1 × T4 RNA ligase buffer and 50 units of T4 RNA ligase (Takara). This reaction mixture was incubated at 16 °C for 18 h. The ligation mixture of 5 μl was then used as a template for the first PCR amplification. The first PCR was set up in a total volume of 25 μl containing 1 × *Taq* polymerase buffer, 400 μM dNTP, 5 units of *Taq* polymerase, 400 nM primer 3, 400 nM primer 4 and 1 M betaine. The reaction was performed for 35 cycles under the following conditions: 1 min at 94 °C, 35 cycles of 20 s at 94 °C, 40 s at 58 °C and 2 min at 72 °C, and 5 min at 72 °C. The resulting PCR product was used as a template for nested PCR. The nested PCR was done with the same reaction mixture except that the 1 μl of template from the first PCR product was used with the primer pairs 2 and 5. The reaction condition for nested PCR was the same as the first PCR except that the amplification cycle was decreased to 20. The final product of the nested PCR was separated on an agarose gel, eluted and sequenced.

Plasmid construction and site-directed mutagenesis

Each of the two alternative 5′-UTRs of dog GS was generated by RT-PCR using primers 7 and 8 or primers 7 and 9 (primer 7, 5′-GGAAGCTTAGAGCCGAGAATGGGAG-3′, primer 8, 5′-GGAAGCTTGGTGGAAGTTGTTCTGGG-3′ and primer 9, 5′-GGAAGCTTGGTGGAAGTTGTTCCCAG-3′, each of which contain 5′-*Hin*dIII restriction sites. The PCR products were digested with *Hin*dIII, purified on gel and subcloned into the *Hin*dIII site of pGL2-promoter vector (Promega). These constructs were sequenced for confirmation and named pGL2P-short and pGL2P-long. Similarly, 5′-UTRs of mouse and human GSs were amplified by RT-PCR and cloned into the pGL2 plasmid. The two ATG codons in 5′-UTR of dog long GS were mutated to TTG sequentially or independently with site-directed primers, as described by Sambrook and Russell [39]. The two uATGs, spaced only 6 bp, in exon 1′ of human GS were altered to TTGs simultaneously. The single uATG of mouse GS was also mutated to TTG. The amplified fragments were subcloned into the *Hin*dIII site of pGL2-promoter vector and sequenced. These constructs were used for the reporter assay.

The splice donor and acceptor sequences of pSPL3 vector (Invitrogen) were amplified by PCR with primers *Kpn*I22 (5′-**GGTACC**ATTCCAGAAGTAGTGAGGAGGC-3′) and *Apa*I19 (5′-**GGGCCC**CACTGCATTCTAGTTGTGG-3′), which contain *Kpn*I and *Apa*I sites (shown in boldface) respectively. The amplified fragment was digested with *Kpn*I and *Apa*I, and subcloned into the corresponding site of pcDNA3.1(+) (Invitrogen), called pcDNAclone. The genomic DNA for dog GS was obtained from the cosmid library by PCR with the primers 1 and 6, covering approx. 3 kb. The PCR product was purified on gel and subcloned into the *Not*I and *Eco*RV sites of pSPL3 or pcDNAclone. The construct derived from pSPL3 was used for the exon-trapping analysis in COS-7 cells. The construct derived from pcDNAclone was used in Madin–Darby canine kidney (MDCK) and SK-N-SH cells. The genomic DNA for human GS was amplified by PCR with the hGSe1-f1 and hGSe3-r1 primers, generating approx. 4.5 kb DNA, and was subcloned into the *Not*I and *Eco*RV sites of pcDNAclone. The partial mouse GS gene, spanning approx. 3.5 kb, was amplified with the mGS-f and mGSint2-r1 (5′-TTGTTGCTGCGCCTCCAGAC-3′, in the intron 2), and ligated into the same sites of pcDNAclone.

Cell cultures and transfection

The animal cell lines of COS-7 (monkey kidney), MDCK (dog kidney), SK-N-SH (human brain neuroblastoma) and Neuro-2A (mouse brain neuroblastoma) were used. The cells were plated on 6-well dishes (35 mm wells) for transfections. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) in a humidified atmosphere with 5 % CO_2 after the addition of 10^5 units/l penicillin, 100 mg/l streptomycin, 0.25 mg/l fungizone and 10 % (v/v) foetal bovine serum (Invitrogen). LIPOFECTAMINE™ Plus reagent (Invitrogen) was used for transfection.

Exon-trapping experiment

Exon-trapping analysis was carried out with the Exon-Trapping System (Invitrogen), following the manufacturer's instructions. Briefly, total cellular RNA was harvested after 36 h of transfection with pSPL3 or pcDNAclone derivatives as described above. RT–PCR was performed with the primers SD6 (5′-TCTGAGTCACCTGGACAACC-3′) and SA2 (5′-ATCTCAGTGGTATTTGTGAGC-3′), recognizing the exonic sequences contained in the vector, and the product was separated on polyacrylamide gels and visualized with ethidium bromide.

Luciferase and β-galactosidase assay

The constructs in pGL2-promoter and pSV-β-galactosidase control vectors (Promega) were co-transfected into MDCK, SK-N-SH and Neuro-2A cells. All cells were harvested in the reporter lysis buffer (Promega) after 24 h of transfection. The luciferase activities were measured using the luciferase assay reagent (Promega) and the luminometer (Microlumat LB96P; EG&G Berthold, Bad Wildbad, Germany). The β-galactosidase activities in the reporter lysis buffer were measured with a spectrophotometer using the β-galactosidase enzyme assay system (Promega).

