Genomics **61,** 298–306 (1999)
Article ID geno.1999.5964, available online at http://www.idealibrary.com on IDEAL®

The *Gas7* Gene Encodes Two Protein Isoforms Differentially Expressed within the Brain

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Received April 19, 1999; accepted August 24, 1999

***Gas7*, a growth arrest-specific gene first isolated from serum-starved NIH3T3 cells, is expressed abundantly in the brain and is essential for the outgrowth of neurites from cultured cerebellar neurons. Here, we report the existence of a *Gas7*-related cDNA, designated *Gas7-cb*, isolated from the mouse cerebellum, and we report the finding that *Gas7-cb* transcripts and protein are expressed at different locations than those of *Gas7*. *Gas7-cb* cDNA differs from the *Gas7* cDNA only in the 5′ region. Its encoded protein shares the same 320 amino acids in its C-terminus with those of *Gas7*. Analyses of the RNA and protein expression of *Gas7-cb* and *Gas7* by RNase protection assay and Western blot indicated that while *Gas7* expression is predominant in the cerebrum and in growth-arrested NIH3T3 fibroblasts, *Gas7-cb* expression is predominant in the cerebellum. Characterization of *Gas7* and *Gas7-cb* RNAs and of the genomic structure of murine *Gas7* cloned in a bacterial artificial chromosome indicated that the *Gas7* gene spans more than 60 kb and consists of at least 15 exons. The 5′-terminus of *Gas7-cb* is located at exon 6a, which is absent in *Gas7* transcripts but is retained in its entirety in *Gas7-cb* transcripts, resulting in the presence of a unique 20-amino-acid sequence at the N-terminus of the Gas7-cb protein. Our results show that the *Gas7* gene encodes two Gas7 isoforms, Gas7 and Gas7-cb, whose expression is differentially regulated within mouse brain.**

INTRODUCTION

Growth arrest-specific (*Gas*) genes are differentially expressed in quiescent cells that enter the G0 state as a result of serum deprivation or growth to confluence (Schneider *et al.*, 1988; Brenner *et al.*, 1989). While preferential expression in G0 is the defining feature of *gas* genes, they are known to have disparate functions. Gas1 blocks the G0-to-G1 transition of fibroblasts in a p53-dependent manner (Del Sal *et al.*, 1995). Gas2, a microfilament-associated protein cleaved by ICE-like protease upon apoptosis, is involved in cytoskeleton rearrangements during the G0-to-G1 transition (Brancolini and Schneider, 1994) and apoptosis (Brancolini *et al.*, 1995). Gas3 (PMP22), a component of myelin in peripheral nerves, regulates the proliferation and differentiation of Schwann cells (for review, see Naef and Suter, 1998) as well as the proliferation and apoptosis of fibroblasts (Zoidl *et al.*, 1997). Gas6, which is a ligand for receptor tyrosine kinases Axl, Rse, and Mer plays a role in the growth or survival of germ cells, fibroblasts, and Schwann cells (Bellosta *et al.*, 1997; Matsubara *et al.*, 1996; Stitt *et al.*, 1995). Gas9 (Lih *et al.*, 1996) was found to be identical to the platelet-derived growth factor α receptor, which has an important role in cellular mitogenic responses and early stage embryogenesis following growth arrest (Pledger *et al.*, 1977; Schatteman *et al.*, 1992).

The *Gas7* gene, whose action was first discovered in growth-arrested NIH3T3 fibroblasts using a promoter-search strategy (Brenner *et al.*, 1989), subsequently was found to be expressed prominently in neural tissues and to be implicated in neurite formation during terminal differentiation of cerebellar Purkinje neurons (Ju *et al.*, 1998). Whereas antisense inhibition of *Gas7* expression impedes neurite outgrowth from maturing Purkinje cells in culture, overexpression of the Gas7 protein promotes neurite outgrowth from undifferentiated Neuro2A neuroblastoma cells (Ju *et al.*, 1998). The mouse *Gas7* gene maps to chromosome 11 (Ju *et al.*, 1998); its human homologue is located in the short arm of chromosome 17 (SHGC-1222, Myers, 1995, Accession No. G13706, unpublished data in Genbank).

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. (*Gas7-cb*) AF133184 and (*Gas7* genomic fragment) AF135442.

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0888-7543/99 $30.00

Here we report that the murine *Gas7* gene encodes two protein isoforms specified by transcript species that are synthesized differentially within different regions of mouse brains. While expression of *Gas7* transcripts and protein is predominant in the cerebrum and in growth-arrested fibroblasts, expression of *Gas7-cb* transcripts and protein is predominant in the cerebellum. Using genomic sequencing, RNase protection assays, and Western blot analysis, we define the exon organization of the *Gas7* gene, the splice junctions of its transcripts, and the tissue-specific expression of Gas7 protein isoforms.

MATERIALS AND METHODS

Cell culture. NIH3T3 cells were cultured in DMEM supplemented with penicillin/streptomycin and 10% calf serum.

Transfection. *Gas7* and *Gas7-cb* cDNAs were cloned in pcDNA3 (Promega) for transfection. The 5′ untranslated region of *Gas7-cb* cDNA was replaced with the 5′ untranslated region of *Gas7* cDNA due to the failure of expression of Gas7-cb protein in NIH3T3 cells with its own natural 5′ untranslated sequence. One microgram of pcDNA3gas7 or pcDNA3gas7-cb cDNA was transfected into each well of NIH3T3 cells (50% confluence) growing in a 6-well plate, using Lipofectamine Plus reagent (Gibco BRL). One and a half days after transfection, the cells were collected, and the proteins were extracted with a buffer containing 10 mM Hepes (pH 7.9), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet-P40, and 0.5% sodium deoxycholate. The protein concentration was measured by a Bio-Rad protein assay.

