The promoter of brain-specific angiogenesis inhibitor 1-associated protein 4 drives developmentally targeted transgene expression mainly in adult cerebral cortex and hippocampus

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Abstract **Restricting transgene expression to specific cell types and maintaining long-term expression are major goals for gene therapy. Previously, we cloned brain-specific angiogenesis inhibitor 1-associated protein 4 (BAI1-AP4), a novel brain-specific protein that interacts with BAI1, and found that it was developmentally upregulated in the adult brain. In this report, we isolated 5 kb of the 5′ upstream sequence of the mouse BAI1-AP4 gene and analyzed its promoter activity. Functional analyses demonstrated that an Sp1 site was the enhancer, and the region containing the transcription initiation site and an AP2-binding site was the basal promoter. We examined the ability of the BAI1-AP4 promoter to drive adult brain-specific expression by using it to drive lacZ expression in transgenic (TG) mice. Northern blot analyses showed a unique pattern of β-galactosidase expression in TG brain, peaking at 1 month after birth, like endogenous BAI1-AP4. Histological analyses demonstrated the same localization and developmental expression of β-galactosidase and BAI1-AP4 in most neurons of the cerebral cortex and hippocampus. Our data indicate that TG mice carrying the BAI1-AP4 promoter could be a valuable model system for region-specific brain diseases.**

Keywords: BAI1-AP4 promoter; Neuron-specific expression; Transgene; Developmental expression

1. Introduction

The use of cell-type-specific promoters is invaluable for generating transgenic (TG) mouse models of human diseases and for testing the effects of overexpressed functional gene products [1]. Spatiotemporally restricted gene targeting is needed for analyzing the functions of various molecules in a variety of biological processes. Neuron-specific enolase (NSE) is developmentally expressed in the brain, with expression in mature neurons and paraneurons [2]. The NSE promoter has been used to generate TG mouse models for brain-specific disease. In TG mice expressing β-galactosidase under NSE promoter control, the expression of the *NSE-lacZ* transgene paralleled the expression of the *NSE* gene with regard to developmental onset, but not postnatal mRNA accumulation [3].

Previously, brain-specific angiogenesis inhibitor 1-associated protein 4 (BAI1-AP4) was isolated as a novel brain-specific protein that interacted with the Refsum disease gene product and BAI1 [4,5]. In situ hybridization analyses of the brain showed specific localization of *BAI1-AP4* to the supragranular layer in the cerebral cortex, hippocampus, dentate gyrus (DG), Purkinje cell layer, and neuronal layers of the retina. The expression level of *BAI1-AP4* increased as the development of the brain progressed. Specifically, *BAI1-AP4* expression was markedly induced after eyelid opening, reached its highest level at 1 month of age, and maintained a high level throughout adult life [6]. BAI1-AP4 showed a unique pattern of spatial expression, being expressed only in certain mature neurons in the adult brain. However, the mechanisms regulating the BAI1-AP4 gene at the promoter level have not yet been reported.

In the present study, we cloned and characterized the BAI1-AP4 gene promoter, and examined the potential of this upstream sequence in producing neuron-specific expression of a heterologous protein (lacZ) in TG mice. We found that the spatial and temporal expression patterns of the lacZ transgene in the cerebral cortex and hippocampus of the TG brain were roughly the same as those of endogenous BAI1-AP4. This TG strategy using the BAI1-AP4 promoter may help in the development of valuable animal brain-specific disease models to study memory and learning disorders.

2. Materials and methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The Ethics Committee of Chonnam National University Medical School approved all experimental protocols.

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Abbreviations: BAI1-AP4, brain-specific angiogenesis inhibitor 1-associated protein 4; HSV-TK, herpes simplex virus-thymidine kinase; NSE, neuron-specific enolase; TG, transgenic

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2.1. Cloning of the BAI1-AP4 promoter gene

To clone the BAI1-AP4 promoter, a 129-cell genomic library was screened with the 5′ portion of BAI1-AP4 cDNA (450 bp). One positive plaque was obtained from this screening, and genomic DNA (~11 kb) was purified and sequenced using the dideoxy chain terminating approach (Fig. 1) as described [5].

2.2. Primer extension

A primer, complementary to nucleotides +28 to +55 of the BAI1-AP4 promoter, was labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. The duplexes were incubated at 42 °C for 1 h in the same buffer plus 10 mM dNTP and 50 U of M-MLV reverse transcriptase (Boehringer–Mannheim). The reaction products were resolved on an 8% denaturing polyacrylamide gel followed by image analysis.