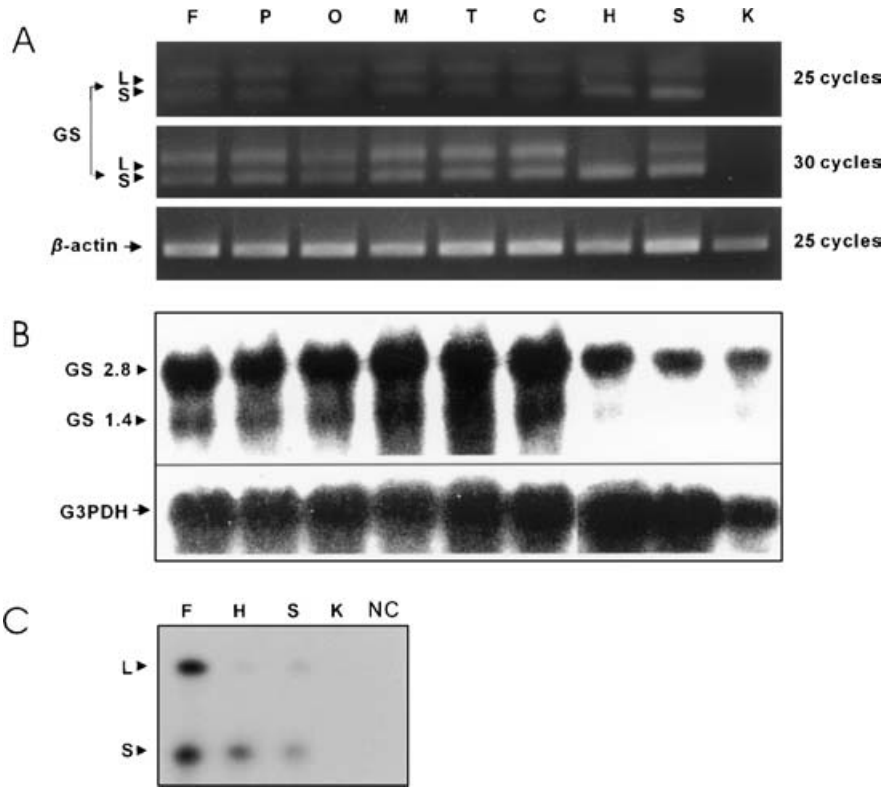

Figure 2 Tissue-specific expressions of the alternative transcripts of dog GS gene

(**A**) Results of semiquantitative RT-PCRs for the dog GS transcripts are shown for nine different dog tissues: F, frontal lobe; P, parietal lobe; O, occipital lobe; M, medulla oblongata; T, thalamus; C, cerebellum; H, heart; S, skeletal muscle; K, kidney. The primers of 1 (Figure 1) and GSe3-r1 are used. The short form (S) is 342 bp in size, and the long form (L) 462 bp. Two kinds of amplification conditions were performed (25 and 30 cycles) and the β-actin transcript was used as the control. (**B**) Northern-blot hybridization to exon 3 of dog GS and to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) for total RNAs from dog tissues. The bands for the GS mRNA are approx. 2.4 and 1.4 kb in size, due to their differences in polyadenylation [10,11]. (**C**) The RPA was performed with 25 μg of total RNA from the frontal lobe (F), heart (H), skeletal muscle (S) and kidney (K). The yeast RNA was used as a negative control (NC). The antisense riboprobe was complementary to the entire exon 1′ (120 bp), exon 2 (179 bp) and the contiguous 49 bp of exon 3.

The β-galactosidase activities were used to standardize the expression of luciferase activities. Total cellular RNAs were prepared from the above cell lysates, which were used for semiquantitative and real-time RT-PCRs.

Real-time RT-PCR

Total RNAs from the cell lysates were reverse-transcribed with oligo(dT)$_{30}$, and the resulting cDNAs were amplified using the LightCycler-DNA Master SYBR Green I (Roche) in which the PCR products were detected in real time. The copy numbers of luciferase and β-actin mRNAs were calculated after obtaining standard curves for the pGL2-promoter vector and the pBluescript II SK(+) harbouring β-actin respectively. In this analysis, plasmid DNA standards were tested over the range of five orders of magnitude, showing excellent linearity (correlation coefficient: $r^2 = 0.998$). The value of luciferase mRNA was divided by that of β-actin, so that the results were reported as a ratio relative to each control sample.

RESULTS

Alternative splicing in the 5′-UTR of dog GS gene

The dog GS gene was shown to have seven exons and six introns, spanning approx. 8 kb, by PCR and sequence analysis of the cosmid harbouring the dog GS gene (D. Shin, unpublished work). A similar organization was found in other mammals [10–12], with an initiation codon (ATG) residing in exon 2 (Figure 1). When 5′-cRACE was performed for mRNA from dog brain to characterize the 5′-UTR of GS gene, it was found that there were two different species of 5′-UTR. The result was confirmed by performing RT-PCR with the primer pairs designed from the results of 5′-cRACE and by sequencing the amplified fragment. The sequences of both 5′-UTRs were identical except that a 120 bp insertion originated from the first intron (GenBank® accession no. AF544243). This 120 bp sequence has the splicing consensus of 'AG' and 'GT' at its 5′ and 3′-ends respectively. Thus it is probable that the novel species of 5′-UTR was generated by an alternative splicing of intron 1. This 120 bp sequence is termed exon 1′, which is contained in the long form of 5′-UTR (Figure 1).

