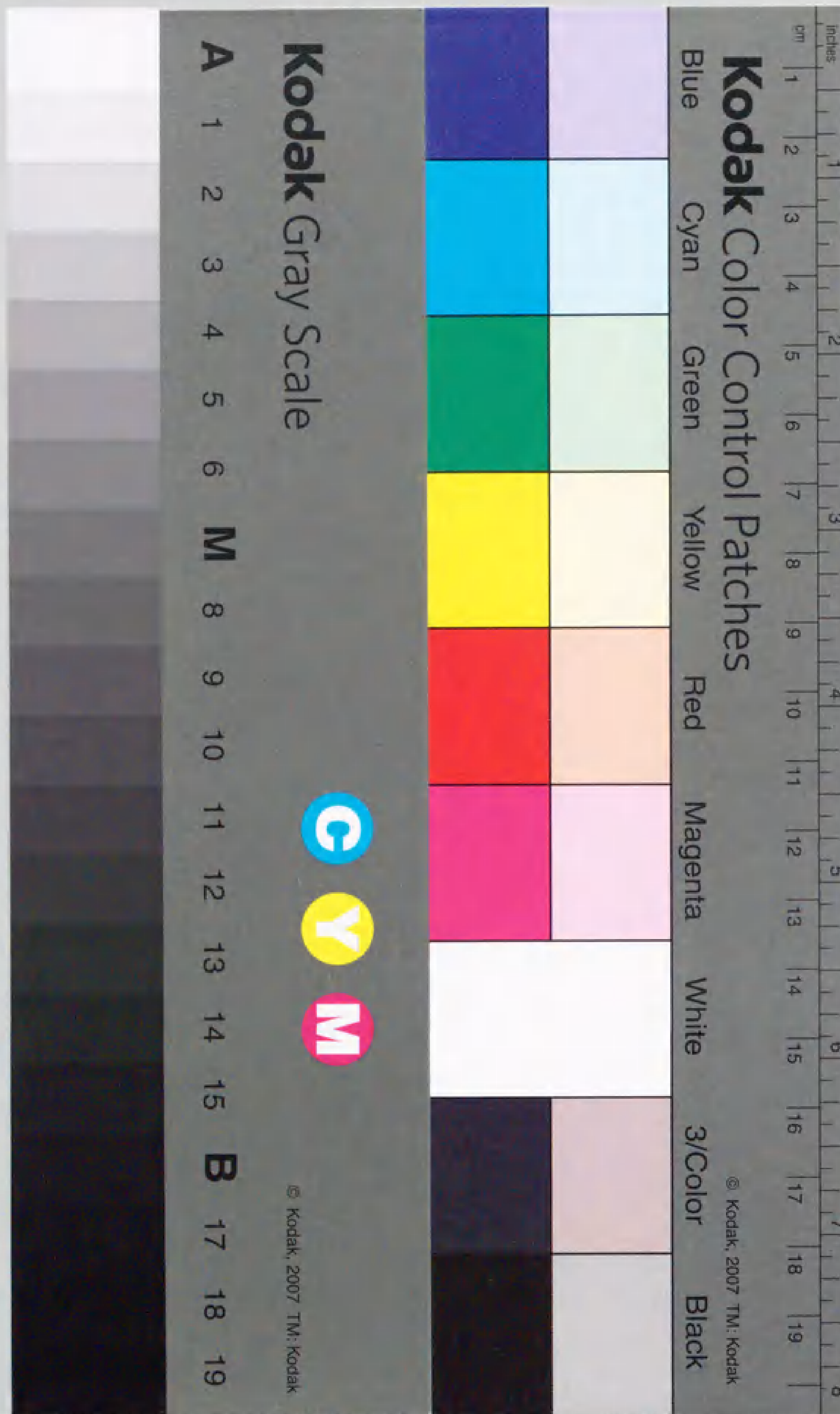

論　文　目　録

岐阜大学

報告番号	甲第383号	氏名	酒井秀樹

主論文

Molecular cloning and differential expression of the genes during glioma cell differentiation

1) Identification of differentially expressed mRNAs during rat C6 glial cell differentiation by mRNA fingerprinting using arbitrarily primed PCR (RAP)　1冊
平成9年6月発行　Neuroscience Letters　229：93〜96

2) Molecular cloning of a cDNA encoding a serine protease homologous to complement C1s precursor from rat C6 glial cells and its expression during glial differentiation　1冊
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印刷中　Brain Tumor Pathology

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NEUROSCIENCE LETTERS

Identification of differentially expressed mRNAs during rat C6 glial cell differentiation by mRNA fingerprinting using arbitrarily primed PCR (RAP)

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Abstract

Differentiation of glial cells is controlled by a complex program of differential expressions of many genes. To identify differentially expressed genes that are involved in rat C6 glial cell differentiation induced by dibutyryl cyclic AMP and theophylline, mRNA fingerprinting using arbitrarily primed PCR (RAP) was used. Four cDNA fragments, that were differentially expressed during differentiation, were isolated. Sequence analysis revealed that one of them, abundantly expressed during differentiation, was homologous to a hamster calcium-dependent serine protease. Another one was highly similar to rabbit dystrobrevin and the other two clones were identical to rat triose phosphate isomerase and calnexin. The results obtained suggest that the expressions of particular genes were changed and that RAP is a useful method to identify genes which are differentially expressed during glial cell differentiation. © 1997 Elsevier Science Ireland Ltd.

Keywords: Cyclic AMP; Differentiation; Gene expression; Glia; mRNA fingerprinting using arbitrarily primed PCR

During brain development, differentiation of glial cells is controlled by a complex program of differential expression of various genes [2,8]. Identification of the molecules implicated in glial cell differentiation is important for understanding the physiological and pathological functions of glial cells in the brain. To date, however, only a limited number of genes involved in glial cell differentiation have been identified.

Rat C6 glial cells have been used as a model system for studies of glial cell development and differentiation [16]. In these cells, the increase in intracellular cyclic AMP (cAMP) concentration by some agents, such as dibutyryl cyclic AMP (dbcAMP) and theophylline, was thought to induce differentiation toward a more mature astrocytic phenotype [12,15,20]. Using this model system, we have recently shown the changes in mRNA expression of phospholipase D, protein kinase C and small GTP-binding protein, Rho A during differentiation [18,19]. To search for genes involved in glial cell differentiation, we have adopted mRNA fingerprinting using arbitrarily primed PCR (RAP) [9,17], a recently developed technique for identifying and cloning differentially expressed genes. cDNA fragments obtained by RAP often correspond to coding regions of mRNA and this method is more convenient than mRNA differential display (DD) [5] in identifying the isolated cDNA fragments by homology analysis with databases. In the present study, we have identified four mRNA species that were up- or down-regulated during C6 glial cell differentiation induced by cAMP.

A C6 glial cell line obtained from the Human Science Research Resources Bank (Osaka, Japan) was treated with 1 mM dbcAMP (Sigma Chemical Co., USA) and 0.25 mM theophylline (Wako Chemical Co., Japan) [10,18,19]. Total RNA was extracted from the cells at 0, 24 and 48 h with Isogen (Nippon Gene, Japan) according to the manufacturer's instruction and was then treated with Massage Clean Kit (GenHunter, USA) to remove contaminating chromosomal DNA. The first strand cDNA was synthesized by reverse transcriptase using random hexamer-mixed primers. Arbitrary primers used in RAP were end-labeled with [γ-^{32}P]ATP. Sequences of these arbitrary primers are listed

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in Table 1. PCR reaction was performed with a thermal cycler (Quick Thermo Personal, Nippon Genetics, Japan) for one cycle of 3 min at 94°C, 5 min at 37°C, 5 min at 72°C followed by 35 cycles of 30 s at 94°C, 1 min at 50°C and 1 min at 72°C. The amplified cDNAs were separated on a sequencing gel followed by autoradiography. Bands of interest were excised from the gels and the DNA was eluted by boiling. The eluted DNA was reamplified by PCR and was subcloned into pMOSBlue T-vector (Amersham Life Science, USA). The nucleotide sequence was determined by dideoxynucleotide termination method. Homology analysis was performed using BLAST algorithm at NCBI, NLM, NIH (Bethesda, MD, USA).

Northern blot analyses were performed to confirm differential gene expression. Twenty micrograms of total RNA was subjected to 1.2% agarose/formaldehyde electrophoresis and transferred onto GeneScreen Plus membranes (DuPont/NEN, USA). The cDNA fragments obtained from RAP and partial cDNA fragments of rat S-100B protein [4] used as probes were labeled with [α-^{32}P]dCTP using Multiprime DNA labeling system (Amersham Life Science). The membranes were hybridized with probes, washed, and exposed to film (Kodak X-OMAT AR).