2.3. Preparation of plasmids for deletion mutants

A 3150-bp fragment containing the 5′ flanking region and transcription start site of the BAI1-AP4 gene was prepared. PCR was used to prepare deletion constructs, with genomic DNA of BAI1-AP4 as template. PCR was performed using specific primers with *Kpn*I and *Sma*I linker sites. Each PCR product was cloned into the pGL3-Basic vector (Promega) at the *Kpn*I and *Sma*I sites. The pGL3-Basic plasmids containing various segments of the 5′ flanking region of the BAI1-AP4 gene were purified by CsCl ultracentrifugation. The resulting plasmids in pGL3-Basic contained various segments of the 5′ region of the BAI1-AP4 gene, including a transcription initiation site (Fig. 2). Plasmids were designated pBAP4-3126, pBAP4-3032, pBAP4-839, pBAP4-741, pBAP4-492, pBAP4-356, pBAP4-276, pBAP4-224, pBAP4-3126 (del −151/+260), pBAP4-224 (del +66/+260), pBAP4-224 (del −11/+260), pBAP4-224 (del −46/+260) and pBAP4-224 (del −91/+260); the plasmids contained BAI1-AP4 nucleotide residues −3126 to +260, −3032 to +260, −839 to +260, −741 to +260, −492 to +260, −356 to +260, −276 to +260, −224 to +260, −3126 to −151, −224 to +66, −224 to −11, −224 to −46 and −224 to −91, respectively (Fig. 2). All constructs were confirmed by sequencing.