Tissue-specific variation of the dog GS transcripts

To examine variation in the splicing of GS, semiquantitative RT–PCRs were performed for RNAs from nine different dog tissues (Figure 2A), using the primers 1 and GSe3-r1 (see the Experimental section). We obtained the expected sizes of 342 bp (S) and 462 bp (L) for short and long UTRs respectively, indicating that the GS is highly expressed in the brain, heart and skeletal muscles, whereas it exists in relatively lower amounts in the kidney. It was noted that the amounts of each species are similar in the brain, but not in other tissues, such that the short form is much more abundant, which is consistent with the results

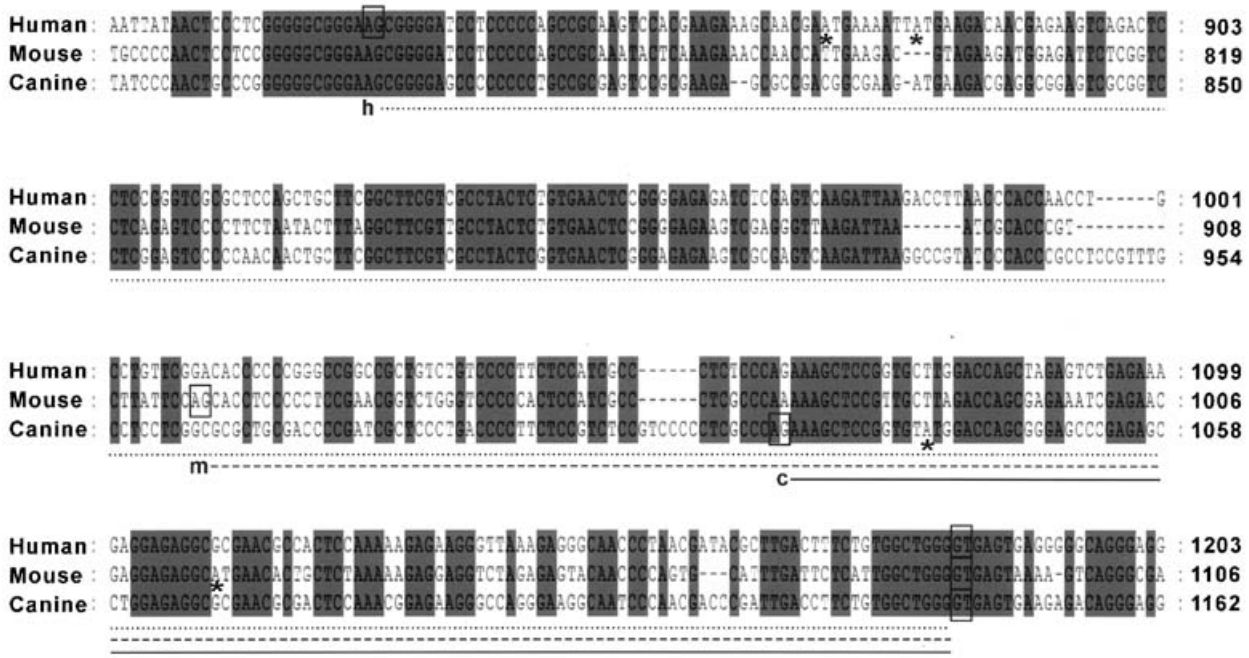

Figure 3 Alignment of sequences around exon 1′

The human, mouse and dog sequences for exon 1′ are aligned using CLUSTAL W [44]. The nucleotides are numbered from the start of intron 1, with conserved nucleotides shaded in grey. · · · · · ·, Human exon 1′; ------, mouse exon 1′; ——, dog exon 1′. The consensus 5′ (GT) and 3′ splice sites (AG) are boxed. The 5′ splice sites are conserved, whereas the 3′ splice sites are variable, resulting in the difference in size of exon 1′. The uATGs are marked with an asterisk underneath.

of RPA (Figure 2C). The expression levels of GS in various tissues were confirmed by Northern-blot hybridizations (Figure 2B). The dog GS transcripts were detected as a major band of 2.8 kb in size and a minor one of 1.4 kb, as reported in other mammals [10,11].

Alternative splicings in the human and mouse GS genes

When the intron 1 sequences of dog, human and mouse GSs were compared, a highly homologous region corresponding to the dog exon 1′ was found (Figure 3), implying that exon 1′ is likely to be present in other mammalian species. To confirm the presence of exon 1′ in human GS, we performed RT-PCR with total RNA from human brain with primers recognizing exon 1 (hGSe1-f1) and exon 3 (hGSe3-r1) of the human GS gene. In the present study, two PCR products of 283 and 639 bp in size were obtained (Figure 4A, lane H). This indicates that the long transcript (L) containing exon 1′ exists, but is expressed at lower levels than the short one (S). By sequencing the PCR products, we were able to characterize exon 1′ of human GS which was 356 bp in size. Coincidently, the GenBank® has reported a sequence (gi:18548260) of GS mRNA containing exactly the same extra UTR.