Western blot. Proteins to be studied were resolved by SDS–PAGE and then transferred to a PVDF membrane with a Bio-Rad semidry transfer cell. The efficiency of transfer was checked by Amido black staining of the membrane. The membrane was blocked with 5% skim milk in TBST (20 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20), followed by incubation with rabbit anti-Gas7 serum or anti-Gas7-cb serum diluted 3000-fold with TBST at room temperature for 1 h. After three washes with TBST, the membrane was incubated with sheep anti-rabbit IgG-POD (Boehringer Mannheim) at room temperature for 30 min. This was followed by another three washes with TBST, prior to development with enhanced chemiluminescence reagents (SuperSignal kit, Pierce).

Production of anti-Gas7-cb antibody. A synthetic peptide, consisting of amino acids 1–20 of Gas7-cb (MGKKMSNMENSFDDGSHLSPC-COOH; synthesized by Beckman Center, Stanford University Medical Center), was conjugated to keyhole limpet hemocyanin (KLH) using an Imject activated immunogen conjugation kit (Pierce). The conjugated peptide–KLH was purified by a gel filtration column included in the kit. The immunogen was mixed with Freund's adjuvant (Gibco BRL) and injected subcutaneously into a rabbit. Two hundred micrograms of immunogen was used for the initial injection, and 100 μg was used for each subsequent booster injection. The anti-Gas7-cb serum used in this study was obtained after the third booster injection.

RNase protection assay. An RNase protection assay was carried out using an RPAII kit (Ambion). Total RNA was isolated from mouse cerebrum, cerebellum, and contact-inhibited NIH3T3 cells using an RNeasy midi kit (Qiagen). Poly(A) mRNA was purified from total RNA using an Oligotex mRNA mini kit (Qiagen). The purified poly(A) mRNA was subsequently used in these experiments. To make the template for synthesizing the *Gas7* probe, a DNA fragment (produced by PCR) covering a region of 44 nucleotides specific to *Gas7* and a region of 105 nucleotides shared by *Gas7* and *Gas7-cb* cDNA (see Fig. 1A) was cloned to the *Eco*RV site of pcDNA3. The template for synthesizing the *Gas7-cb* probe was similarly made by cloning the DNA fragment containing a region of 46 nucleotides specific to *Gas7-cb* and a region of 105 nucleotides shared by *Gas7* and *Gas7-cb* cDNA (Fig. 1A) to pcDNA3. Both templates were linearized by *Eco*RI and transcribed in a mixture (50 μl) containing 10 units of Sp6 RNA polymerase (Promega), 1 μg of template DNA, 5 mM DTT, 40 units of RNasin (Promega), 1× TSC buffer (Promega), 0.6 mM ATP/CTP/GTP, 12 μM cold UTP, and 50 μCi of [α-^{32}P]UTP (3000 Ci/mmol, NEN). The reaction was carried out at 37°C for 2 h, followed by destruction of the DNA with 10 units of RNase-free DNase I at 37°C for 15 min. The probes were gel-purified. Two micrograms of poly(A) mRNA and 1.5×10^5 cpm probe were used for each RNase protection assay. The protected RNA fragments were analyzed with a 6% PAGE containing 7 M urea.

Cloning and characterization of Gas7-cb cDNA. The *Gas7* and related cDNA, *Gas7-cb*, were isolated from a newborn mouse brain cDNA library cloned in Lambda ZAP II vector (Stratagene) using *Gas7* cDNA as the probe. To isolate the 5′ region of *Gas7-cb* cDNA, a 5′ RACE kit purchased from Life Technologies was used. The first-strand cDNA was synthesized by reverse transcription using 20 μg of mouse cerebellum total RNA as the template and a *Gas7*-specific oligonucleotide (5′ AGC TTA GCC AGG TTC TTT GC 3′) as the primer. A poly(C) tail was added to the 3′ end of the first-strand cDNA by terminal deoxynucleotidyl transferase. The double-stranded cDNA was synthesized by PCR using the first-strand cDNA as the template and using the anchor primer and another *Gas7*-specific oligonucleotide (5′ TGC CTT TCA GTT GCT TCT GC 3′) as primers. The double-stranded cDNA was then cloned into a PCRII TA cloning vector (Invitrogen) and sequenced with a T7 Sequenase II reagent kit (USB). The *Gas7-cb* cDNA was reconstructed by replacing the 5′ region of *Gas7* cDNA (*Xho*I–*Bam*HI fragment) cloned in pSP72NOT (Promega) with the 5′ region of *Gas7-cb* (*Xho*I–*Bam*HI fragment) cloned in PCRII.

Chromosomal localization of Gas7 by fluorescence in situ hybridization. DNA prepared from the BAC clone was labeled with digoxigenin–dUTP by nick-translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblast cells in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI.

Characterization of the Gas7 genomic structure. A genomic clone containing the *Gas7* gene was isolated from the BAC Mouse ES-129/SvJ genomic library (Genome Systems Inc.) by screening with a probe corresponding to nucleotides 1–1086 of the *Gas7* cDNA. Several approaches were applied to analyze this genomic clone. For identification of the exon/intron boundaries, a sublibrary was first created from this clone. The sublibrary of the BAC clone was prepared by two types of partial restriction-enzyme digestions to ensure adequate cloning coverage: *Sau*3AI and Jon's blunt cocktail (*Alu*I, *Hae*III, *Rsa*I) (protocol supplied by H.-C. Chi, University of California, Irvine). After gel electrophoresis, restriction fragments (averaging 2.5–3.5 kb in size) were cloned into pBluescript II KS (Stratagene) at the *Bam*HI site for *Sau*3AI fragments and the *Eco*RV site for the fragments derived from the digestion with Jon's blunt cocktail. In both cases the pBluescript vector was treated with calf intestinal phosphatase. The sublibrary was screened by hybridization using *Gas7* cDNA as a probe. The positive clones were subjected to DNA sequence analyses using the T3 and T7 universal primers derived from pBluescript or internal primers derived from the inserts of these clones. Some of the DNA sequences were obtained by direct sequencing of the BAC clone using a cycle sequencing protocol (Amersham Life Science) and *Gas7* cDNA-specific primers. The divergence of the cDNA sequences from the genomic sequence was used to identify the exon/intron junctions as shown in Table 1. Next, to characterize introns of less than 5 kb, a pair of primers complementary to the exon margins were used in a PCR amplification with the BAC clone as the template. The PCR products were cloned into pGEM-T vector (Promega), and the end sequences of inserts confirmed the position of these fragments in genomic DNA. Finally, for