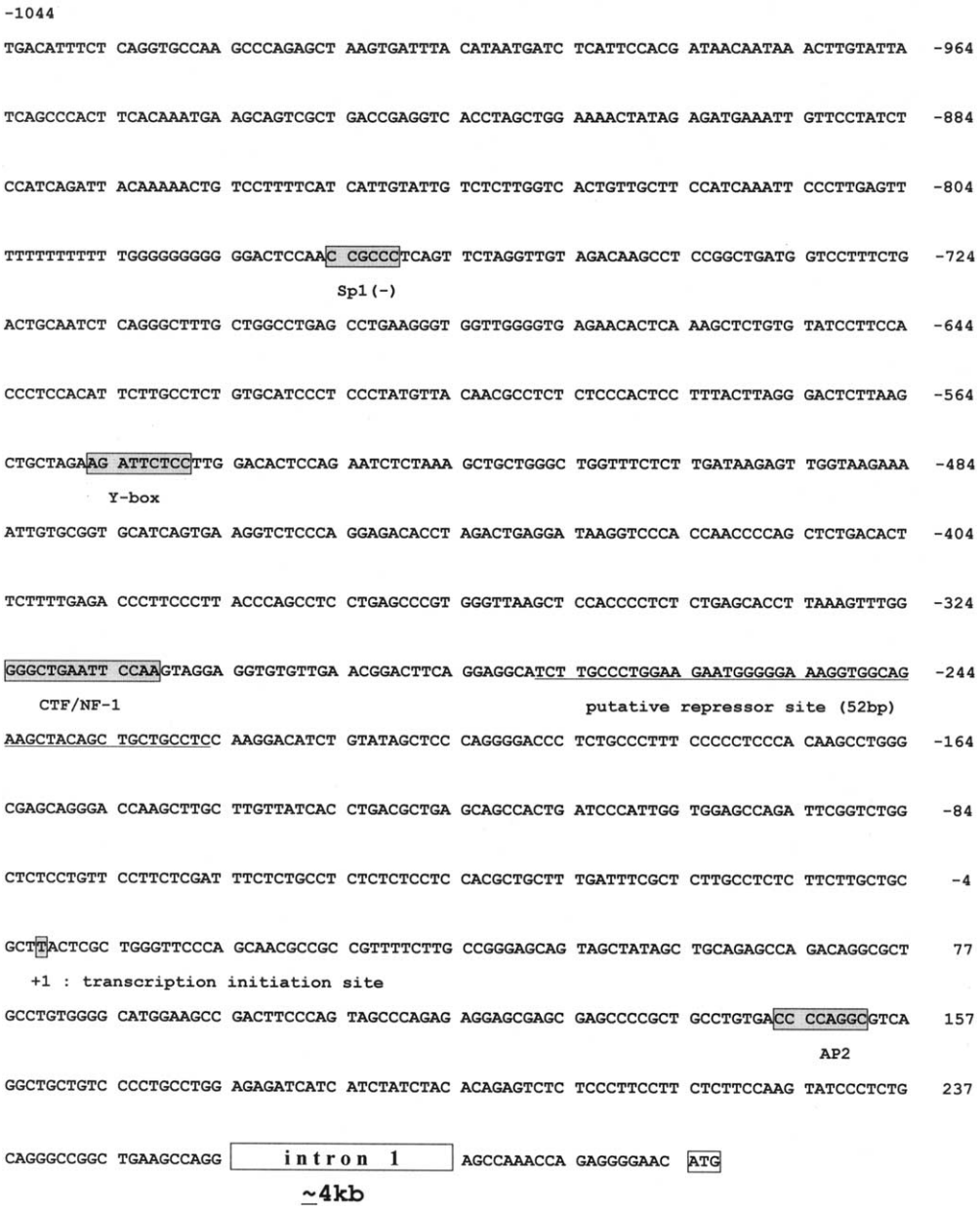

Fig. 1. Partial nucleotide sequence of the BAI1-AP4 promoter. The transcription initiation site is boxed and the beginning of the transcription start site is numbered +1. Nucleotide numbers are based on transcription initiation at +1. The consensus sequences of the nuclear protein-binding sites (Sp1, Y-box, CTF/NF-1, and AP-2) are boxed. The putative repressor factor-binding site is underlined.

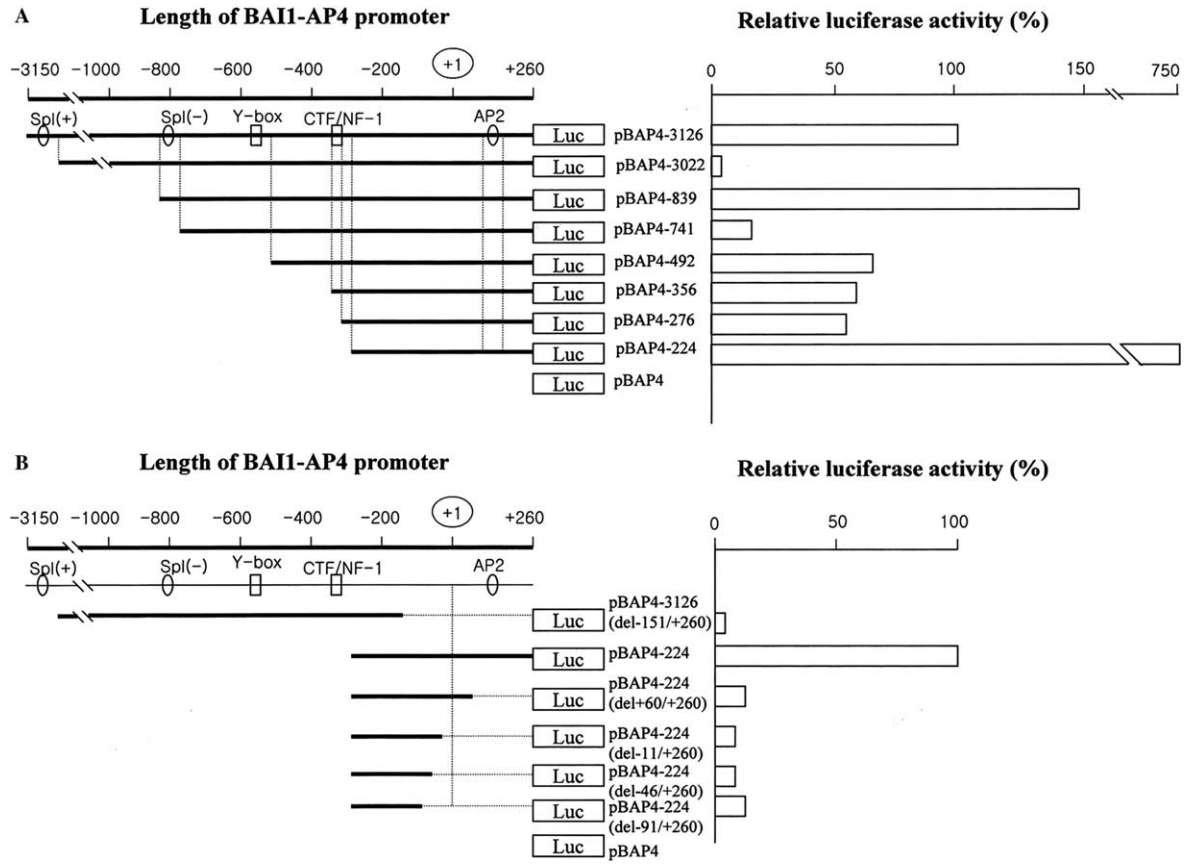

Fig. 2. The location of the binding sites for transcription factors and the transcriptional activities of various deletion constructs of the BAI1-AP4 promoter. Deletion constructs of the BAI1-AP4 promoter were fused to the luciferase reporter gene and transfected into 293HEK cells. After incubation, the cells were assayed for luciferase activity. (A) The luciferase activity of the constructs containing all elements (pBAP4-3126) was taken as 100% and the luciferase activity of each construct is expressed as a relative percentage. (B) The luciferase activity of the pBAP4-224 was counted as 100% and the luciferase activity of each construct is expressed as a relative percentage.