For mouse, we conducted RT-PCR with total RNA from the mouse brain. In the present study, the primer pair of mGS-f (residing in exon 1) and mGS-r (in exon 2) generated only a single product (114 bp; Figure 4B, lane 1). However, we obtained products of 164 and 140 bp with primers (mGS120-f and mGS120-r) designed to recognize the putative exon 1′ of the mouse gene (Figure 4B, lanes 2 and 3), indicating that the sequence of exon 1′ is transcribed. The mouse exon 1′ was characterized further by PCR analysis as a 168 bp DNA.

Despite the variation in size of exon 1′ in different species, the splicing in favour of the long transcript is generally suppressed. The degrees of suppressions are variable depending on species, at least in brain tissues, implying that the expression of the long UTR may be regulated by a species-specific splicing mechanism. When we compared the splicing junctions of different exon 1′s, it was found that they showed a significant variation in their sequences of 3′ splicing acceptor, which appear to be responsible for alteration in splicing efficiency (Figure 3).

Splicing variation was confirmed with the exon-trapping system in different cell types

To demonstrate the splicing of GS from a clone containing the dog GLUL genome, we conducted the exon-trapping experiments using COS-7, MDCK and SK-N-SH cell lines. The genomic fragment of dog GS of approx. 3 kb, amplified by PCR from exon 1 (primer 1) to intron 2 (primer 6), was cloned into pSPL3 or pcDNAclone with the exon-trapping cassette (see the Experimental section for details), which were transfected into the three cell lines. After 36 h of incubation, total RNA was prepared and subjected to RT-PCR with SD6 and SA2 primers recognizing the exonic cassette in the vector. The results confirm the pattern of alternative splicing demonstrated by RT-PCR (above) and also indicate that the levels of the long transcripts varied in different cell types. The level of transcript containing exon 1′ (21.8 % of the total as in Figure 5A) is higher in MDCK compared with that in SK-N-SH (16.6 %). The splicing in SK-N-SH appears to be slightly more efficient than that in COS-7. Similarly, the human genomic fragment for GS, comprising exon 1 (hGSe1-f1) to exon 3 (hGSe3-r1) of approx. 4.5 kb, was cloned into pcDNAclone and transfected into MDCK or SK-N-SH cells. In this case, the splicing towards the long transcript was more efficient in SK-N-SH cells (20.5 %) than in MDCK cells (13.3 %; Figure 5B). Exon-trapping experiment with the mouse GS, containing exon 1

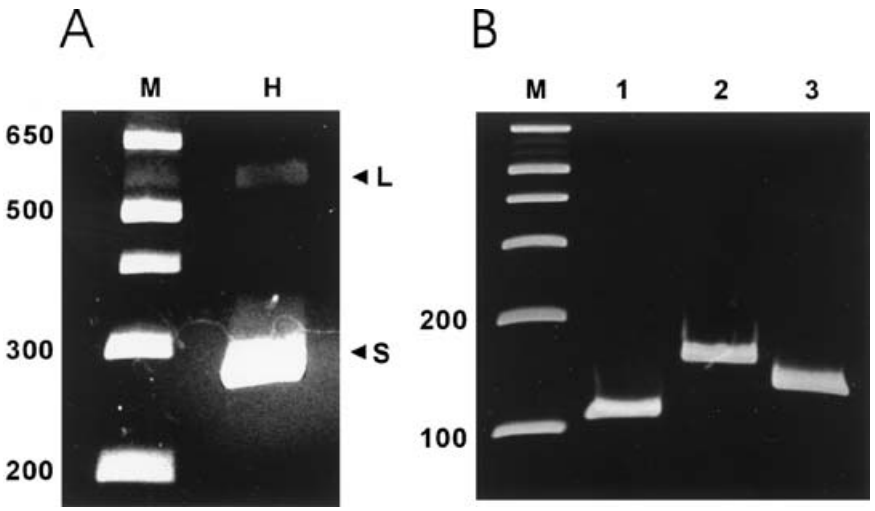

Figure 4 Alternative splicings in the human and mouse GS genes

(**A**) RT-PCR was performed for the sample of human brain, the mixture of parietal lobe, cortex and white matter. The short form (S; 283 bp) is much more abundant relative to the long one (L; 639 bp). Lane M is the DNA marker with size in base pairs. (**B**) Results of RT-PCR for the whole mouse brain sample. The 114 bp band in lane 1 was obtained with a primer pair of mGS-f (for the exon 1) and mGS-r (the exon 2), and the one (164 bp) in lane 2 with primers of mGS-f and mGS120-r1 (the putative exon 1′). The band (140 bp) in lane 3 was obtained with the primers of mGS120-f1 (the putative exon 1′) and mGS-r. Since the long form of mouse GS was not detected in lane 1, the RT-PCRs for lanes 2 and 3 were almost saturated. Lane M is the size marker.