Using dbcAMP treatment, C6 cells underwent morphological changes to astrocyte-like shape as previously described [18,19]. Northern blot analysis demonstrated that mRNA of S-100B protein, which is often used as a marker of glial cell differentiation [20], reached the maximal level 24 h after treatment (data not shown).

mRNA populations from the control and differentiated C6 glial cells were subjected to RAP with eight different arbitrary primers. Approximately 600 bands ranging from 200 to 500 bp size were successfully amplified. Although most of the bands did not display significant changes during differentiation, the intensities of several bands were altered, suggesting differential expression of particular genes. Eight cDNA fragments, which showed changed intensities, were selected and subcloned. Differential expression was confirmed by Northern blot analyses using these cDNA fragments as probes. No positive signals were obtained by two cDNA fragments (C6B1 and C6D2). Two others (C6F1 and C6H1) recognized the corresponding mRNAs but their message levels were unchanged during differentiation. However, the other four cDNA fragments hybridized to the corresponding mRNAs and their patterns of expression were verified by Northern blot analysis (Fig. 1). The expressions of mRNA recognized by the cDNA fragments, termed C6A2 and C6G1, were up-regulated during differentiation, whereas expressions of mRNA of C6C6 and C6E3 were down-regulated. The BLAST sequence homology analysis revealed that C6E3 and C6G1 clones corresponded to the partial sequences of rat calnexin (Genbank accession number L18889, nucleotide 1567–1776) and rat triose phosphate isomerase (TPI) (L36250, nucleotide 1–359), respectively. C6A2 and C6C6 clones were homologous to hamster calcium-dependent serine protease (CASP) (corresponding to X16160, nucleotide 262–627) and rabbit dystrobrevin (corresponding to S78779, nucleotide 1–233), respectively (Table 1).

RNA fingerprinting using RAP or DD is a potentially useful method to detect altered gene expression in complex RNA populations [3,5,9,13,17], although high incidence of false positive was often observed. In this study, out of eight candidates four false positives were observed. However,

Table 1

Homology analysis of cDNA fragments obtained by RAP

Clone	Arbitrary primers	Expression (RAP)	Sizes of RAP fragments, bp	Expression (Northern blotting)	mRNA sizes, kb	Sequence homology
C6A2	rST-2U	Up	365	Up	3.0	5′UTR + ORF of Hamster calcium-dependent serine protease (85%)
C6B1	rST-6U	Down	400	No signal	–	Not sequenced
C6C6	rST-3D	Down	358	Down	2.8	ORF of Rabbit dystrobrevin (81%)
C6D2	rST-5D	Down	275	No signal	–	Not sequenced
C6E3	hPK-AU	Down	395	Down	4.0, 3.8[a]	ORF + 3′UTR of rat calnexin (100%)
C6F1	hPK-AU	Up	250	Unchanged	3.5	Not sequenced
C6G1	hPK-AD	Up	359	Up	1.6	5′UTR + ORF of rat triose phosphate isomerase (100%)
C6H1	hPK-B1U	Up	500	Unchanged	3.4	Not sequenced

Sequences of arbitrary primers used for RAP are as follows:
rST-2U; 5′-AGGAGGAACCAGGAGCCCATTG-3′ rST-2L; 5′-AGCAGCAGGTGAGCAGCAGGA-3′
rST-3D; 5′-TGAGACTGGGCACTGGGCAAGC-3′ rST-5D; 5′-GTAATAGGGGTTCAGGGTGTTC-3′
rST-6U; 5′-GCAGCTGTCGGTCAAAGAGCAC-3′ hPK-AU; 5′-ATCCGCAGTGGAATGAGTCCTTTACAT-3′
hPK-AD; 5′-TTGGAAGGTTGTTTCCTGTATTCAGAG-3′ hPK-B1U; 5′-TTTTGGCATGTGTAAGGAAAACATCTGGGA-3′
UTR, untranslating region; ORF, open reading frame.
[a]Single cDNA fragment recognized transcripts of two sizes.

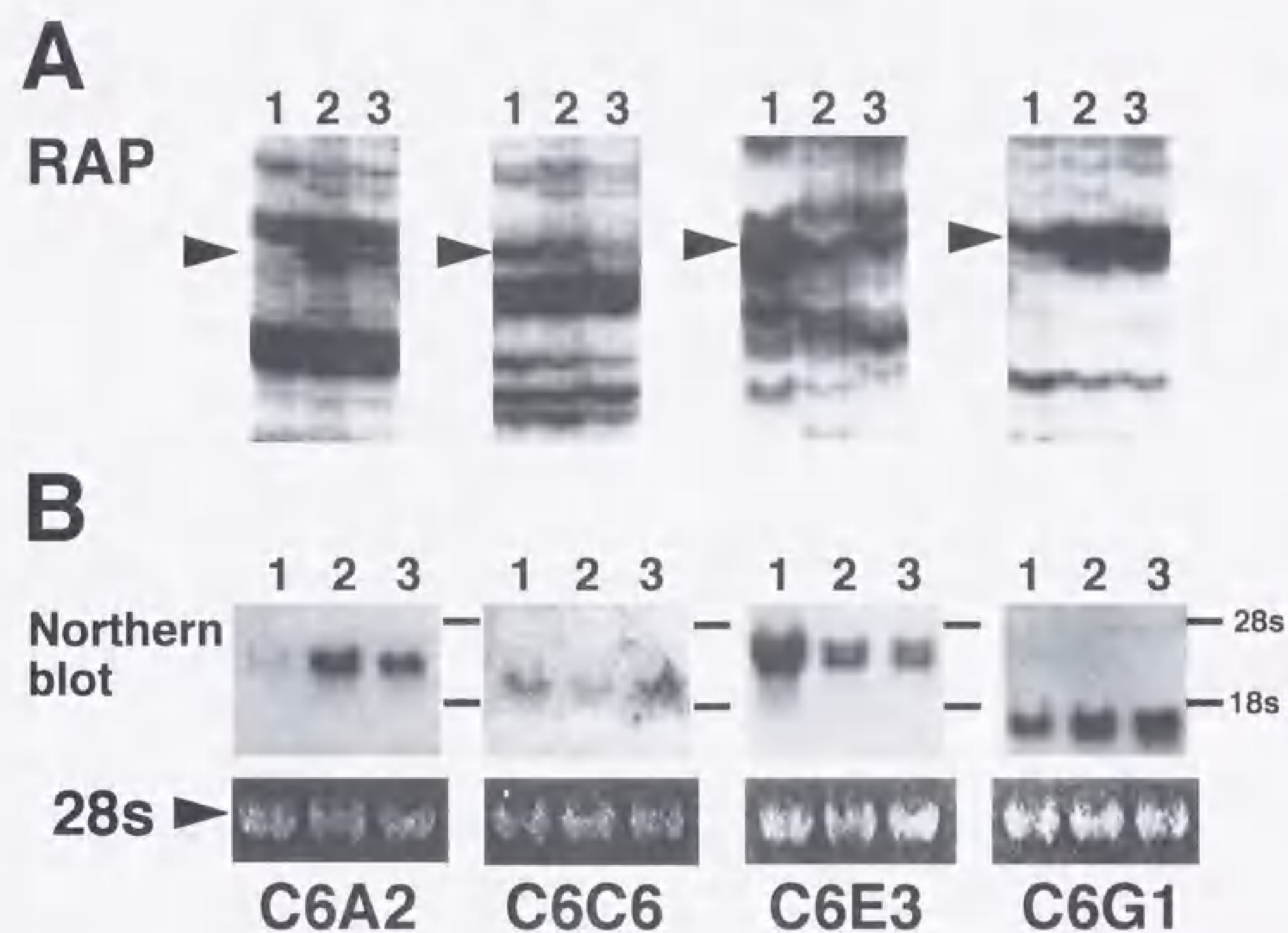

Fig. 1. RAP fingerprinting and Northern blots of four differentially expressed cDNA fragments (C6A2, C6C6, C6E3 and C6G1). (A) Autoradiogram of RAP during C6 glial cell differentiation. Bands representing differential gene expression by RAP are marked by arrowheads. Two micrograms of total RNA from the untreated control (lane 1) and dbcAMP-treated (lane 2: 24 h, lane 3: 48 h) cells were subjected to RAP with ^{32}P-labeled arbitrary primers as described in Materials and Methods. (B) Northern blots of cDNA fragments obtained by RAP. Twenty micrograms of total RNA from normal untreated (lane 1) and dbcAMP-treated (lane 2: 24 h, lane 3: 48 h) cells were subjected to Northern blot analysis with ^{32}P-labeled cDNA fragments (C6A2, C6C6, C6E3 and C6G1) obtained by RAP as probes. Positions of 28S and 18S rRNAs are indicated. Gels were stained with ethidium bromide.

these can be eliminated by careful Northern blotting and/or further improvements of the protocols [13]. Compared with DD, RAP tends to produce cDNA fragments which frequently correspond to open reading frame (ORF) of mRNA [3,13]. Indeed, four cDNA fragments obtained by the current RAP analysis contained the ORFs of corresponding mRNAs (Table 1).

C6E3 and C6G1 were identical to the genes of rat calnexin and TPI, respectively. Calnexin is a molecular chaperone that is involved in maturation and transport of a variety of glycoproteins in the endoplasmic reticulum [6]. TPI catalyzes interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The differentiation of C6 cells induced by cAMP results in arrest of cell growth and suppression of other malignant features of transformed cells [15]. Expression of calnexin and TPI may suggest the altered regulation of protein maturation and glycolytic metabolism, respectively.