2.4. Construction of a BAI1-AP4-lacZ fusion

To generate the BAI1-AP4 promoter-lacZ transgene, a *Not*I–*Not*I restriction fragment carrying 3475 bp of lacZ cDNA from the pCMVβ vector was cloned into the pUCBM20 vector. A *Xho*I–*Not*I restriction fragment of approximately 9.0 kb was subcloned directly upstream of the lacZ cDNA. This fragment contained 5033 bp of 5′ flanking sequence and 276 bp of BAI1-AP4 (encompassing exons 1–2, up to but not including the initiation codon) and ~4.0 kb of intron 1 (Fig. 3A). The SV40 early region transcription termination/polyadenylation site was inserted downstream from the lacZ cDNA insert.

2.5. Luciferase assays

Human embryonic kidney (293HEK) cells were grown in DMEM supplemented with 10% heat-inactivated FBS at 37 °C in 5% CO_2 incubator. Transfection of each plasmid DNA (2 μg) into 293HEK cells was performed with the non-liposomal lipid FuGENE 6 (Boehringer–Mannheim) following the manufacturer's protocol. The cells were cultured at 37 °C in 5% CO_2 for 36 h. For luciferase assays, cells were harvested, pelleted at 14,000 rpm, washed in cold phosphate-buffered saline (PBS), resuspended in lysis buffer, and incubated at room temperature for 20 min. Cell debris were removed at 14,000 rpm (4 °C) and the supernatant was collected in a new tube. Luciferase activity was measured via luminometer, for 50 s at 25 °C immediately following the automatic injection of substrate. The proteins of mouse tissues were prepared as described [4].

2.6. Generation and maintenance of TG mice

FVB/N6 mice (Dae Han Laboratory Animal Research Center, Korea) were used as donors and recipients for the generation of TG mice. To generate the BAI1-AP4 promoter-lacZ transgene, a ~13-kb fragment composed of the BAI1-AP4 promoter, lacZ cDNA, and SV40 polyadenylation sequences was purified by agarose gel electrophoresis followed by Qiaquick Gel Extraction Kit (Qiagen). This fragment was used for pronuclear injection of FVB/N6 oocytes [7]. Injected eggs were transferred to pseudopregnant FVB females using standard techniques [8]. Heterozygous TG progenies (F_1) were obtained by breeding the founders (F_0) to FVB/N6 wild-type mice. The F_1 male and female littermates were bred back to FVB/N6 wild-type mice to produce F_2 progenies, and the positive F_2 generations were used for the studies. TG mice carrying the lacZ gene were identified by PCR amplification of tail genomic DNA using primers 5′-ATCCCCACAGGAGCCAAACCAGAG-3′ (sense) and 5′-TCTTCGCTATTACGCCAG-3′ (antisense), which amplify a 280-bp fragment spanning the junction between the BAI1-AP4 promoter and the lacZ gene. Three lacZ TG founders were produced.

2.7. Northern blot analyses

To isolate total RNA, various mouse tissues were homogenized with a polytron homogenizer in 4.0 M guanidine thiocyanate and 1% β-mercaptoethanol. The RNA was purified by centrifugation through 5.7 M CsCl as described [4]. RNA samples were quantitated by spectrophotometry at 260 nm. For Northern blot analysis, total RNA (10 μmg) was denatured with glyoxal, separated by size on 1.0% agarose gels, and transferred to Genescreen (DuPont). The probes used were a 1250-bp *Not*I–*Eco*RV fragment (5′ portion) derived from the lacZ cDNA and a part of BAI1-AP4 cDNA (nucleotide residues 1–415). Probes were radiolabeled by nick translation and hybridizations and signal visualizations were performed as described [4]. The integrity of the RNA samples was established by Northern blot analysis with a mouse GAPDH or β-actin probe.