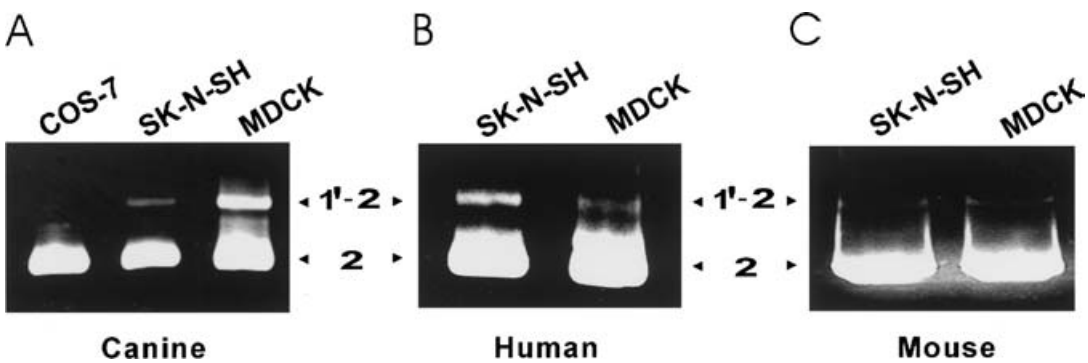

Figure 5 Dependence of alternative splicing on cell type

The splicings of dog GS introns were analysed in COS-7, SK-N-SH and MDCK cells (**A**) using the exon-trapping system. The levels of the long forms relative to the short ones are different according to cell type. An exon-trapping analysis was conducted for the human (**B**) and mouse (**C**) GS introns in SK-N-SH and MDCK cells. Types of the resulting exons are designated in the middle; exon 1′ joined with exon 2 (1′–2) and the exon 2 alone with the exon 1′ skipped (2).

to intron 2 of approx. 3.5 kb, demonstrated similar degrees of splicing in SK-N-SH (4.9 %) and MDCK cells (4.8 %; Figure 5C) to generate the long UTR, implying a species-specific variation in GS splicing.

Translational effect of the extra 5′-UTR

5′-UTR has been suggested to be involved in the regulation of translation [26–30]. A preliminary analysis of RNA secondary structure [41] for the two different transcripts of dog GS revealed that the secondary structure of the long form has an estimated free energy of − 104.5 kcal/mol (1 kcal = 4.184 kJ), which is much lower than that of the short form (− 43.3 kcal/mol in Table 1). In addition, the longer transcript has an ATG at its 5′-UTR. The 5′-UTRs of human and mouse GSs also share these features, which are summarized in Table 1.

To assess the effect of the extra 5′-UTR on translation, the two different 5′-UTR fragments of dog GS were cloned into the simian virus 40 (SV40) promoter contained in pGL2 downstream.

Table 1 Propensity of forming a secondary structure involved with the extra 5′-UTRs of GS genes

Origin of GS	Size of 5′-UTR (bp)	No. of upstream ATGs (location)*	Free energy (kcal/mol)†
Dog short form	127	1 (− 117)	− 43.3
Dog long form	247	2 (− 120, − 237)	− 104.5
Human short form	109	0	− 54.1
Human long form	465	2 (− 317, − 326)	− 173.5
Mouse short form	105	0	− 17.5
Mouse long form	273	1 (− 83)	− 69.2

* Counted from the translation start to 'A' residue.
† Calculated with the MFOLD program [41].

When transfected into SK-N-SH and Neuro-2A cell lines, the expressions of the short 5′-UTR were found to be approx. 20- and 10-fold higher than those of the long 5′-UTR (Figure 6A), indicating that the extra 5′-UTR has a negative effect. In MDCK