TABLE 1

***Gas7* Gene Has Canonical Splice Junctions**

Exon	Size (bp)	3′ Splice site (intron/exon)	5′ Splice site (exon/intron)	Intron size (kb)
4	81	cctttgac**ag**/AGACCACCTG	CCTACAACAG/**gt**aagagcca	>13
5	86	gtccccac**ag**/TGAATGGATA	TGATTCCCAG/**gt**aaggggct	>7
6ab	336	5′ UTR	CACCATTACC/**gt**aagtgggc	7.5
6b	54	ttctcccc**ag**/AACCTGGGAT	CACCATTACC/**gt**aagtgggc	7.5
7	90	taccctgc**ag**/ATCAACTGTG	CTACTTTTGG/**gt**aggtaccg	2.2
8	116	ttcttccc**ag**/GCGGACAAGA	TCCGGGAAAG/**gt**gagctcaa	3.5
9	75	ctctttgc**ag**/GATAAAGATT	AGGAGGAAGG/**gt**gagttggg	?
10	79	cctgttgc**ag**/CTCCTTGGGA	CTCTGCCAAG/**gt**aaccctcc	3.5
11	129	tccttcac**ag**/CTCCACAGCG	AGTGGAGAAG/**gt**gagaggcc	1.3
12	124	cccgctcc**ag**/GCCCGCAAAG	ACTCAGGCCG/**gt**gagcccat	?
13	80	cttttccc**ag**/GAGATGACCT	GACCACACTG/**gt**atgtgatc	1.4
14	99	tccttggc**ag**/GAACTGGAGC	CAACCAAAGC/**gt**gagttccc	0.8
15	1619	ccccttgc**ag**/ACAGTCGAGC	3′ UTR	

longer introns, the BAC clone was digested with restriction enzymes and probed with the oligonucleotides of the sequences adjacent to the region of interest. When the restriction fragments most likely containing the region of interest were found, they were cloned into pBluescript, mapped, and sequenced.

Southern blot analysis. Four micrograms of genomic DNA isolated from mouse 129/SvJ ES-cells was digested for 6 h with 35 units of restriction enzymes (as indicated in Fig. 5), separated in a 0.5% agarose gel, and transferred to Zeta-probe Blotting Membrane (Bio-Rad) according to the vendor's instructions. The probes were prepared by random-primed radiolabeling using [α-^{32}P]dCTP as described (Feinberg and Vogelstein, 1983). Templates for making probes 1 and 2 were prepared by PCR amplification. The template for PCR was the BAC clone and pairs of primers used for probe 1 and probe 2 were 5′ AGT TCC CAC CTC TCT CAT TGT 3′, 5′ AAG GAA CAG GGA CTC TGA CT 3′ and 5′ CCT TCT GAA TGA CAC AAC CC 3′, 5′ ACC TCG GAC TCT GCT GCA CT 3′, respectively. The template for third probe contained a 0.75-kb