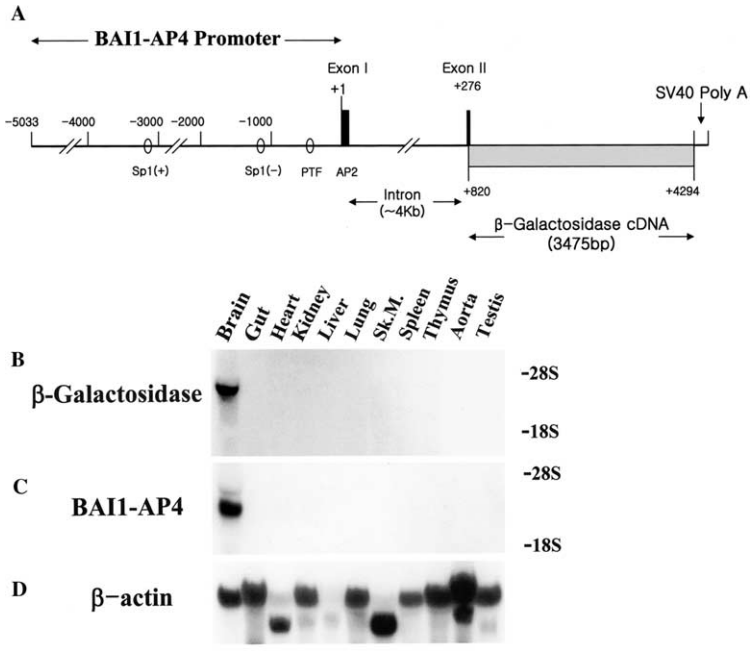

Fig. 3. Structure of the *BAI1-AP4-lacZ* transgene constructs and Northern blot assay of β-galactosidase and *BAI1-AP4* expression in TG mouse tissues. (A) The *BAI1-AP4-lacZ* transgene consists of the entire coding sequence of the mouse lacZ cDNA (3475 bp) cloned downstream of the 9-kb BAI1-AP4 promoter. The SV40 early region transcription terminator and polyadenylation site were inserted downstream from the lacZ cDNA. The transcription factor-binding sites [Sp1, AP-2, and a putative transcription factor (PTF)], considered to be important to the expression pattern, are circled. (B) Total RNAs isolated from various tissues of adult TG mice were hybridized with a β-galactosidase cDNA probe. (C) The same blot was rehybridized with a BAI1-AP4 cDNA probe. (D) For each blot, RNA fidelity was confirmed by rehybridization with a β-actin probe.

2.8. In situ hybridization

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the brain was fixed by in vivo perfusion of the abdominal aorta with 4% paraformaldehyde in PBS for 10 min. The brain was excised and then immersed in the same fixative for 3 h at 4 °C. The blocks were washed in PBS, dehydrated in a graded series of ethanol washes, and embedded in paraffin. Tissue sections (6 μm) were mounted on gelatin-coated glass slides. Sense and antisense cRNA probes specific for the mouse BAI1-AP4 were generated from the recombinant molecules (nucleotide residues 1–415) using T3 and T7 RNA polymerases in the presence of digoxigenin-11-UTP (Boehringer–Mannheim). These probes were also used as c-DNA probes in the Northern blot analysis. In situ hybridization was performed as described previously [4].

2.9. β-Galactosidase staining and immunohistochemistry of TG brain

TG brain tissue was stained as whole mounts using a β-galactosidase staining kit (Specialty Media) as follows. The fixed brain was washed with PBS at 4 °C. Staining was carried out at 37 °C for 16 h in a solution of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) at a final concentration of 0.4 mg/ml with 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6 \cdot 6H_2O$, and 2 mM $MgCl_2$ in PBS. After staining, coronal or horizontal sections of TG brain tissue were rinsed with PBS and photographed as whole mounts. These samples were then embedded in paraffin, cut at 50 μm, de-paraffinized, counterstained with neutral red (0.5%), and photographed.

Immunohistochemistry was performed using an immunoperoxidase procedure (VECTA ABC Kit, VECTOR Lab). The X-gal-stained brain tissue sections (50 μm) were treated with 3% H_2O_2 in 60% methanol for 15 min to quench endogenous peroxidase activity. After washing, the sections were blocked in PBS containing 5% normal horse serum for 1 h. The sections were incubated for 12–14 h with the monoclonal antibody for neuronal nuclei (NeuN, Chemicon) diluted in PBS with 0.3% bovine serum albumin. The sections were then rinsed, incubated sequentially for 30 min each with the biotinylated secondary antibody and the ABC reagents, followed by a 5-min incubation with the DAB, and photographed on a light microscope.

3. Results and discussion

3.1. Nucleotide sequence of the BAI1-AP4 promoter

We sequenced the BAI1-AP4 gene promoter from residue −1044 and identified the location of several putative transcription factor-binding sites (Fig. 1). There were several consensus nuclear protein-binding sites; one inverted Sp1-binding site, a Y-box-binding site, a CTF/NF1-binding site, one AP2-binding site, and a putative repressor factor-binding site. The transcription initiation site was identified by primer extension analysis with mouse brain mRNA as a template. There was no TATA box motif.