C6A2 and C6C6, although they are only partial cDNA fragments, probably represent rat CASP and dystrobrevin genes, respectively, since their sequences were >80% homologous to the corresponding regions of each reported counterpart. Dystrobrevin is a dystrophin-associated protein localized in neuromuscular junctions, but its precise function in central nervous tissues including glial cells is unknown at present [1]. CASP is a member of the serine protease family and identical to the complement subcomponent C1s in hamster [11]. Some members of serine protease family, such as tissue- and urokinase-type plasminogen activators and thrombin, have been demonstrated to be implicated in various physiological processes in nervous tissues, e.g. neurite outgrowth, formation and reorganization of synapses, and cell migration [7,14]. The temporal expression of C6A2 preceded or well correlated with outgrowth of neurite-like processes during C6 cell differentiation induced by cAMP. Therefore, it is tempting to speculate that the protease encoded by C6A2 might partake in glial differentiation.

Although potential functional roles of the genes isolated by RAP remain to be disclosed, their differential mRNA expression leads us to predict the possible involvement in differentiation process. To this end, cDNA cloning and functional analysis should be done and are under current progress in our laboratory.

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Molecular cloning of a cDNA encoding a serine protease homologous to complement C1s precursor from rat C6 glial cells and its expression during glial differentiation

Key Words: mRNA fingerprinting using arbitrarily primed polymerase chain reaction (RAP); Gene expression; Serine protease; Cyclic AMP

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Abbreviations: dbcAMP, dibutyryl cyclic AMP: EGF, epidermal growth factor: GAPDH, glyceraldehyde 3-phosphate dehydrogenase: GFAP, glial fibrillary acidic protein: PCR, polymerase chain reaction: RACE, rapid amplification of cDNA ends: RAP, mRNA fingerprinting using arbitrarily primed PCR: RT-PCR, reverse transcriptase-polymerase chain reaction: tPA, tissue-type plasminogen activator: uPA, urokinase-type plasminogen activator

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and Genbank nucleotide sequence databases with the following accession number D88250.

Abstract

A cDNA of rat C6 cells was cloned, which was considered to be involved in glial cell differentiation induced by dibutyryl cyclic AMP and theophylline. The cDNA fragment of the gene, termed *r-gsp*, has been originally isolated by mRNA fingerprinting using arbitrarily primed polymerase chain reaction (RAP), and was homologous to complement C1s precursors of hamster and human. It encodes a protein of 694 amino acids containing a potential signal peptide, an EGF-like domain surrounded by two complement C1r/C1s related repeats, and a putative trypsin-type serine protease domain. Since the hamster and human C1s, and a protein coded by *r-gsp* shared high similarity in primary structure, *r-gsp* gene could encode a C1s counterpart of rat. Messenger RNA expression of this gene was markedly increased during cyclic AMP-induced glial cell differentiation. Its expression profile was well correlated with those of glial fibrillary acidic protein (GFAP) and S100B, which are known as glial differentiation markers. It was, moreover, observed that the *r-gsp* expression in brain increased considerably after birth like those of S100B and GFAP. The results presented here suggest that rat C1s gene would be also implicated in glial differentiation besides the complement cascade.

1. Introduction

Differentiation of glial cell is controlled by a complex program of differential expressions of various genes. Differential expression of numerous genes is regulated in spatially and/or temporarily limited manners during the glial development and differentiation (Raff, 1989). It is of importance to identify the genes implicated in glial differentiation to understand biological characters of glial and glioma cells. To study differential gene expression in this complex process, we have recently adopted mRNA fingerprinting using arbitrarily primed polymerase chain reaction (RAP) to rat C6 glial cell differentiation induced by cAMP (Sakai et al., 1997). RAP is a potentially useful method to detect altered gene expression in complex RNA populations (Welsh et al., 1992). Several differentially expressed cDNA fragments were obtained by RAP. One of them, designated C6A2 fragment, was homologous to the corresponding regions of hamster and human complement subcomponent C1s precursors. Complement C1s is a member of serine protease family and a key component to activate the classical pathway of the complement cascade (Tosi et al., 1987).

In this paper, we report the isolation and cloning of a full cDNA containing C6A2 fragment, designated *r-gsp* and then demonstrate its increased expression during glial differentiation and brain development.

2. Materials and Methods

2.1. Cell culture

A C6 cell line was obtained from Human Science Research Resources Bank (Osaka, Japan). C6 cells were maintained as previously described

(Yoshimura et al, 1996 and 1997). For differentiation, cells were treated with 1 mM dibutyryl cyclic AMP (dbcAMP) (Sigma, USA) and 0.25 mM theophylline (Wako, Japan) in serum-free D-MEM. Control treatments were carried out in the absence of both serum and these agents.

2.2. mRNA fingerprinting using arbitrarily primed PCR (RAP)

Two μg of DNase-treated total RNA, which was extracted from the cells at various differentiation stages, was reverse-transcribed using random hexamer-mixed primers. PCR conditions of RAP were described in detail in the previous report (Sakai et al., 1997). The amplified cDNAs were separated on sequencing gels followed by autoradiography. Bands of interest were excised from the gels and the DNA was eluted by boiling. The eluted DNA was reamplified, subcloned and sequenced. Homology analysis was performed using BLAST algorithm at NCBI, NLM, NIH (Bethesda, MD, USA). Differential expression displayed by RAP was confirmed by Northern blot analysis using these cDNA fragments as probes. One clone (365 bp), termed C6A2 fragment, was homologous to the corresponding regions of hamster and human complement subcomponent C1s precursors (Sakai et al., 1997). The primer sequence used in RAP to obtain C6A2 fragment was 5'-AAGAGGAACCAGGAGCCCATTG-3'.

2.3. Identification of full-length r-gsp cDNA

First, to obtain a cDNA fragment encoding serine protease domain of *r-gsp* product, two oligonucleotide primers for PCR were synthesized based on the cDNA sequence near the highly conserved amino acid regions (His^{482} and Ser^{638}) in the serine protease domain of hamster C1s. The primer sequences were 5'-CTGACGGCAGCTCACG-3' (sense primer) and 5'-

CCCACCGCTGTCCCC-3' (antisense primer). By RT-PCR using these primers, the cDNA fragment encoding serine protease domain of *r-gsp* product, termed SP fragment, was isolated from differentiated C6 cells. Next, to identify nucleotide sequence of *r-gsp* full-length cDNA, additional RT-PCR and 5'- and 3'- rapid amplification of cDNA ends (RACE) (Frohman, 1993) were performed using nucleotide primers derived from the C6A2 and SP cDNA fragments. The cDNA fragments obtained by PCR and RACE were subcloned and sequenced. Finally, to ensure that assemblage of all of the pieces generated during cloning created a genuine contiguous transcript, high fidelity PCR amplification of the entire coding region using primers based on 5'- and 3'- noncoding sequences was performed by *Pfu* DNA polymerase (Stratagene, USA). The cDNA fragments obtained by high fidelity PCR amplification were subcloned followed by nucleotide sequencing and restriction enzyme mapping.

2.4. Northern blot analysis, RT-PCR and RT-PCR Southern hybridization

Total RNA extracted from C6 cells at various differentiation stages was subjected to Northern hybridization. The C6A2 fragments obtained by RAP and the fragment containing full-length coding region of *r-gsp* gene by high fidelity PCR were used for Northern hybridization as probes. In addition, mRNA expressions of S-100B protein (Kuwano et al., 1984) and glial fibrillary acidic protein (GFAP) (Condroelli et al., 1994) were examined as markers of glial differentiation. Furthermore, to examine the expression of *r-gsp* mRNA in various tissues, total RNAs extracted from various organs of an adult rat were subjected to Northern hybridization and RT-PCR analysis. The primer sequences used for RT-PCR amplification of *r-gsp* gene were 5'-TTACTGTGGCAATGGATTCC-3' (AF primer: sense primer corresponding to nucleotide 1011-1030) and 5'-GTAGTAATATGGCTCTTCGC-3' (BR primer: anti-sense, 1421-1440). PCR reaction was performed for 28 cycles of

30 sec at 94°C, 1 min at 60°C and 1 min at 72°C. Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed for 22 cycles at 94°C for 30 sec, at 60°C for 1 min and at 72°C for 1 min. Sequences of the primers used for PCR of GAPDH are 5'-TGAAGGTCGGTGTGAACGGATTTG-3' (sense) and 5'-TGATGGCATGGACTGTGGTCATGA-3' (anti-sense).