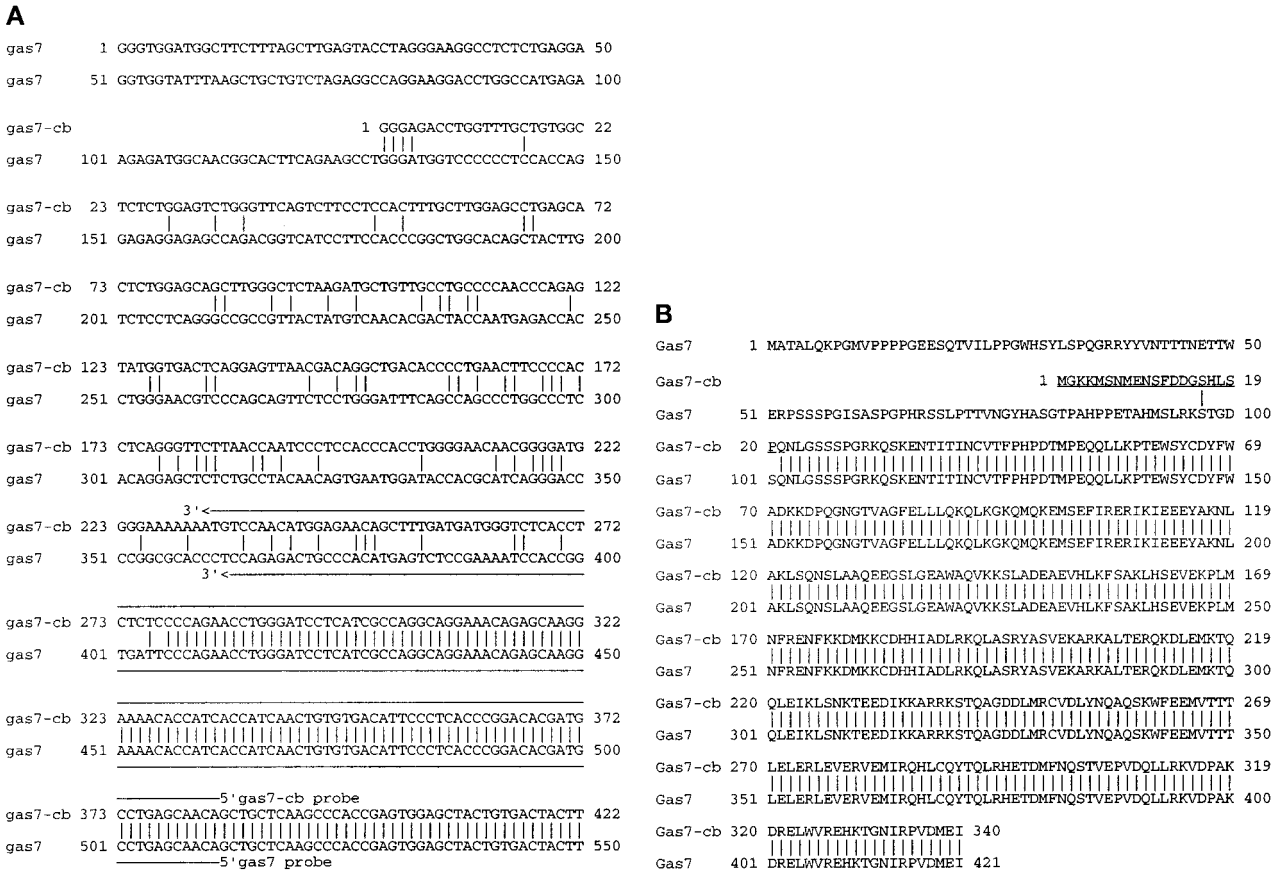


FIG. 1. (**A**) Comparison of the *Gas7* and *Gas7-cb* cDNA. Only the 5′ regions of the cDNA are shown. For complete sequences, see Accession Nos. U19860 (*Gas7*) and AF133184 (*Gas-cb*) in GenBank. The antisense *Gas7* and *Gas7-cb* probes used in the RNase protection assay (see Fig. 2) are indicated by arrows. (**B**) The predicted amino acid sequences encoded by *Gas7* and *Gas7-cb* cDNA. The sequence of the peptide used as the antigen for producing the *Gas7-cb* antibody (see Fig. 3) is underlined.

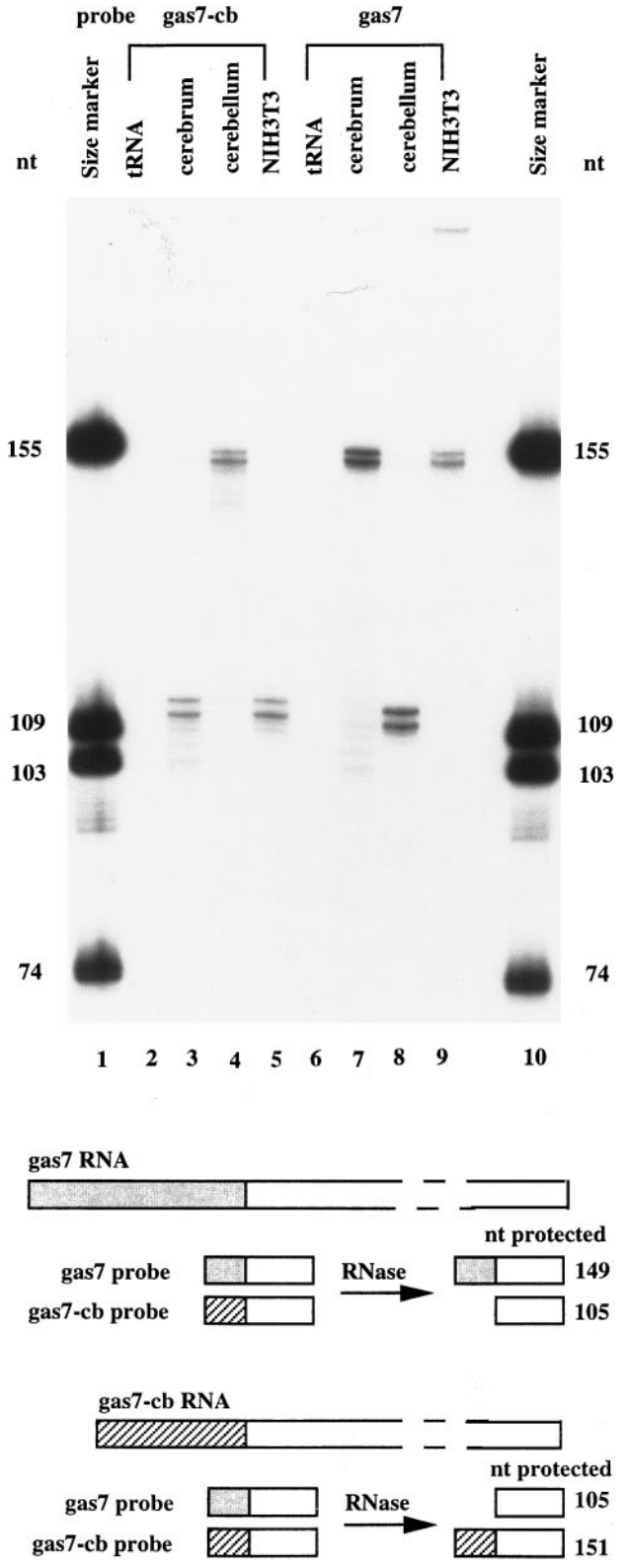


FIG. 2. RNase protection assay with the RNA isolated from the cerebrum, cerebellum, and growth-arrested NIH3T3 cells. Poly(A) mRNA isolated from the tissues or cells indicated was analyzed with the *Gas7* and *Gas7-cb* probes. As shown in the diagrams (**bottom**), these two probes included a region of 105 nucleotides shared by *Gas7* and *Gas7-cb* (white boxes) and a region specific to *Gas7* (shaded boxes) or *Gas7-cb* (hatched boxes). Hybridization of the *Gas7* probe to the *Gas7* RNA and *Gas7-cb* RNA was intended to protect 149- and 105-nucleotide probe fragments, respectively, from the RNase digestion while hybridization of the *Gas7-cb* probe to the *Gas7* RNA and *Gas7-cb* RNA was intended to protect 105- and 151-nucleotide probe fragments, respectively.

*Xba*I–*Eco*RI DNA fragment that was derived from a *Gas7* genomic fragment isolated from the NIH3T3 cells (Ju *et al.*, 1998). Hybridizations were performed using standard protocol (Sambrook *et al.*, 1989).