3.2. Transcriptional activity of the BAI1-AP4 promoter gene

Our previous report showed a unique developmental pattern of strong BAI1-AP4 expression in the adult brain, but weak ubiquitous expression of BAI1-AP4 was observed in all embryonic tissues examined [4]. In this study, we used embryonic 293HEK cells, because they have the transcription factors corresponding to those observed in the BAI1-AP4 promoter and also have good transfection efficiency. To elucidate the role of each element, several fragments of the promoter were prepared and the transcriptional activities of these constructs were determined by luciferase assay after transfection into 293HEK cells.

The promoter activity of the construct pBAP4-3126 was much higher than that of pBAP4-3022 and the activity of pBAP4-839 was much higher than that of pBAP4-741 (Fig. 2A). These findings suggest that the regions containing the Sp1-binding site and the inverted Sp1-binding site are the major enhancers of promoter activity. Sp1 is a ubiquitous

transcription factor and controls numerous eukaryotic genes including housekeeping genes, signal pathway-induced genes, and tissue-specific genes [9,10]. However, there were no differences in the promoter activities among pBAP4-741, pBAP4-492, and pBAP4-356, indicating that the Y-box-binding site and the CTF/NF1-binding site did not affect reporter expression in these cells.

In contrast, the promoter activity of the construct pBAP4-276 was much lower than that of the pBAP4-224, indicating that residues −276 to −25 might contain a putative repressor-binding site (Fig. 1). A putative transcription factor, though it is not yet identified or cloned, may bind to this element in a manner similar to the RE1-silencing transcription factor, which helps restrict neuronal traits to neurons by blocking expression in non-neuronal cells [11,12].

The region in the pBAP4-224 construct containing the AP2-binding site and the transcription initiation site has potent transcriptional activity (Fig. 2B). The construct without this region [namely pBAP4-3126 (del −151/+260)] exhibited almost no transcriptional activity. The activities of the constructs without the AP2-binding site and/or the transcription initiation site [namely pBAP4-224 (del +66/+260), pBAP4-224 (del −11/+260), pBAP4-224 (del −46/+260), and pBAP4-224 (del −91/+260)] were markedly decreased. These findings indicate that the region containing the transcription initiation site and the AP2-binding site is the basal promoter for the expression of the BAI1-AP4 gene. The AP-2 transcription factor is believed to play a crucial role in the control of gene expression in response to cell differentiation signals within neural crest and epidermal cell lineages, and it is reported to be a critical regulatory molecule required for vertebrate development [13]. AP-2 is also a developmentally regulated transcription factor expressed in ectodermal cell lineages and essential for neural tube morphogenesis, and constitutively regulates several genes that have fundamental roles during development.

In the NSE promoter, the important upstream sequences are the cAMP response element and the AP-1-, AP-2-, and Sp1-binding sequences. In intron 1 of NSE gene, a cAMP response element, E2F-binding sequence, early growth response-1-binding motif, and a neuron-specific element-like sequence were found [14]. These findings suggested that the neuron-specific enhancement of NSE-CAT reporter expression is regulated by the combination of the effects of the 5′ upstream region and intron 1 of the NSE gene. Taken together, we hypothesize that AP-2 is important to the BAI1-AP4 promoter for developmentally regulated expression, whereas the expression of BAI1-AP4 gene is restricted to neurons by some other putative transcription factor. The cloning and characterization of this repressor is under investigation.

3.3. Expression of the BAI1-AP4-lacZ fusion gene and creation of TG mice expressing β-galactosidase in the brain

The BAI1-AP4 promoter was fused to lacZ (Fig. 3A). To test whether the BAI1-AP4 promoter-lacZ fusion gene is functional in eukaryotic cells, this transgene construct was transfected into HEK293 embryonic kidney cells. These cells were chosen because the BAI1-AP4 gene was ubiquitously expressed at a low level in embryonic tissues. Enzymatically active β-galactosidase was expressed in transfected 293 cells, as determined by the X-gal staining of cells (data not shown). Thus, the BAI1-AP4 promoter-transgene construct does function in these cells.

To ascertain whether the BAI1-AP4 promoter would specifically drive *lacZ* reporter gene expression in the brain, TG mice were generated using a ∼13-kb gene fragment that contains the BAI1-AP4 promoter, lacZ cDNA, and SV40 polyadenylation sequences. TG mice were identified by PCR amplification of tail genomic DNA with primers that amplify a 280-bp region spanning the junction between the BAI1-AP4 promoter and the lacZ cDNA (data not shown). Three BAI1-AP4/lacZ TG founders were produced and these founder mice gave rise to offspring in crosses with wild-type FVB mice. The PCR analysis of the progeny showed that the lacZ TG DNA was inherited with constant copy numbers. All founders transmitted the introduced *BAI1-AP4-lacZ* gene in a Mendelian fashion (about 50% heterozygotes).