To examine expression of *r-gsp* gene during development of brain, total RNA from whole brains of rats before and after birth was subjected to RT-PCR and RT-PCR Southern analysis. Whole brains were removed from fetal and neonatal rats at embryonic days 18 (E18), postnatal days 0 (P0), 7 (P7) and 14 (P14). Sequences of the primers used for RT-PCR were 5'-AGACTCTGTGGCCAGAGGTCC-3' (C6A2A primer: sense, 505-525) and 5'-GGACCCGACATACATCACTGG-3' (C6A2B primer: anti-sense, 1711-1731). The first strand cDNA synthesized from two μg of total RNA was amplified using these primers on the condition as follows: 28 cycles of 30 sec at 94°C, 1 min at 60°C and 1 min at 72°C. The PCR products were transferred onto a nylon membrane and hybridized with an internal probe (corresponding to nucleotide 1010-1440) of *r-gsp* cDNA. Expressions of S-100B protein and GFAP were also examined using RT-PCR as markers of glial differentiation. Sequences of the primers used for these PCR amplifications are as follows; S100B, 5'-AAGTCCACACCCAGTCCTCTCT-3' (sense) and 5'-CTCACTCATGTTCAAAGAACTC-3' (anti-sense); GFAP, 5'-GATGGAGCGGAGACGTATCACC-3' (sense) and 5'-TCACTTCTGCCTCAGGGTGCCG-3' (anti-sense). PCR reactions of S100B and GFAP were for 28 cycles at 94°C for 30 sec, at 60°C for 1 min and at 72°C for 1 min.

3. Results and Discussion

3.1. Isolation of full-length r-gsp cDNA by RT-PCR and RACE

Several cDNA fragments differentially expressed during C6 glial cell differentiation were obtained by RAP. One of them, termed C6A2 fragment was homologous to hamster and human C1s genes. In order to obtain the full-length cDNA clone, approximately 1 x 10^6 plaques of a rat whole brain cDNA library (Stratagene, USA) were screened with C6A2 fragment as a probe. However, no positive clones were isolated with this method, suggesting its scarce expression in adult rat brain. Therefore, RT-PCR and RACE were performed to obtain full-length cDNA. To correct nucleotide mismatches probably caused by misreading by *Taq* polymerase, nucleotide sequences and restriction enzyme mapping were compared among plural subcloned cDNA fragments. Moreover, nucleotide sequences of cloned cDNA fragments were confirmed by the cDNA fragment obtained by high fidelity *Pfu* DNA polymerase. Finally, a continuous cDNA fragment containing 2924 nucleotides was obtained. Since we demonstrated that C6A2 fragment hybridized with a transcript of 3.0 kb in the previous report (Sakai et al., 1997), the continuous nucleotide sequence was considered to cover nearly full-length *r-gsp* cDNA.

3.2. Primary structure of cDNA and deduced amino acid sequences of r-gsp

Fig. 1 shows the restriction enzyme map, the nucleotide and deduced amino acid sequences of the cloned *r-gsp* cDNA. The cDNA contained an open reading frame of 2082 bp that started with an ATG codon at position 247, followed by the second ATG triplet at position 265. However, since the sequence near the first ATG agrees with the consensus sequence for initiation described by Kozak (1991) (Fig.1B), translation of *r-gsp* mRNA probably

starts at the first ATG. The cDNA could encode a protein made up of 694 amino acids including a typical signal peptide sequence of 21 amino acids in the N-terminal end. The 3'-noncoding region contained a potential polyadenylation sequence, AATAAA, which was located at 23 nucleotides upstream from the putative polyA$^+$ tail. The sequence of C6A2 fragment obtained by RAP corresponded to the nucleotide number 251 - 561 of the full-length cDNA (Fig.1B, shown by the bold and italic letters).

Alignment of serine protease domain of the predicted amino acid sequence of *r-gsp* with those of other serine proteases is shown in Fig. 2A. The key amino acid residues conserved in serine proteases are found in *r-gsp* product, located at His^{481}, Asp^{535}, and Ser^{637} (Fig.1B and 2A, indicated by closed triangles). The deduced amino acid sequence of *r-gsp* in full sequence has 84%, 75%, and 21% identities with those of hamster, human C1s, and rat trypsinogen I, respectively. The alignment and primary structure of regions surrounding these key residues are well conserved. Furthermore, putative glycosylation sites of *r-gsp* were located at amino acid residues 180-182 and 412-414 (Fig.1B). Two glycosylation sites have also been found in hamster and human C1s precursors (Kinoshita et al., 1989; Tosi et al., 1987), positions of which well corresponded to those of putative glycosylation sites of *r-gsp*. In addition, the number and alignment of all cysteine residues of *r-gsp* product totally matched those of hamster and human C1s precursors. Since the C1s precursors and *r-gsp* product shared the high similarities of primary structures and the same domains, a protein coded by *r-gsp* gene could be a C1s counterpart of rat.

Hamster and human C1s precursors have shown to contain an epidermal growth factor (EGF) precursor-like region between two repeated domains (C1r/C1s-related domains) (Kinoshita et al., 1989; Tosi et al., 1987). *r-gsp* product also contains an EGF precursor-like region (position 137-177, double-underlined sequences in Fig.1B), which exhibited high identity (98%)

to that of hamster C1s (Fig.2B). Many members of serine proteases, such as plasminogen activators, blood coagulation factor VII, IX, X, XII and protein C (Furie and Furie, 1988), also have EGF-like domains in their N-terminal portions. This domain is thought to be related with Ca^{2+} binding and to play important roles in cell growth, differentiation, and development in various tissues including brain (Handford et al., 1991). EGF-like domain of hamster C1s is considered to be a regulatory subunit with Ca^{2+} binding site (Kinoshita et al., 1989). The EGF-like domain of *r-gsp* product has a high homology with hamster and human counterparts, suggesting that this domain would be an essential region for regulation of their functions.

Some members of extracellular serine proteases, such as tissue- and urokinase-type plasminogen activators (tPA and uPA) (Sumi et al., 1992), thrombin (Dihanich et al., 1991), calpain (DeLuca et al., 1993), and neuropsin (Chen et al., 1995), have been reported to be expressed in brain tissues and play important roles in neuronal development and activity. However, to our knowledge, there is little or no information about the functions of C1s protease in nervous tissues and cultured glial cells. The principal physiological role of C1s has been known to activate the classical pathway of the complement cascade. However, its multipotential functions have recently been demonstrated. For example, C1s protease cleaves type I and II collagen (Yamaguchi et al., 1990), major histocompatibility complex class I antigen (Eriksson and Nissen, 1990), β_2-microglobulin (Nissen et al., 1990), and matrix metalloprotease 9 (Sakiyama et al., 1994). Toyoguchi et al. (1996) have demonstrated that C1s is involved in chondrocyte differentiation and cartilage remodeling. Thus, C1s protease has various important functions besides the complement cascade.

3.3 Expression of r-gsp gene during C6 glial cell differentiation and brain development, and its tissue distribution

In development and maturation of nervous tissues, some proteases and their proteolytic activities may be important for formation, retraction, and rearrangement of neuronal and glial cytoprocesses (Sumi et al., 1992). In the present study, it was shown that C6 cells initiate extension of the cytoprocesses following treatment with dbcAMP/theophylline, and most of cells were changed to astrocytic-shape with long processes at 24 h (Fig.3A). The temporal profile of morphological changes was well compatible with the increase in the expression level of glial differentiation markers (S100B and GFAP) (Fig.3A and B). This finding has led us to speculate that a protease encoded by *r-gsp* gene would be implicated in the morphological change at certain specific stage during differentiation in glial cells. In this context, we have examined the expression of *r-gsp* during C6 differentiation.

Northern blot analysis revealed that the C6A2 fragment obtained by RAP and the fragment contained full-length coding region of *r-gsp* cDNA obtained by high fidelity PCR as probes were hybridized to only one transcript derived from differentiated C6 cells (Fig.3B). Expression of the 3.0 kb transcript corresponding to *r-gsp* was at low level in untreated control cells, and was then gradually up-regulated during differentiation after treatment with dbcAMP/theophylline. Such altered mRNA expression of *r-gsp* gene was well correlated with those of S100B and GFAP, known as a glial differentiation markers (Kuwano et al., 1984 and Condroelli et al., 1994). Treatment of cells with serum free-medium without dbcAMP did not promote expression of *r-gsp* (Fig.3C). Its induction by dbcAMP was enhanced by addition of theophylline. These results indicate that altered expression of *r-gsp* was due to cAMP-induced glial differentiation but not caused by simple serum deprivation.

Moreover, expression of *r-gsp* in various tissues was examined by Northern hybridization (Fig.4A) and RT-PCR analysis (Fig.4B). The *r-gsp*

gene was expressed in various organs; liver, spleen, heart, lung, kidney, small intestine, testis, and skeletal muscle. In brain tissues of adult rat, the corresponding bands were hardly observed due to faint intensity by Northern blotting but clearly demonstrated by RT-PCR. Although cDNA cloning of rat C1s has not yet been performed, the tissue distribution in rat organs has been examined by an immunohistochemical study with cross-reactions of monoclonal antibodies against hamster C1s (Sakiyama et al., 1991). The distribution profile of rat C1s well matched with that obtained by the present study. These findings also support the notion that the protein encoded by *r-gsp* is rat C1s. In addition, to gain further support for the involvement of *r-gsp* in glial differentiation, the changes of its expression were investigated during brain development. Total RNA was extracted from whole brains of rats at E18, P0, P7 and P14. The expression levels of *r-gsp* were determined by RT-PCR followed by Southern blot analysis. As shown in Fig.5, the level of *r-gsp* mRNA increased during brain development like those of S100B and GFAP, which are known to be up-regulated during glial differentiation. These results provided additional evidence suggesting the possible implication of *r-gsp* in brain development.