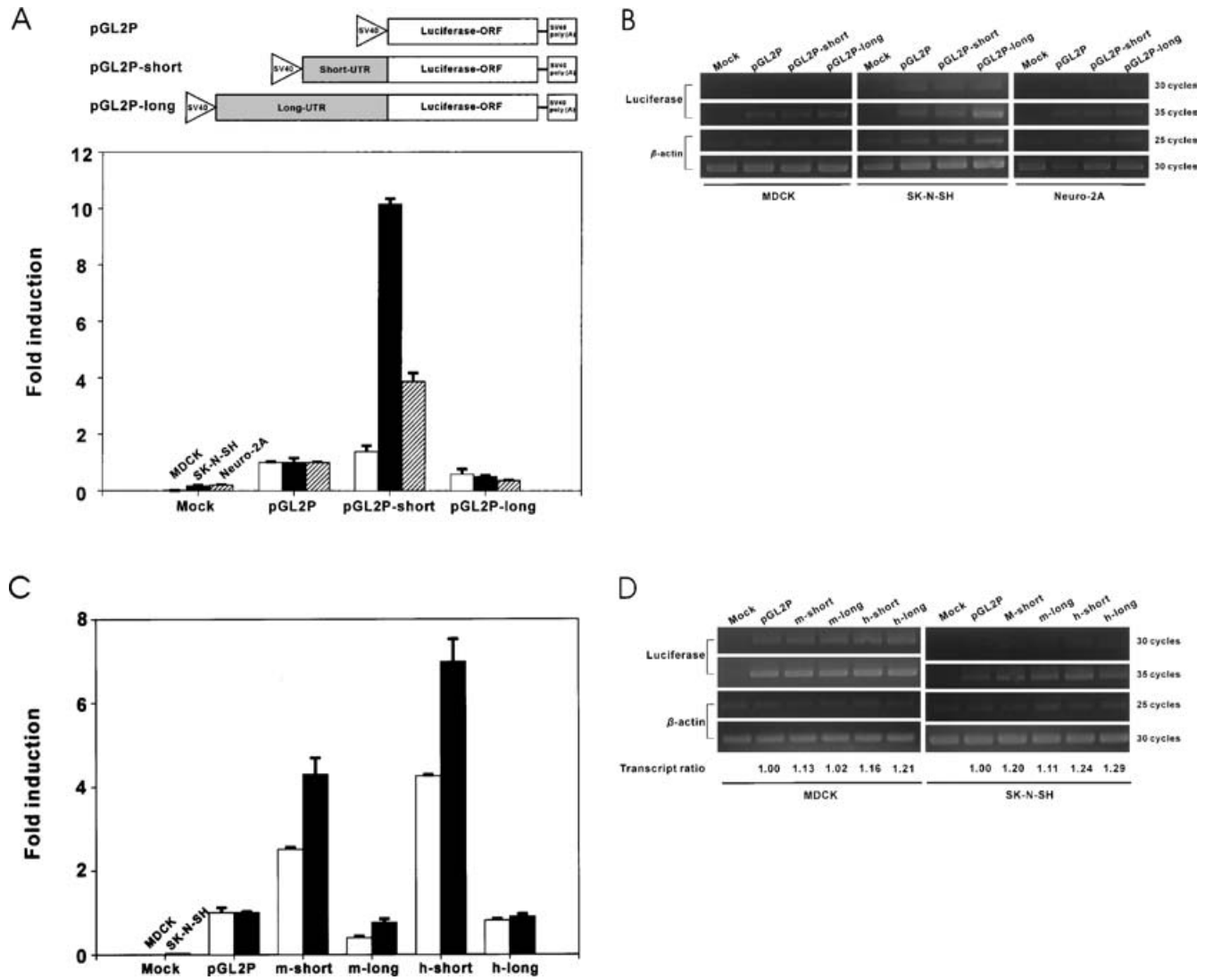

Figure 6 Effects of different GS 5′-UTRs on reporter expression

(**A**) Fold inductions of luciferase activities in MDCK, SK-N-SH and Neuro-2A cells are shown. Samples are the negative control (Mock), pGL2-promoter vector itself (pGL2P), the dog short-UTR construct (pGL2P-short) and the dog long-UTR construct (pGL2P-long). Results are presented as fold induction relative to that of pGL2-promoter vector. Variations in different luciferase experiments are normalized using β-galactosidase activities from the co-transfected pSV-β-galactosidase vectors. Results are expressed as means $\pm$ S.D. from three independent transfections. (**B**) Total RNAs from transfected cells were reverse-transcribed with oligo(dT)$_{30}$, and the cDNA product was used for semiquantitative RT–PCR. Two sets of amplification cycles were performed (30/35 cycles for luciferase and 25/30 cycles for β-actin). (**C**) Luciferase activities of mouse and human UTRs in MDCK and SK-N-SH cells are shown as fold induction relative to pGL2P. Samples are the short UTR of the mouse GS gene (m-short), the long UTR of mouse (m-long), the short UTR of the human GS gene (h-short) and the long UTR of human (h-long). (**D**) Semiquantitative and real-time RT-PCRs were conducted with total RNA from transfected cells. Relative copy numbers of luciferase transcripts were determined by the real-time RT-PCR and are presented relative to that of pGL2P (transcript ratio).

cells, this effect due to the presence of exon 1′ was much lower, perhaps resulting from a difference in cell type. To standardize the transfection efficiencies, the enzyme activities from the co-transfected pSV-β-galactosidase plasmids were used. The possibility of transcriptional effect was ruled out by detecting almost equal amounts of luciferase mRNA by semiquantitative RT-PCR analysis (Figure 6B). The negative effect of the extra 5′-UTR on translation was also observed in the mouse and human GS genes, in which the decreases in expression were approx. 6- and 8-fold in SK-N-SH cells respectively (Figure 6C). Again, the differences were smaller in MDCK cells. Similar levels of luciferase mRNAs were detected in both semiquantitative and real-time RT-PCRs (Figure 6D), implying that the transcriptions were unaffected.

We examined the effect of uATGs on the dog exon 1′ by introducing changes in their sequences without significantly influencing their secondary structures (Figure 7A). The results indicate that the ATG to TTG mutation in exon 1′ has some effect on the expression levels, increasing them by 5–6-fold in SK-N-SH cells compared with wild-type. The effect due to an alteration in the exon 1 ATG was far less, showing only 1.6-fold compared with wild-type. Similar changes in the uATGs in the human and mouse exon 1′ result in slight increases of up to 2-fold (Figure 7B). Although the exon 1′ ATG appears to contribute to the suppression of GS translation, the level of translation in the mutant transcript is still far below that of the short transcript lacking exon 1′, indicating that uATG is not the sole determinant for the translational suppression.