3.4. Expression of the BAI1-AP4-lacZ transgene in mouse brain

To determine the extent of transgene expression, we performed Northern blot analysis of various tissues from heterozygote offspring of the three founders using a lacZ cDNA probe. A transcript of the expected size (∼4.0 kb) was observed in brain tissue of heterozygotes, but not in other tissues (Fig. 3B). BAI1-AP4 signal was also obtained from the same blot using a BAI1-AP4 cDNA probe (Fig. 3C). However, no *lacZ* transgene transcript was detected in any tissues from non-TG littermates (data not shown). These results indicate that the *BAI1-AP4-lacZ* transgene is expressed only in the brain and expression of the *BAI1-AP4-lacZ* transgene parallels the expression pattern of *BAI1-AP4*.

3.5. Developmental expression of lacZ-transgene in the TG mouse brain

The temporal regulation of *lacZ*-transgene expression was analyzed by Northern blot analysis. BAI1-AP4/lacZ TG mouse brain RNAs were isolated at sequential time points after birth. The lacZ probe detected a very low level of the ∼4.0-kb transgene transcript at the 1st day after birth (Fig. 4A). The BAI1-AP4 signal was also obtained from the same blot using a BAI1-AP4 cDNA probe (Fig. 4B). During neonatal development, the *lacZ* mRNA level gradually increased, showing an expression pattern roughly similar to that of the endogenous *BAI1-AP4* mRNA. The expression level of the *lacZ* transgene and *BAI1-AP4* mRNAs appeared to increase as the development of the brain progressed, and reached their highest levels 4 weeks after birth. The *lacZ* transgene expression decreased slightly after 1 month and this level was maintained through adult life (Fig. 4A). No *lacZ* transgene transcript was detected in brain from non-TG littermates at any age (data not shown). These results indicate that the expression level of *lacZ* appeared to increase as the development of the brain progressed, and *BAI1-AP4* and β-*galactosidase* expression overlap in the developing nervous system.

The developmental time course of NSE expression in the brain is very similar to that of BAI1-AP4 gene, both showing gradually increased expression from birth to adulthood. However, when we compared the β-galactosidase reporter gene expression in the brain between TG mice under NSE and BAI1-AP4 promoters, we found some differences. In the NSE/lacZ TG mouse brain, expression of the *NSE-lacZ* transgene did not parallel the *NSE* gene with regard to postnatal mRNA accumulation [3]. The endogenous NSE mRNA level increased considerably during the postnatal period, while the NSE/lacZ mRNA remained at the neonatal level. Moreover, lacZ mRNA was already ex-

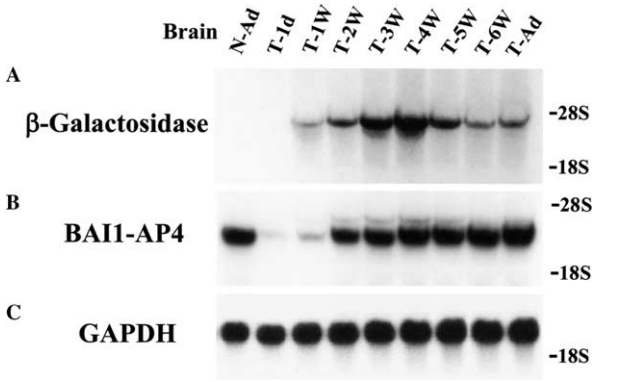

Fig. 4. The developmental pattern of β-galactosidase and *BAI1-AP4* expression in the BAI1-AP4/lacZ TG mouse brain. (A) Total brain RNAs were isolated at various stages of mouse development and hybridized with a β-galactosidase cDNA probe. (B) The same blot was rehybridized with a BAI1-AP4 cDNA probe. (C) RNA fidelity was confirmed by rehybridization with a GAPDH probe. N-Ad, normal adult brain RNA from non-TG mouse; T-1d, TG brain of postnatal day 1; T-1W, TG brain of postnatal day 7; T-2W, TG brain of postnatal day 14; T-3W, TG brain of postnatal day 21; T-4W, TG brain of postnatal day 28; T-5W, TG brain of postnatal day 35; T-6W, TG brain of postnatal day 42; T-Ad, 3-month old TG adult brain.

pressed at the adult level in the prenatal period (embryonic day 18.5). In contrast, BAI1-AP4/lacZ was expressed after birth, and the expression level of both *BAI1-AP4* and β-*galactosidase* appeared to increase as the development of the brain progressed until 4 weeks after birth. However, compared with the developmental expression of endogenous *BAI1-AP4*, a slightly lower level of β-*galactosidase* was maintained from 4 weeks through adulthood. It seems that the developmental reporter gene expression from the 5-kb BAI1-AP4 promoter did not perfectly match the endogenous BAI1-AP4 gene pattern, indicating that further extension of the 5′ upstream sequence would be necessary for a BAI1-AP4/lacZ construct to perfectly reproduce endogenous expression. Collectively, our results indicate that the BAI1-AP4/lacZ TG brain is more appropriate than the NSE/lacZ TG brain to study the mechanisms by which activation of gene expression occurs in the adult brain.