RESULTS

A Novel Gas7-Related cDNA Was Isolated from the Brain

Previously, using the *Gas7* cDNA as the probe for multitissue Northern blot analysis, we detected abundant expression of *Gas7* mRNA in mouse brain, where the cerebellum was the area of the brain richest in *Gas7* expression (Ju *et al.*, 1998). Alternative splicing of *Gas7* mRNA was observed in NIH3T3 cells (Ju *et al.*, 1998), which prompted us to identify other *Gas7* mRNA species from the brain tissues by screening of a mouse brain cDNA library and by 5′ RACE using RNA extracted from the cerebellum. In addition to *Gas7*, in the mouse brain cDNA library, we found a *Gas7*-related cDNA, which was later designated *Gas7-cb* (Fig. 1). Sequencing of the 5′ RACE products proved the presence of *Gas7-cb* mRNA in the cerebellum. Comparison of the *Gas7* and *Gas7-cb* cDNAs shows that the nucleotide region 1–405 of *Gas7* is replaced by a different sequence of 277 nucleotides in *Gas7-cb* (Fig. 1A). As a result, *Gas7-cb* encodes a 38-kDa protein with its amino acids 21–340 identical to amino acids 102–421 of Gas7 (Fig. 1B).

Gas7 and Gas7-cb mRNAs Are Expressed Differentially in the Brain

Different protein isoforms can be expressed differentially in different tissues. Since we found both the *Gas7* cDNA and the *Gas7-cb* cDNAs in the brain cDNA library, we further checked their mRNA expression in the cerebrum, cerebellum, and contact-inhibited NIH3T3 cells by RNase protection assay. Two RNA probes, *Gas7* and *Gas7-cb* (Fig. 2), were used to examine the purified poly(A) mRNA, as described under Materials and Methods. The *Gas7* RNA probe was designed to yield protected fragments of 149 or 105 nucleotides when hybridized to the *Gas7* or *Gas7-cb* mRNA, respectively. *Gas7-cb* RNA probe was expected to yield protected fragments of 105 and 151 nucleotides when hybridized to the *Gas7* and *Gas7-cb* mRNA, respectively.

As shown in Fig. 2, the *Gas7* probe produced major fragments of about 149 nucleotides when hybridized to the mRNAs isolated from the cerebrum and NIH3T3 cells (Fig. 2, lanes 7 and 9), indicating the predominance of *Gas7* mRNA species in these cells. On the other hand, the same probe produced major fragments

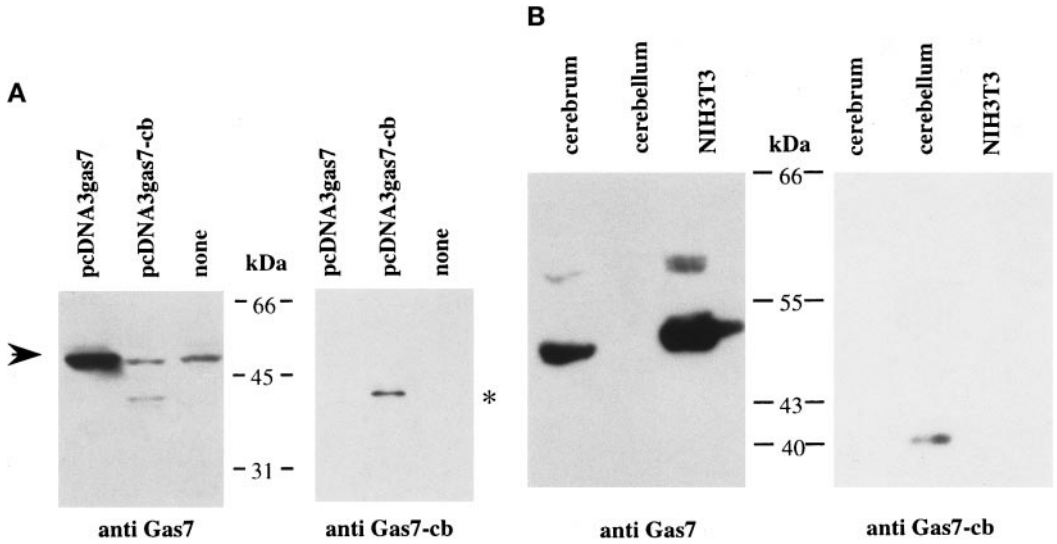


FIG. 3. (**A**) Western blot with *in vivo* synthesized Gas7 and Gas7-cb, using the Gas7 and Gas7-cb antibodies. The *Gas7* cDNA and *Gas7-cb* cDNA cloned in pcDNA3 were transfected into NIH3T3 cells. One and a half days after transfection, 10 μg of proteins extracted from transfected and untransfected cells was examined by Western blot. The membrane was first probed with the Gas7-cb antibody (also described in Fig. 1B), which detected an approximately 40-kDa band (indicated by an asterisk, **right**) only in the sample transfected with pcDNA3*gas7*-cb. The membrane was stripped of the probe and then probed again with the Gas7 antibody, which detected both the endogenous Gas7 and the transfected Gas7 (indicated by an arrow, **left**). The Gas7 antibody detected the transfected Gas7-cb as well, but to a much lesser extent. (**B**) Western blot with the extracts from the cerebrum, cerebellum, and growth-arrested NIH3T3 cells. 15 μg of proteins from each of the tissues and the contact-inhibited NIH3T3 cells was probed by the Gas7 and Gas7-cb antibodies. The Gas7-cb antibody detected a band of about 40 kDa in the cerebellum sample (**right**) while the Gas7 antibody detected bands of approximately 48 kDa in the cerebrum and NIH3T3 samples (**left**).

of about 105 nucleotides when the cerebellum mRNA was examined (Fig. 2, lane 8), indicating the predominance of *Gas7-cb* in the cerebellum. The fragments of the *Gas7-cb* probe protected by the cerebrum and NIH3T3 mRNA were nearly 105 nucleotides in length (Fig. 2, lanes 3 and 5) while those protected by the cerebellum mRNA were nearly 151 nucleotides in length (Fig. 2, lane 4). These results agree with the conclusion that the *Gas7* mRNA species is predominant in the cerebrum and contact-inhibited NIH3T3 cells while the *Gas7-cb* mRNA species is predominant in the cerebellum. The negative control of tRNA gave no protection to either probe (Fig. 2, lanes 2 and 6).