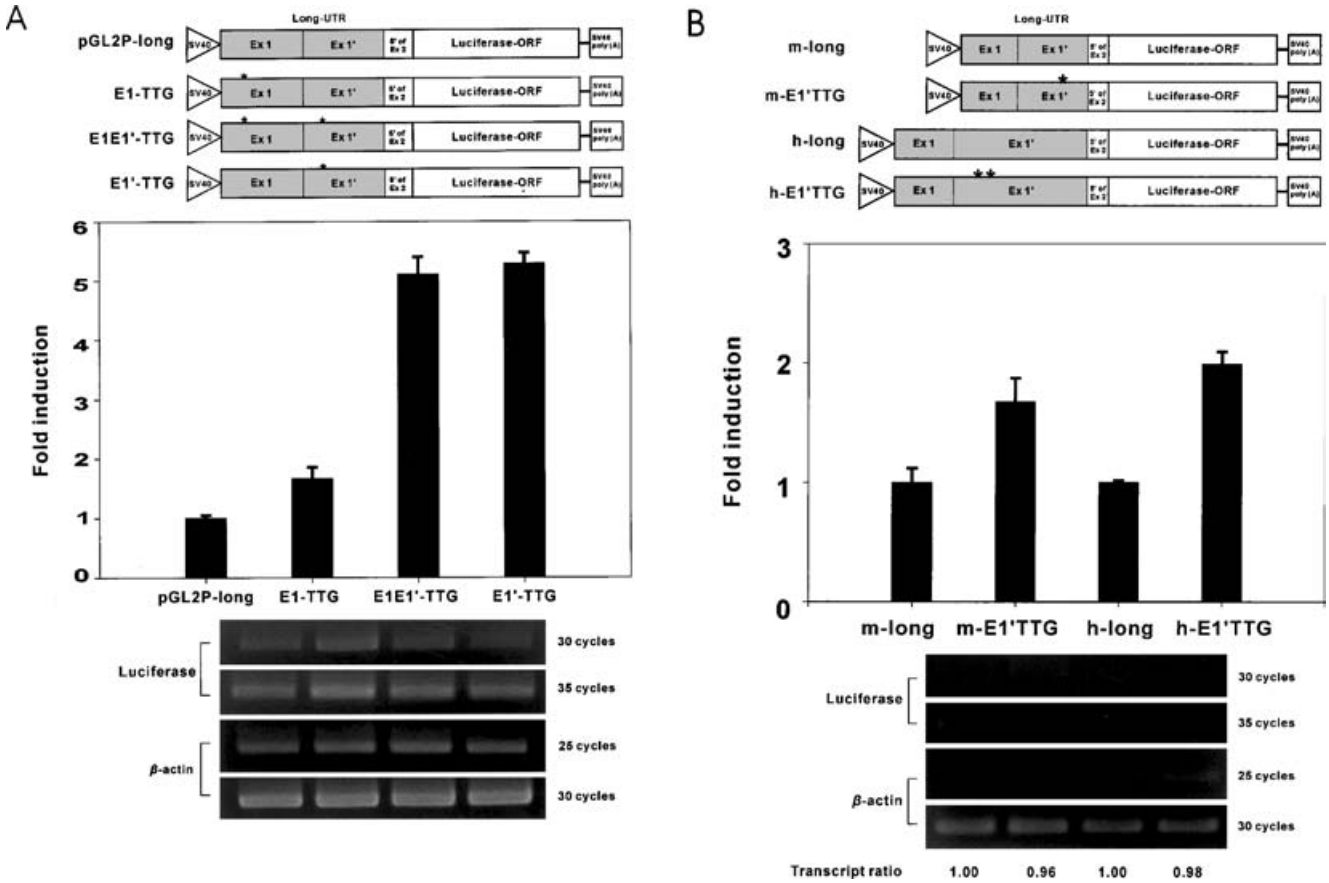

Figure 7 Mutational changes and effects of uATGs on translational suppression monitored by reporter assay

(**A**) Three different mutational constructs of pGL2P-long were generated; an ATG to TTG change in the dog exon 1 (E1-TTG), double changes of ATGs in both exons 1 and 1′ (E1E1′-TTG), and a single ATG change in exon 1′ (E1′-TTG). Mutational constructs were transfected into SK-N-SH cells. Results are presented as fold induction of luciferase activity relative to that of wild-type long UTR (pGL2P-long), after normalization using the β-galactosidase activities derived from co-transfected pSV-β-galactosidase vector as a standard. Results are expressed as means $\pm$ S.D. from three independent transfections. Total RNAs from transfected cells were reverse-transcribed with oligo$(dT)_{30}$, which was used for semiquantitative RT–PCR. Two sets of amplification cycles were performed (30/35 cycles for luciferase and 25/30 cycles for β-actin). (**B**) Similarly, two constructs derived from m-long and h-long sequences were made by changing uATG into TTG in exon 1′, named m-E1′TTG and h-E1′TTG. Luciferase activities were presented as a fold relative to that of the corresponding wild-type long-UTR construct tested in SK-N-SH cells. On the other hand, semiquantitative and real-time RT-PCRs were conducted for the total RNA from the transfected cells. The relative copy numbers of luciferase transcript obtained by the latter are shown (transcript ratio).