The data obtained in this work suggest possible implication of r-gsp in C6 glial cell differentiation. However, its expression level was low in brain tissues of adult rat. Induction of gene expression of r-gsp may be more enhanced than that of normal astrocytes in rat brain, since C6 cells have transformed phenotypes. It is also possible that *r-gsp* mRNA may express in limited cells in brain tissues of adult rat. The serine proteases, such as plasminogen activators (Sumi et al., 1992) and prothorombin (Dihanich et al., 1991) which are originally thought to function in extra-nervous tissues, appeared to be expressed in brain in spatially and/or temporarily limited manners. Previously, we have demonstrated that the expression of *r-gsp* reached a maximum level at 24h after treatment of C6 cells with cAMP and

then decreased (Sakai et al., 1997). Thus, it could be suggested that *r-gsp* mRNA would also be expressed in spatially and/or temporarily limited manners in rat brain. Therefore, ontogenic analysis of developing rat brain using immunohistochemistry or *in situ* hybridization should be required to reveal neurobiological functions of the protein coded by *r-gsp*.

4. Conclusion

In the present study, we have cloned *r-gsp* gene encoding a protein homologous to hamster and human complement C1s, which are members of extracellular serine protease family. The mRNA expression of *r-gsp* gene was up-regulated during cAMP-induced glial differentiation and brain development of rat. Its expression profile was well correlated with those of S100B and GFAP. Messenger RNA expression of *r-gsp* was observed in various tissues, even in lesser extent in adult brain tissues. These results suggest that *r-gsp* gene would be implicated in glial differentiation in spatially and/or temporarily limited manners. For understanding the roles of this protease in glial differentiation, further studies of immunohistochemical analysis or *in situ* hybridization during brain development are required.

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Figure legends

Fig.1: Restriction enzyme map, the nucleotide and deduced amino acid sequences of *r-gsp* cDNA. (**A**) Restriction enzyme map of *r-gsp* cDNA. The open box represents the open reading frame. (**B**) Nucleotide and deduced amino acid sequences of *r-gsp*. The nucleotide sequences shown by bold italic letters indicate the C6A2 cDNA fragment obtained by RAP. The putative translation initiation site and polyadenylation signal sequence are indicated by underlines. The dotted and double underlines are the potential signal peptide sequence and the EGF-like domain, respectively. Amino acid residues marked with closed triangles represent key amino acids conserved in trypsin-type serine proteases. Two Asn residues marked with closed circles are the possible glycosylation sites.

Fig.2: Comparisons of serine protease domains and EGF-like domains of *r-gsp* product with other C1s precursors. (**A**) Alignment of the serine protease domains of the predicted amino acid sequence of *r-gsp* gene with those of other serine proteases. Conserved amino acid residues are highlighted in black. Amino acid residues marked with closed triangles represent key amino acids of trypsin-type serine proteases. Two oligonucleotide primers for RT-PCR for serine protease domain (SP-U and SP-L) are synthesized based on the cDNA sequence near the highly conserved amino acid regions (shown by overlaid dots) in the serine protease domain of hamster C1s. (**B**) Comparisons of EGF-like domains of the deduced amino acid sequences of *r-gsp* with other C1s and mouse EGF precursors. Conserved amino acids are highlighted in black.

Fig.3: Changes of cell shape and *r-gsp* mRNA expression during C6 cell differentiation. (**A**) Morphological changes of C6 glial cells during

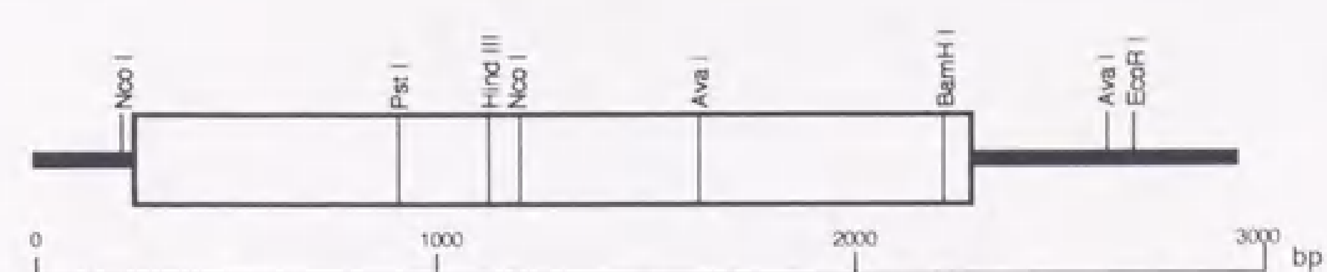
differentiation. Photographs were taken under a phase-contrast microscope (X100). **a**: Untreated control cells. **b** and **c**: Cells treated with 1mM dbcAMP and 0.25 mM theophylline for 12 h and for 24 h, respectively. (**B**) Northern blot analysis of *r-gsp* expression during C6 glial cell differentiation. Twenty μg of total RNA from normal untreated cells (0 h) and dbcAMP-treated (6, 12, 18, 24 h) cells was subjected to Northern hybridization probed with ^{32}P-labeled cDNA fragments (**a**: C6A2 fragment, **b**: the fragment containing full-length coding region of *r-gsp*, and rat GFAP and S100B). The blots were also hybridized with a rat GAPDH probe for the purpose of quantitation. The positions of ribosomal RNAs are indicated on the right. (**C**) Induction of *r-gsp* mRNA by dbcAMP/theophylline. Twenty μg of total RNA were isolated from C6 cells cultured in different conditions; growth medium (containing serum) or combinations of dbcAMP and theophylline in absence of serum. The membranes were hybridized with ^{32}P-labeled C6A2 fragment as a probe.

Fig.4: Expression of *r-gsp* gene in various rat tissues. (**A**) Northern blot analysis of *r-gsp* mRNA. Twenty μg of total RNA from a variety of organs of a young adult rat was subjected to Northern blot analysis. For positive control, total RNA (20 μg) extracted from differentiated C6 cells was included. C6A2 fragment was ^{32}P-labeled and used as a probe for hybridization. The membrane was also hybridized with a rat GAPDH probe for the purpose of quantitation. The positions of ribosomal RNAs are indicated on the right. (**B**) RT-PCR of *r-gsp* in various rat tissues. The first strand cDNAs synthesized from two μg of total RNA from various rat tissues were amplified using AF and BR primers. Gels were stained by ethidium bromide.

Fig.5: Changes of *r-gsp* mRNA expression during development of rat brain. Two μg of total RNA from whole brains of fetal and neonatal rats (E18, P0, P7 and P14) was subjected to RT-PCR analysis. Differential

expression of *r-gsp* was demonstrated by a autoradiogram of RT-PCR Southern analysis. The PCR products amplified by C6A2A and C6A2B primers (corresponding to nucleotide 505-1731) were transferred onto a nylon membrane and hybridized with a ^{32}P-labeled cDNA fragment (corresponding to nucleotide 1010-1440) as an internal probe. In addition, gene expression of GFAP, S100B protein and GAPDH was examined by RT-PCR. Gels were stained by ethidium bromide.

A



B

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   1 GGAGGTATCGAGGAAGAGAGAACAGGGAGGTGGGGCGGAGGTTCCTCGCAGAGCCTCTGGAGCCGCAGGGGCTTCACGGCATGACCAGAA   90
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 181 GTATTTGTCAGATAAAGACGGCCAGCCCGGCTGCCCTTTACCTCCAAGTCAGAGATCCAGAGAGCCATGGGCAAATCGCCAGAGATGTGG  270
   1                                                                    M  G  K  S  P  E  M  W    8

 271 TGCTTTGTCTTCTTTTCTCTTTTGGCATCGTTTTCTGCTGAGCCTACCATGTATGGGGAGATCCTGTCCCCTAATTATCCCCAGGCGTAC  360
   9  C  F  V  F  F  S  L  L  A  S  F  S  A  E  P  T  M  Y  G  E  I  L  S  P  N  Y  P  Q  A  Y   38

 361 CCCAATGAGGTCGTGAAAACTTGGGACATAGAAGTCCCAGAGGGGTTTGGGATTCACCTTTACTTCACCCATCTGGACATGGAGCTGTCA  450
  39  P  N  E  V  V  K  T  W  D  I  E  V  P  E  G  F  G  I  H  L  Y  F  T  H  L  D  M  E  L  S   68

 451 GAGAACTGTGCATACGACTCAGTGCAGATAATCTCAGGAGGTATCGAGGAAGAGAGACTCTGTGGCCAGAGGTCCAGCAAGAGTCCCAAC  540
  69  E  N  C  A  Y  D  S  V  Q  I  I  S  G  G  I  E  E  E  R  L  C  G  Q  R  S  S  K  S  P  N   98