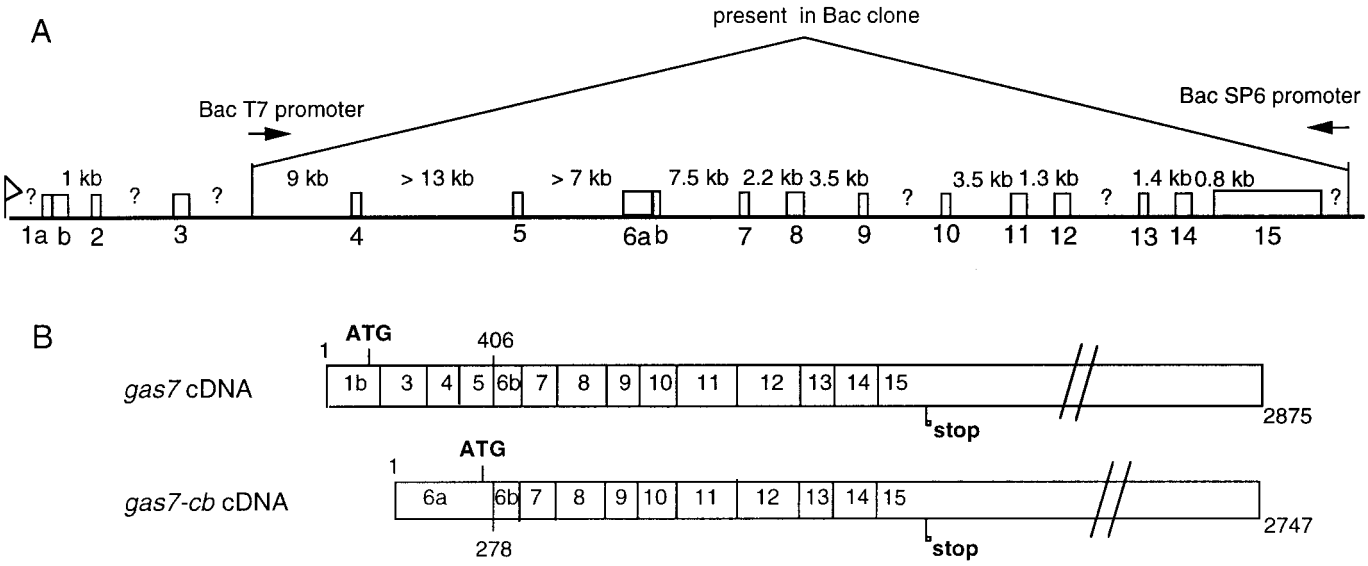
Western Blot Confirmed the Differential Expression of Gas7 and Gas7-cb

The differential expression of *Gas7* and *Gas7-cb* was checked at the protein level by Western blot. The specificity of the Gas7-cb antibody was tested with the *in vivo* synthesized proteins (Fig. 3A) as described under Materials and Methods. The Gas7-cb antibody detected only the Gas7-cb protein but not the other proteins (Fig 3A, right), proving the specificity of the antibody. Another antibody, Gas7, which was produced using full-length Gas7 as the antigen (Ju *et al.*, 1998), reacted strongly to endogenous NIH3T3 Gas7 and transfected Gas7 but reacted poorly to Gas7-cb (Fig 3A, left). Gas7 and Gas7-cb share a common region of 320 amino acids but differ in their N-terminal regions. It is possible that the Gas7 antibody reacts mainly to the N-terminal region of Gas7. Using Gas7 and Gas7-cb antibodies, the tissue-specific expression of Gas7 and Gas7-cb was examined by Western blot analysis (Fig. 3B). The Gas7-cb antibody revealed a band in the cerebellum sample of about 40 kDa, close to the predicted size of the protein encoded by the *Gas7-c*b cDNA (Fig. 3B, right). The Gas7 antibody revealed a major band of approximately 48 kDa in the cerebrum and NIH3T3 samples. The 40-kDa band in the cerebellum sample was also detected by the Gas7 antibody when the film was exposed for a longer period (data not shown), suggesting its relevance to Gas7. These results confirm the RNA analysis data showing that Gas7-cb is predominant in the cerebellum and Gas7 is predominant in the cerebrum and NIH3T3 cells.

The Gas7 Gene Encodes Both Gas7 and Gas7-cb cDNA

The BAC clone isolated from the BAC Mouse ES-129/SvJ genomic library (Genome Systems Inc.) using the *Gas7* cDNA probe contained an insert longer than 100 kb. From the DNA sequence analysis and PCR analysis described under Materials and Methods, we mapped regions that encode Gas7 and Gas7-cb.

Comparison of cDNA and genomic DNA sequences revealed the exon organization of the *Gas7* gene (Fig. 4). The splicing site sequences (Table 1) agree with the known consensus sequence (Breathnach and Chambon, 1981). The *Gas7* gene in the BAC clone contains 12 exons and spans more than 60 kb. The present 12 exons, which encode nucleotides 244–2875 of the *Gas7* cDNA, represent 91.5% of the full-length *Gas7* cDNA.



C

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GCAGCTTGGG CTCTAAGATG CTGCTGCCTG CCCCAACCCA GAATATGGTG ACTCAGGAGT TAACGACAGG
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tggagtgtgc tgtgtgca
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FIG. 4. (**A**) Genomic structure of the *Gas7* gene. Exons are depicted as rectangles; sizes of introns are indicated above the solid line. The sizes of exons and introns are not drawn to scale. The part of the *Gas7* gene present in the BAC clone and the orientation of the gene relative to the T7 and Sp6 promoters in the vector are shown. (**B**) Exon organization of *Gas7* and *Gas7-cb* cDNA. The sequence analyses of *Gas7* genomic DNA, *Gas7* cDNA, and *Gas7-cb* cDNA showed that exons 6a and 6b form the first exon of *Gas7-cb* while in *Gas7* exon 5 joins exon 6b. The locations of the translation initiation and stop codons are shown. (**C**) The sequence of the genomic DNA fragment (GenBank Accession No. AF135442) that contains exon 6ab. These data were obtained from direct sequencing of the BAC clone and the insert of J1-24, a clone of the library containing the fragments of the restriction enzyme (*Alu*I, *Hae*III, *Rsa*I) digested BAC clone. Lowercase letters indicate intron sequences, and uppercase letters denote exon 6ab, where exon 6b is shown in boldface type.