DISCUSSION

During the study of GS in dog brain, we found that an alternative GS transcript was generated by RNA splicing. Although the genomic structure of dog GS, consisting of seven exons and six introns, is similar to that of other mammals, no evidence of alternative splicing has been presented so far. In the present study, we demonstrated that the long transcript with an extra 5′-UTR also exists in mouse and human brains. The overall sequence identities among the intron 1s of human (2623 bp after deleting non-homologous internal sequences of 305 bp), dog (2617 bp) and mouse (2663 bp) are in the range of 44–61 %. Similar degrees of identity, between 51 and 62 %, are found in the intron 1a region (approx. 800 bp; Figure 1), upstream of exon 1′ (356 bp in human sequence). In contrast, the degree of similarity in the exon 1′ region (approx. 400 bp) is considerably higher, showing 66–75 % identity among the three species (Figure 3). Nevertheless, the junctions of exon 1′ are quite different, resulting in a size variation of exon 1′ in different species. The size of human exon 1′ (356 bp) is three times larger than that of dog exon 1′ (120 bp). Although the three different exon 1′s share the same 5′ splice sites at the 3′-end of the exon 1′ (Figure 3), sequences of the 3′ acceptor regions are very dissimilar in different species. Thus it is probable that the variation in the 3′ acceptor sites cause a change in the efficiency of alternative splicing. Indeed, considerable differences in splicing were observed among the three species (Figures 2 and 4), although further verification would be necessary to distinguish species-specific differences in splicing machinery.

Alternative splicing is known to be regulated in a cell-type-specific manner [21], as in the case of exon 1′ in the GS gene. The exon-trapping system using several cell lines revealed that the relative level of the long form to the short form is variable in different cell types, such that the level of dog long transcript is much higher in MDCK cells than in SK-N-SH or COS-7 cells. This tendency is reversed in human long transcript, where the processing in SK-N-SH cells is higher than that in MDCK cells. The splicings of the mouse transcripts are nearly identical in both SK-N-SH and MDCK cells. This implies that the splicing efficiency towards the long transcript is, to some extent, species-specific. However, we cannot completely rule out the possibility of an exclusive involvement of a cell-type-specific factor in the alternative splicing of GS.

The monitoring of alternative splicing in different tissues by semiquantitative RT-PCRs indicated that an increased amount of

long UTR is found in the brain and also in skeletal muscle and liver (results not shown), but not in other tissues, in which the short form is more abundant. The levels of GS transcription are also variable in different tissues of dog; the level of GS transcription in the brain is relatively higher than that in the kidney. Unlike dog brain, alternative splicing was barely detected in human and mouse brains, implying that there might be an active role of the long GS transcript exclusively for dog brain. Although the long transcripts of mouse and human GS genes are present in much lower levels than that of dog in our experiments, it is possible that they are spatially or developmentally enriched [4]. Indeed, GS is produced in a restricted region of mouse liver, forming a thin rim of cells around the central veins [16]. The other advantage of having this translationally repressed form of transcript would be that cells could accomplish more immediate regulation of GS expression instead of going through the whole process of transcription.

The free energies of forming secondary structures involved with the long UTRs are also variable. Long UTRs of dog, human and mouse GS have the differences in free energy of -61.2, -119.4 and -51.7 kcal/mol respectively relative to their short UTRs (Table 1). Since we could not find structures showing lower free energies, formed with the scrambled sequences of the same composition and length, it was thought that the lower free energies of long UTRs are inherent in their sequences. It was proposed that a formation of extensive secondary structure between the cap and the authentic ATG codon has a negative effect on the initiation of protein translation [28]. The inhibition of translation due to a stem-and-loop structure involving the 5′-UTR occurs with a free energy range of -50 to -60 kcal/mol. The secondary structure formed in this region prevents 40 S ribosomes from binding to or migration along mRNA. In this regard, the free energy differences resulting from the presence of long UTRs in GS genes are likely to be above the threshold of inhibiting translation.

We found that exon 1′ in the 5′-UTR affected the translation of downstream coding sequence in transfected cell lines. In the present study, the short transcript of GS lacking exon 1′ also enhanced the expression of the reporter enzyme when compared with the control containing only a small spacer sequence (<30 bp) in its 5′-UTR. This difference might result from an increase in leader length between the transcription start and the initiation codon [28], and also from the putative internal ribosome entry site element (GCCC tetranucleotide repeat located at the distal end of exon 1; Figure 1) contained in the short form of 5′-UTR. As a matter of fact, the translational efficiency *in vitro* was increased by the introduction of an unstructured leader sequence, probably facilitating the loading of extra 40S ribosomal subunits [28]. The translational repression by exon 1′ seems partly due to the presence of uATG, which is consistent with a previous study indicating that the long 5′-UTR with uATG had a weak start context [42]. One of the reasons is that a premature translation from uATG may initiate mRNA decay [43]. However, another uATG in exon 1, which is shared by both the short and long forms of GS, had a minor effect on protein translation for reasons unknown. The mutational changes in the ATG contained in the mouse and human exon 1′ exhibited similar degrees of translational enhancement when compared with the change in dog exon 1′.

Besides the post-transcriptional regulation of GS by exon 1′, transcriptional enhancers or suppressors are reported to be contained in the first intron [15,19]. The active centres of regulatory sequences, which are involved in the cell-type specificity of expression, are within the first intron of the rat GS gene at positions of approx. $+350$, $+700$ and $+1150$ from the transcriptional start site [4]. The $+1150$ region overlaps with the exon 1′ sequence and, thus, it may be involved in transcriptional as well in translational regulations. Our findings on translational regulation appear to suggest a novel type of regulatory mechanism relevant to diverse cellular needs of GS activity, particularly in the brain.

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