 541 TCCCCCACTGTAGAAGAGTTTCAATTCCCATACAATAGGCTCCAGGTGGTCTTTACGTCAGACTTCTCCAACGAGGAACGGTTTACTGGC  630
  99  S  P  T  V  E  E  F  Q  F  P  Y  N  R  L  Q  V  V  F  T  S  D  F  S  N  E  E  R  F  T  G  128

 631 TTTGCAGCGTATTACTCAGCCGTAGATGTAAATGAATGCACAGACTTTACAGATGTCCCTTGCAGCCACTTCTGCAATAACTTCATTGGT  720
 129  F  A  A  Y  Y  S  A  V  D  V  N  E  C  T  D  F  T  D  V  P  C  S  H  F  C  N  N  F  I  G  158

 721 GGATACTTCTGCTCCTGCCCCCCAGAATACTTCCTCCACGATGACATGAGGACTTGTGGGGTCAACTGTAGTGGGGATGTATTCACTGCC  810
 159  G  Y  F  C  S  C  P  P  E  Y  F  L  H  D  D  M  R  T  C  G  V  N  C  S  G  D  V  F  T  A  188
                                                                     ●
 811 TTGATTGGGGAGATCGCAAGTCCCAATTATCCCAACCCATACCCGGAGAACTCAAGGTGTGAATACCAGATTCGGCTGCAGGAGGGCTTC  900
 189  L  I  G  E  I  A  S  P  N  Y  P  N  P  Y  P  E  N  S  R  C  E  Y  Q  I  R  L  Q  E  G  F  218

 901 CGACTGGTGTTGACTATCCGGAGAGAAGATTTTGATGTGGAACCAGCGGACTCAGAGGGGAACTGCCACGACAGTTTGACTTTTGCTGCA  990
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 991 AAAAACCAACAGTTTGGTCCTTACTGTGGCAATGGATTCCCTGGACCTCTAACTATTAAAACCCAGAGCAATACTCTTGATATTGTCTTT 1080
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1081 CAAACTGACCTAACGGGGCAAAATAAAGGCTGGAAGCTTCGTTACCATGGAGATCCCATCCCCTGTCCCAAAGAAATCAGTGCTAATTCT 1170
 279  Q  T  D  L  T  G  Q  N  K  G  W  K  L  R  Y  H  G  D  P  I  P  C  P  K  E  I  S  A  N  S  308

1171 ATCTGGGAGCCCGAAAAGGCAAAATACGTGTTCAAAGATGTCGTGAAGATAACCTGTGTGGATGGATTCGAAGTTGTGGAGGGAAATGTT 1260
 309  I  W  E  P  E  K  A  K  Y  V  F  K  D  V  V  K  I  T  C  V  D  G  F  E  V  V  E  G  N  V  338

1261 GGCTCAACATCATTCTATTCCACTTGTCAAAGCAACGGACAGTGGAGCAATTCCAGGCTAGAGTGTCAACCTGTGGACTGTGGTGTTCCA 1350
 339  G  S  T  S  F  Y  S  T  C  Q  S  N  G  Q  W  S  N  S  R  L  E  C  Q  P  V  D  C  G  V  P  368

1351 GAACCCATTGAGAATGGTAAAGTTGAAGACCCAGAAGACACTGTATTCGGCTCCGTCATCCACTACACGTGCGAAGAGCCATATTACTAC 1440
 369  E  P  I  E  N  G  K  V  E  D  P  E  D  T  V  F  G  S  V  I  H  Y  T  C  E  E  P  Y  Y  Y  398

1441 ATGGAACAGGAAGAAGGCGGAGAGTATCACTGTGCTGCTAATGGGAGCTGGGTGAATGACCAGCTGGGTGTCGAGCTTCCAAAATGTATT 1530
 399  M  E  Q  E  E  G  G  E  Y  H  C  A  A  N  G  S  W  V  N  D  Q  L  G  V  E  L  P  K  C  I  428
                                          ●
1531 CCAGTCTGTGGAGTACCCACCGAGCCCTTTAAAGTACAGCAGAGGATATTTGGAGGATACTCTACAAAGATTCAAAGTTTTCCTTGGCAG 1620
 429  P  V  C  G  V  P  T  E  P  F  K  V  Q  Q  R  I  F  G  G  Y  S  T  K  I  Q  S  F  P  W  Q  458

1621 GTCTACTTTGAGTCCCCCCGAGGTGGCGGGGCTCTTATCGATGAGTACTGGGTGCTGACGGCCGCTCACGTTGTGGAGGGAAACTCTGAC 1710
 459  V  Y  F  E  S  P  R  G  G  G  A  L  I  D  E  Y  W  V  L  T  A  A  H  V  V  E  G  N  S  D  488
                                                                     ▲
1711 CCAGTGATGTATGTCGGGTCCACACTTCTGAAAATAGAGCGGTTGAGAAATGCCCAGAGGCTCATCACTGAACGTGTGATTATTCATCCC 1800
 489  P  V  M  Y  V  G  S  T  L  L  K  I  E  R  L  R  N  A  Q  R  L  I  T  E  R  V  I  I  H  P  518

1801 AGCTGGAAACAAGAGGACGACCTGAATACACGGACAAATTTTGACAATGACATTGCCCTGGTGCAGCTCAAAGACCCTGTGAAAATGGGA 1890
 519  S  W  K  Q  E  D  D  L  N  T  R  T  N  F  D  N  D  I  A  L  V  Q  L  K  D  P  V  K  M  G  548
                                                   ▲
1891 CCCACTGTTGCCCCCATCTGCCTGCCAGAAACCTTCTCAGACTACAACCCCTCAGAGGTTGACCTGGGGCTGATCTCTGGGTGGGGCCGA 1980
 549  P  T  V  A  P  I  C  L  P  E  T  F  S  D  Y  N  P  S  E  V  D  L  G  L  I  S  G  W  G  R  578

1981 ACAGAGATTAGAACCAATGTTATTCAACTCAGAGGGGCGAAGTTACCCATAACATCTTTAGAAAAGTGCCAGCAGGTGAAAGTGGAAAAC 2070
 579  T  E  I  R  T  N  V  I  Q  L  R  G  A  K  L  P  I  T  S  L  E  K  C  Q  Q  V  K  V  E  N  608

2071 CCGAAAGCGAGGTCAAACGACTATGTTTTCACTGACAACATGATCTGTGCTGGGGAAAAGGGTGTGGACAGCTGTGAAGGTGACAGCGGA 2160
 609  P  K  A  R  S  N  D  Y  V  F  T  D  N  M  I  C  A  G  E  K  G  V  D  S  C  E  G  D  S  G  638
                                                                                         ▲
2161 GGGGCTTTTGCTCTGCCGGTCCCCAATGTCAAGGACCCCAAATTCTATGTGGCTGGCCTGGTGTCCTGGGGAAAAAGTGTGGGACCTAT 2250
 639  G  A  F  A  L  P  V  P  N  V  K  D  P  K  F  Y  V  A  G  L  V  S  W  G  K  K  C  G  T  Y  668

2251 GGGATCTACACAAAGGTAAAGAACTACGTGGACTGGATCCTGAAAACTATGCAGGAGAATAGTGGGCCCAAGAAGGACTGATCCGTAGTA 2340
 669  G  I  Y  T  K  V  K  N  Y  V  D  W  I  L  K  T  M  Q  E  N  S  G  P  K  K  D  *           694

2341 ACAACACCCCTCCAGGACTAGCAAGGTCATTTTTCTCAGATCCTGGGACGGTCCCATTATTTCAAAATGATGGAGAGAGGGTGTGGGAGC 2430
2431 ATGGTTAACGTTGAACATGATTGTCAAGAAGCCTGCTTGGAGGCAGAGTTGATCACTGAGCCGTGTTGGTTATTCAGTTGCTATTGCTAA 2520
2521 CAACATGCGGAAGCCTTTCTGTCTTGCTTCATCCCACAGGGATATCTTAAACGATTTCCCCCTCATTTAACCCGCTTGAAATCCTTATTG 2610
2611 CTTACAGTAAAGCATGTTTCCAATCTGGTTCTGGCTGCTCGAGAGCCCAGAAGGAGAGGGAAATTTGAGGGTATTTTGTCATGGAATTCA 2700
2701 GGCATCGACAGGTTGTCTGAAACACTATGCAGTCAGGGAACACAGCCTTTTTTCTAAGTGAGATTTACCCAATAGCTGGAAGTCAGAATT 2790
2791 GACTACCTTAGCTTTCCTTTGTGAGTTGTTTCAATATGTTCCCTAGAAATTAGTTTTCTTATAATCCTCCTTTGTATCATACAATGTAAT 2880
2881 GACTTAATAAAAGAGAAATTGAACATTGAAAAAAAAAAAAAAAAA                                             2924
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Fig.1 H. Sakai et al

A

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                                             .....
Rat       r-gsp        463  SPRGGGALI-DEYWVLTAAHV------VE-G--NSDPVMYVGSTLLKIERLRNAQ  507
Hamster    C1s         464  FPRAGGALI-GEHWVLTAAHV------VE-G--NSDPSMYVGSTSVRMENLANVQ  508
Human      C1s         457  NPWAGGALI-NEYWVLTAAHV------VE-G--NREPTMYVGSTSVQTSRLAKSK  501
Rat trypsinogen I       44  YHFCGGSLINDQ-WVVSAAHCYKSRIQVRLGEHNIN-VLE-GDEQF-I----NAA   91
                                          ▲