The 5′ region of the *Gas7* gene is missing from this genomic clone. We had previously sequenced a part of the 5′ region of the *Gas7* gene located upstream of an *Escherichia coli lacZ* gene inserted into the NIH3T3 chromosome as a result of our promoter search (Ju *et al.*, 1998). The sequenced region contains 2 additional exons. Furthermore, a sequence present in *Gas7* cDNA is still missing from this region and the BAC clone. These data indicate that the region of the *Gas7* gene absent from the BAC clone consists of at least three exons (as shown in Fig. 4). Nucleotides 1–336 of *Gas7-cb* cDNA correspond to the entire exon 6, i.e., 6a and 6b, of the *Gas7* genomic DNA (Fig. 4C). However, in *Gas7* cDNA, exon 5 is spliced directly to exon 6b, and as a result, exon 6a is absent.

To reveal the localization of the *Gas7* locus in mouse chromosomes, using a *Gas7* probe derived from the BAC clone, we performed fluorescence *in situ* hybridization (FISH) on normal metaphase chromosomes derived from mouse embryo fibroblast cells. The initial experiment resulted in specific labeling of a medium-sized chromosome that was believed to be chromosome 11 (data not shown), which is consistent with a previous report (Ju *et al.*, 1998). Subsequently, a probe specific for the centromeric region of chromosome 11 was cohybridized with the *Gas7* probe, confirming that *Gas7* is located as single locus on chromosome 11 (Fig. 5). Furthermore, the *Gas7* gene was found to hybridize specifically at a position that is 50% of the distance from the heterochromatic–euchromatic boundary to the terminus of chromosome 11 (Fig. 5; a total of 80 metaphase cells were analyzed with 72 exhibiting spe-

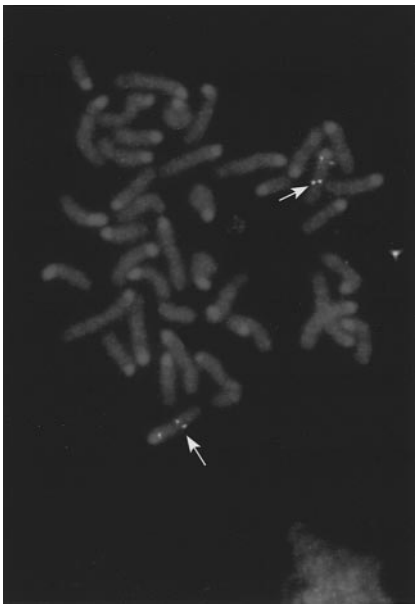

FIG. 5. Chromosomal localization of the *Gas7* gene. The BAC clone containing *Gas7* genomic DNA was labeled with digoxigenin–dUTP by nick-translation, mixed with sheared mouse DNA, and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts, as described (Stokke *et al.*, 1995). Specific hybridization signals were detected using fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI. A total of 80 metaphase cells were analyzed with 72 cells exhibiting specific labeling (indicated with arrows). A hybridization signal labeled by a probe specific for the centromeric region of chromosome 11 (Shi *et al.*, 1997) is also revealed.

cific labeling). Although FISH reveals the locus of a gene, Southern analysis provides better resolution in determining gene copy number and in detecting the presence of related genes (for related information see Davies and Tilghman, 1990). We performed Southern blot analysis on the genomic DNA purified from mouse strain 129/SvJ. Three DNA probes derived from various regions of the *Gas7* genomic DNA were used in these experiments (see Materials and Methods). As shown in Fig. 6, in each Southern blot analysis only one single band was detected for each restriction enzyme cleaved genomic DNA. These results indicate that *Gas7* is very likely a single-copy gene, which agrees with the results mentioned above that the same gene encodes *Gas7* and *Gas7-cb*.

DISCUSSION

Genes encoding a diverse array of protein isoforms via tissue-specific transcription and developmentally regulated alternative mRNA splicing are widespread in mammals (Suter *et al.*, 1994; Khvotchev and Sudhof, 1998; Ashiya and Grabowski, 1997). Analysis of *Gas7* genomic structure and of *Gas7* and *Gas7-cb* mRNA indicates that the same locus encodes both *Gas7* and *Gas7-cb*. The 5′ end of mouse *Gas7-cb* is present in the genomic sequence (entire exon 6), while the 5′ end (exon 1) of mouse *Gas7* is located far upstream. The nearest TATA box (TATAAAA) is 270 bp upstream of the first nucleotide of *Gas7-cb*. No consensus splice acceptor sequence (AG) is adjacent to the first nucleotide of *Gas7-cb*. Our findings suggest that mouse *Gas7-cb* and *Gas7* may be transcribed from different promoters, but the possibility that *Gas7* and *Gas7-cb* mRNA species result from alternative splicing is not excluded. Interestingly, gene regulation by the use of different promoters has been reported for *Gas3* (*PMP22*), in which one promoter is used in neuronal cells while the other is used in nonneuronal cells, resulting in the production of tissue-specific transcripts (Desarnaud *et al.*, 1998; Suter *et al.*, 1994).