Rat       r-gsp        508  RLITERVIIHP--S-WKQEDDLNTRTNFDNDIALVQLKDPVKMGPTVAPICLPET  559
Hamster    C1s         509  KLTTDRVIIHP--G-WKPGDDLSTRTNFDNDIALVRLKDPVKMGPTVSPICLPGT  560
Human      C1s         502  MLTPEHVFIHP--G-WKLLEVPEGRTNFDNDIALVRLKDPVKMGPTVSPICLPGT  553
Rat trypsinogen I       92  K-I----IKHPNYSSWT----LN---N---DIMLIKLSSPVKLNARVAPVALP--  129
                                                        ▲

Rat       r-gsp        560  FSDYNPSEVDLG---LISGWGRTEIRTNVIQLRGAKLPITSLEKCQQVKVENPKA  611
Hamster    C1s         561  SSEYEPSEVDLG---LISGWGRTERRNIVIQLRGAKLPVTSLEKCRQVKEENPKA  612
Human      C1s         554  SSDYNLMDVDLG---LISGWGRTEKRDRAVRLKAARLPVAPLRKCKEVKEEKPTA  605
Rat trypsinogen I      130  -SACAPA----GTQCLISGWGNTLS--N-----GVNNPDL-LQ-C--VDA--PVL  166

                                                          .....
Rat       r-gsp        612  RSN-D----YVF--TDNMICAG--EKGVDSCEGDSGGAFALPV--PNVKDPKFYV  655
Hamster    C1s         613  RAD-D----YVF--TSNMICAG--EKGVDSCQGDSGGAFALPV--PNVRDPKFYV  656
Human      C1s         606  DAE-A----YVF--TPNMICAGG-EKGMDSCKGDSGGAFA--VQDPNDKT-KFYA  649
Rat trypsinogen I      167  -SQADCEAAYPGEITSSMICVGFLEGGKDSCQGDSGG----PV----VCN-----  207
                                                           ▲
```

B

```
Rat       r-gsp        137  DVNECTDFTDVPCSHFCNNFIGGYFCSCPPEYFLHDDMRTC  177
Hamster    C1s         137  DVNECTDFTDVPCSHFCNNFIGGYFCSCPPEYFLHDDMRNC  177
Human      C1s         131  DINECTDFVDVPCSHFCNNFIGGYFCSCPPEYFLHDDMKNC  171
Mouse EGF precursor    362  DVNECAT-QNMGCTLGCENTPGSYHCTCPTGFVLLPDGKQC  401
```

Fig.2 H.sakai et al

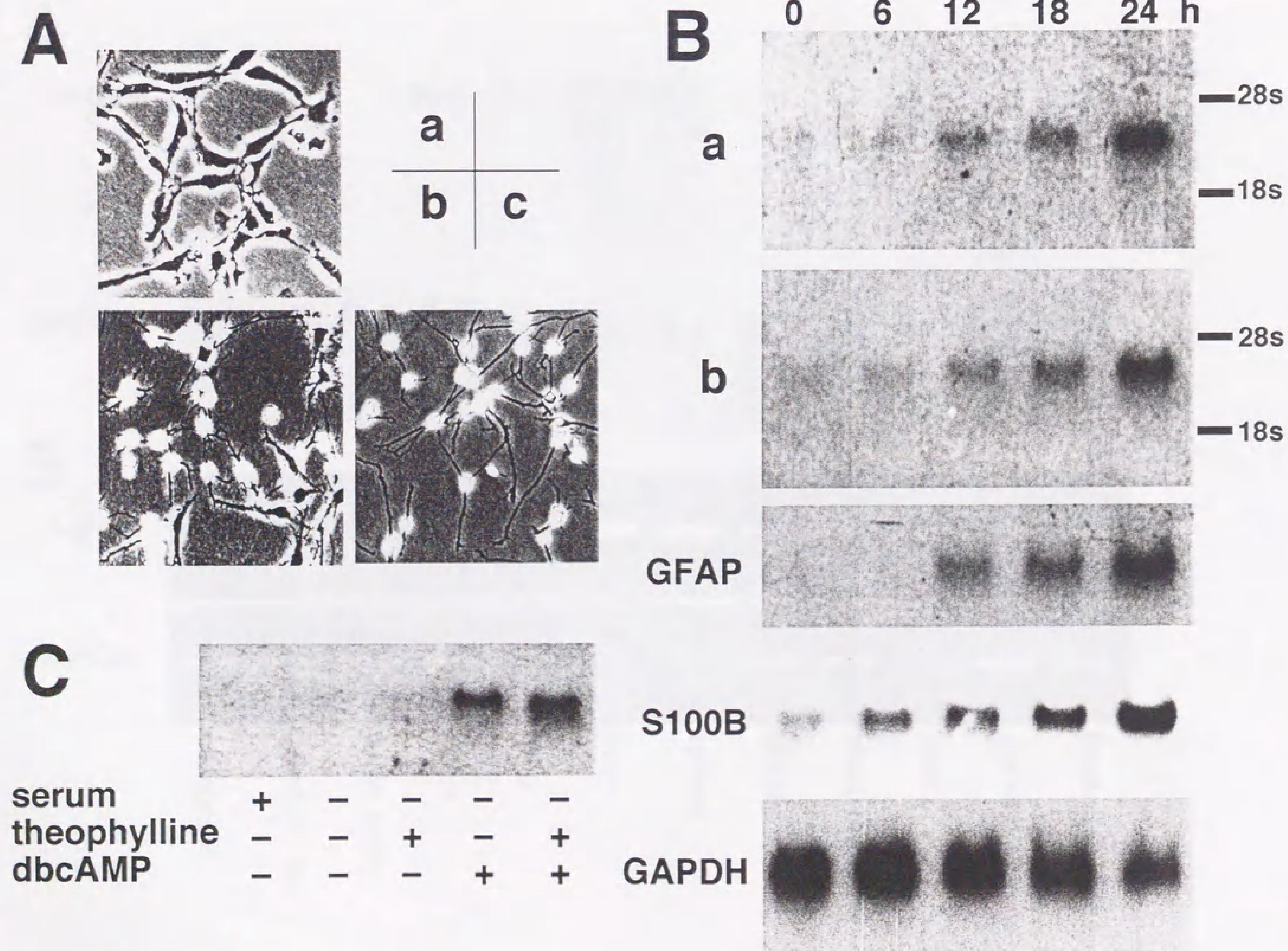


Fig.3 H. Sakai et al

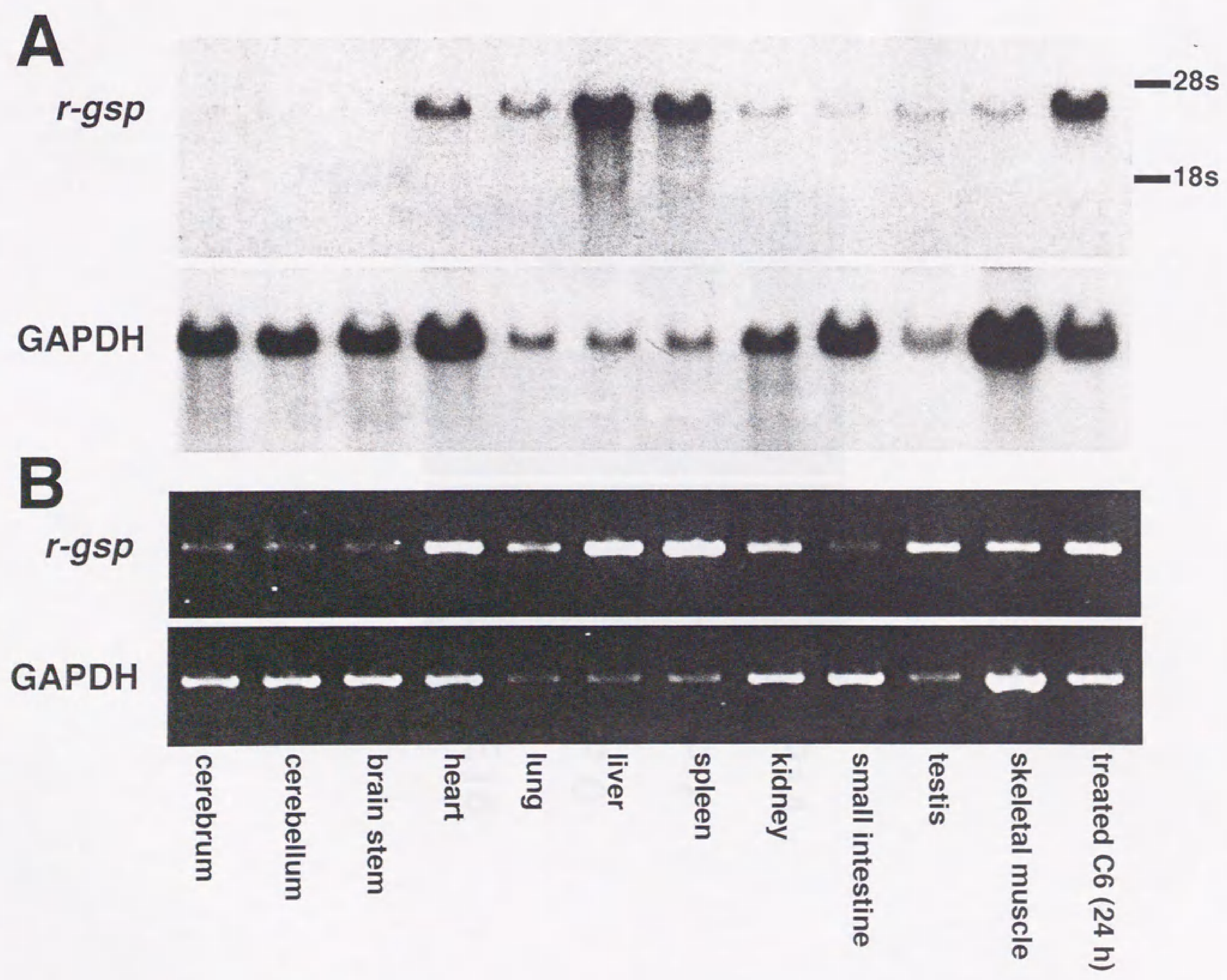
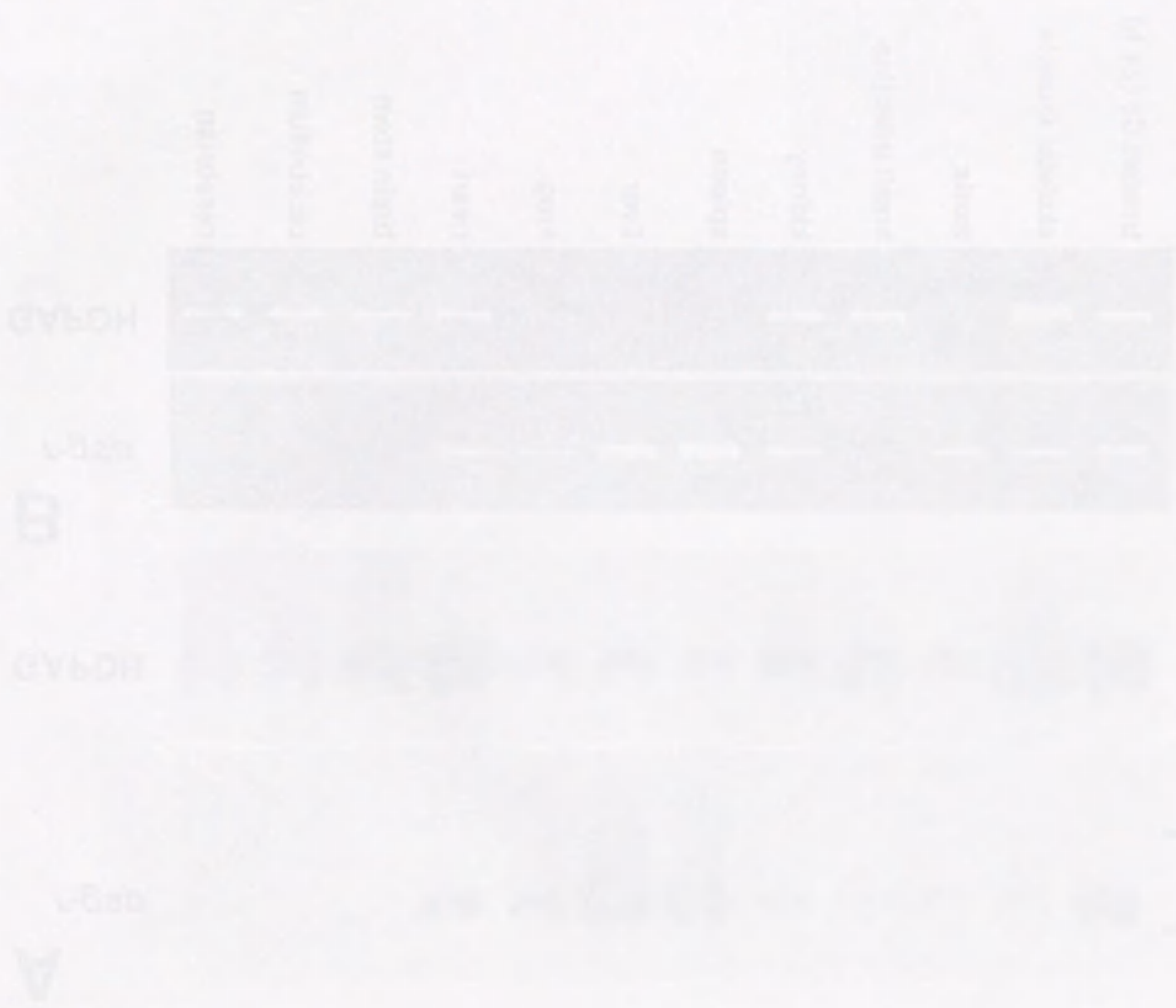


Fig.4 H. Sakai et al



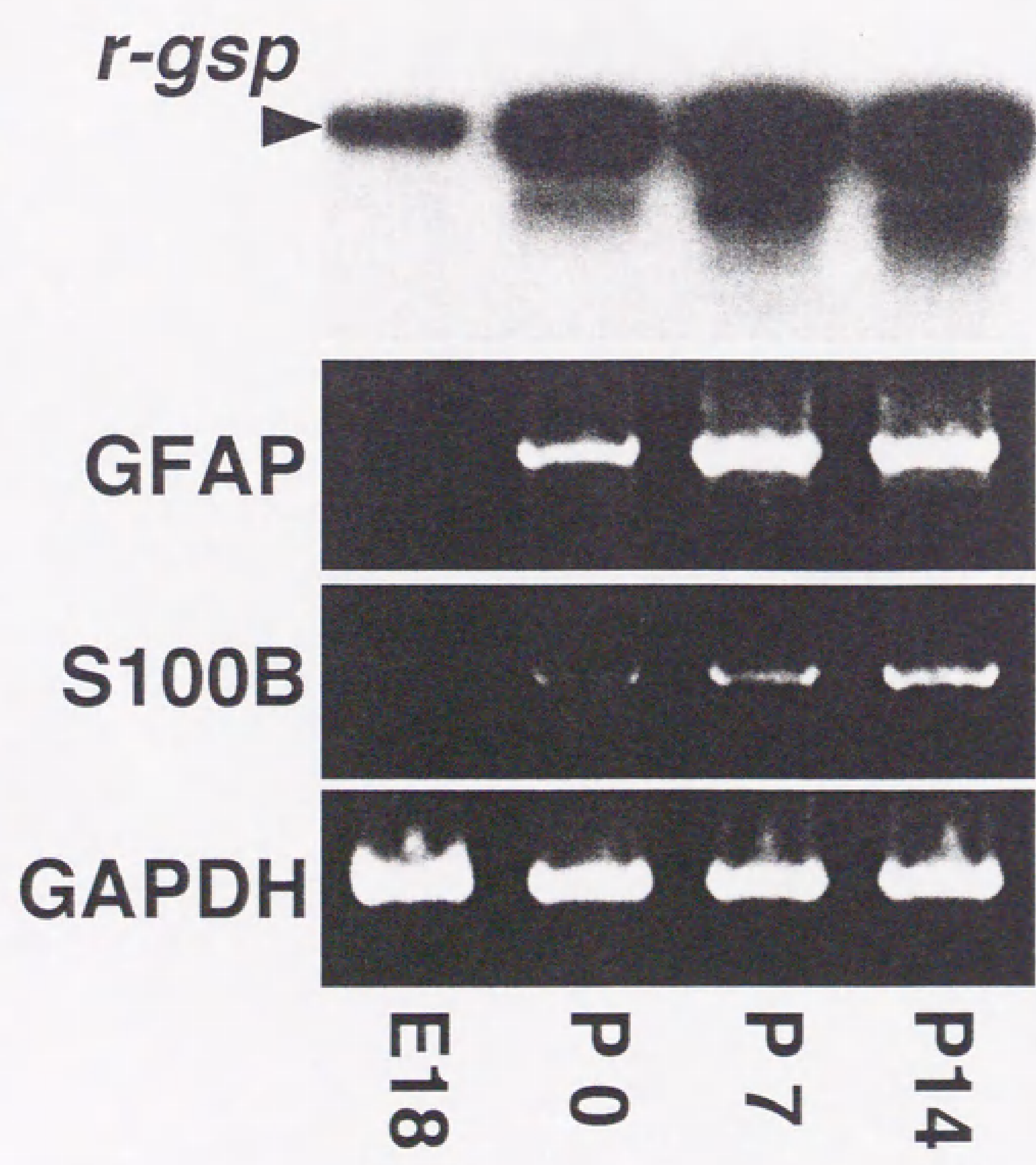


Fig.5 H. Sakai et al