A human cDNA homologue of *Gas7* isolated by Ohara *et al.* (1999, unpublished sequence in Genbank, Accession No. AB007854), shows 72.9% similarity to mouse *Gas7* cDNA, and the predicted protein sequences have 98.3% similarity. During the present

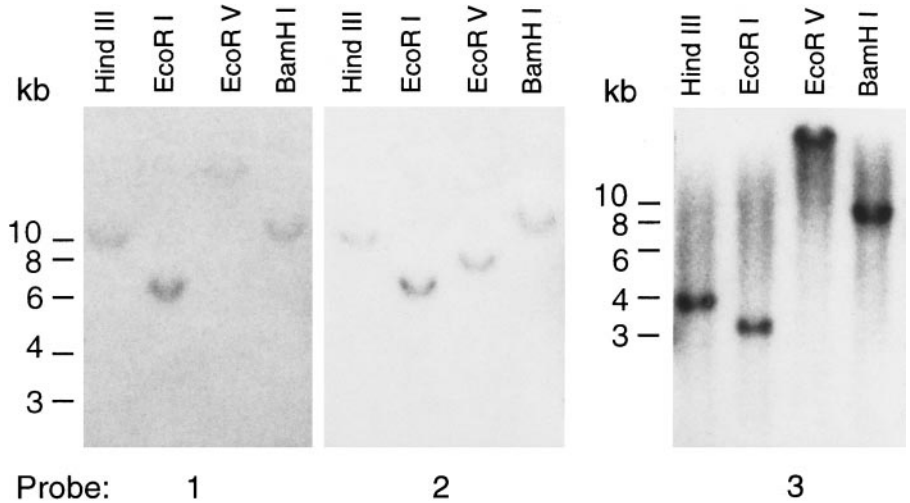

FIG. 6. Southern blot with genomic DNA from mouse 129/SvJ ES cells. Genomic DNA, digested with the restriction enzymes indicated, was probed with three DNA fragments (shown as probes 1, 2, and 3). Probes 1 (0.8 kb) and 2 (1 kb) are located 1.65 and 0.4 kb upstream of exon 4, respectively. Probe 3 (0.75 kb) covering part of exon 1 and the intron downstream was derived from a *Gas7* genomic DNA fragment isolated from NIH3T3 cells (Ju *et al.*, 1998). Size markers are indicated on the left side of the autoradiographs.

study, we obtained a human homologue of *Gas7-cb* from the cerebellum (Chao, 1999, unpublished sequence in Genbank, Accession No. AJ224876), suggesting that both of the Gas7 protein isoforms we identified in murine tissues are also made in human tissues. Mouse *Gas7-cb* has 74.9 and 98.8% similarity to its human homologue at the nucleotide and predicted protein sequence levels, respectively. Comparison of the human *GAS7* genomic sequence (Birren *et al.*, 1998, unpublished sequence in Genbank, Accession No. AC005747) with human *GAS7* and *GAS7-cb* cDNA shows that the exon/intron organization of *Gas7* is conserved between human and mouse.

Previously, using anti-Gas7 antibodies, we detected strong immunohistochemistry reactivity in the cerebellum (Ju *et al.*, 1998). In the present study, it is shown that this anti-Gas7 antibody reacts less strongly to cerebellum Gas7-cb analyzed by Western blot. It is possible that while the anti-Gas7 antibodies strongly react to the N-terminal region of denatured Gas7 (as in the case of Western analysis), they recognize the C-terminal region of a folded native Gas7 or Gas7-cb protein (as in the case of immunohistochemistry analysis). Prolonged exposure of films for RNase protection and use of large amounts of extracts in Western blot analysis showed that *Gas7* mRNA and protein are present in small amounts in the cerebellum and that *Gas7-cb* mRNA and protein are also present in limited amounts in the cerebrum (data not shown). Thus, while expression of *Gas7* is predominant in the cerebrum and that of *Gas7-cb* is predominant in the cerbellum, the specificity of expression is not absolute.

The *Gas7* gene is a novel gene originally isolated from growth-arrested NIH3T3 cells. Its overexpression in Neuro2A neuroblastoma cells resulted in the production of extensive cellular processes (Ju *et al.*, 1998), suggesting that Gas7 may participate in the cytoskeleton reorganization during the formation of cellular processes. When cultured cerebellar neurons were treated with antisense oligonucleotides complementary to sequences in the translation initiation regions of *Gas7* and *Gas7-cb*, neurite outgrowth was inhibited (Ju *et al.*, 1998). As the oligonucleotides employed in those experiments knocked out both proteins encoded by *Gas7* and *Gas7-cb*, whether the observed inhibition of neurite outgrowth resulted from interference with expression of *Gas7*, *Gas7-cb*, or both remains to be revealed. The N-terminal region of Gas7, absent in Gas7-cb, contains a sequence (amino acids 2–30) similar to a region in transcription factor Oct-2 (Wirth *et al.*, 1991) and a region in synapsins Ia/b (Südhof *et al.*, 1989, Accession Nos. M27924 and M27812), which tether synaptic vesicles to cytoskeletons (Sudhof, 1995) and induce the formation of elongated processes when overexpressed in neuronal (Han *et al.*, 1991) or fibroblast (Han and Greengard, 1994) cells. The N-terminal region also contains a WW motif (amino acids 22–60) whose prominent feature is the presence of two conserved tryptophans (W) (Sudol, 1996). However, both *Gas7* and *Gas7-cb* were able to promote the formation of long processes when overexpressed in NIH3T3 cells (unpublished data). The WW motif is widely involved in protein interactions (Sudol, 1996). If this motif plays a role in Gas7 function by mediating the interaction between Gas7 and other proteins, Gas7 and Gas7-cb may have different biological functions.

ACKNOWLEDGMENTS

We thank Dr. Hang-Chang Chi for providing helpful information for analyzing the BAC clone, Dr. Chuck C.-K. Chao for comparison of human genomic sequence with the human *Gas7* cDNA, and Drs. Stanley N. Cohen, Michael O. Poulter, Dmitri A. Bessarab, and Chih-Jian Lih for helpful discussions. We also thank Ying-Jiun Chen and Cheng-Hang Liu for plasmid constructions. This study was supported by a Frontier Research Award from the National Science Council of Taiwan, Republic of China, to S. Lin-Chao (NSC 87-2312-B-001-017). E.M.L. was supported by the Academia Sinica, Taiwan; B.-R.S. received a postdoctoral fellowship from the National Science Council, the Republic of China.

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