3.6. Localization of lacZ-transgene and neuron-specific expression of β-galactosidase in the brain

Having established that expression of the *lacZ*-transgene is targeted to the brain, the distribution of β-galactosidase activity in the BAI1-AP4/lacZ TG mouse brain was determined by incubation of sectioned brain tissue with X-gal. Incubation with X-gal gave negligible background staining in brain tissue from non-TG mice. β-Galactosidase was expressed throughout the whole cerebral cortex (Fig. 5A), with a particularly high level present in the supragranular layer (Fig. 5C), hippocampal formation (Fig. 5A and C), and cerebellar cortex (Fig. 5B and C). It was weakly expressed in the trigeminal, abducent, facial,

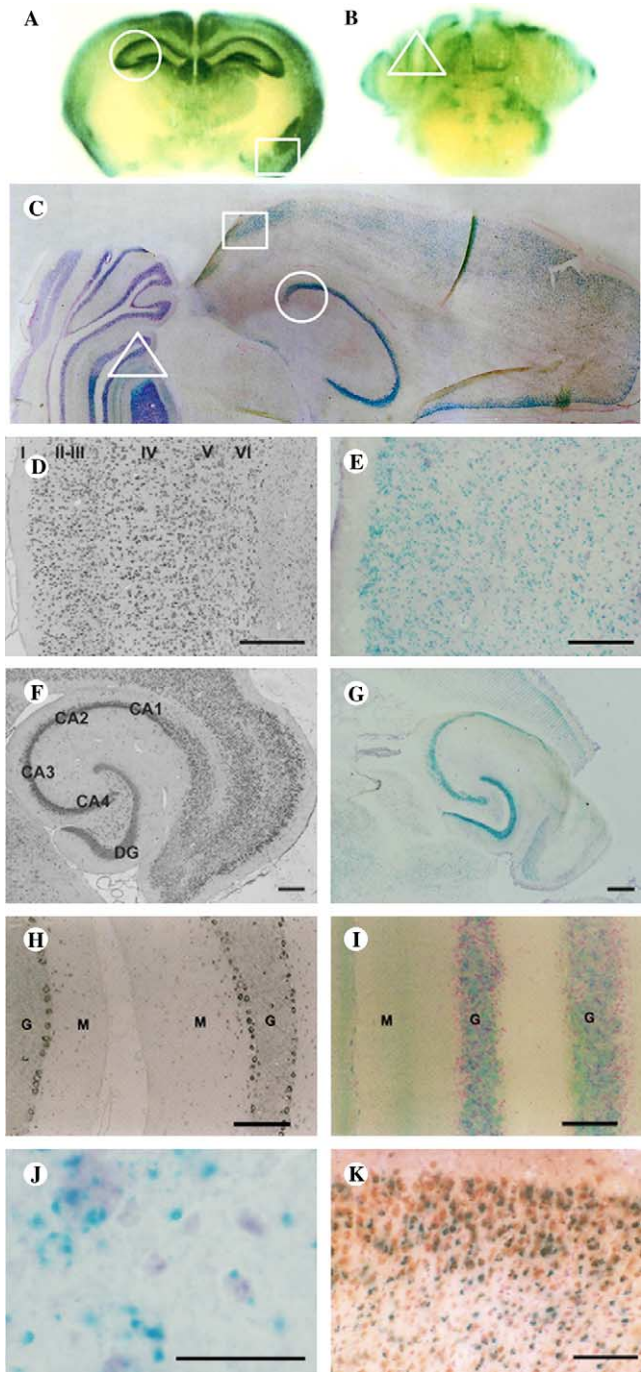

Fig. 5. Histological detection of β-galactosidase and endogenous BAI1-AP4 in neurons of several regions of the BAI1-AP4/lacZ TG adult mouse brain. β-Galactosidase activity in coronal (A,B) and horizontal (C) sections of TG brain tissue was examined by incubation with X-gal. The β-galactosidase was highly expressed throughout the whole cerebral cortex (squares; A,C), all fields of hippocampus proper (circles; A,C), the DG (A). It was moderately expressed at the cerebellum (triangles; B,C), but weakly in the several nuclei of midbrain (B). At higher magnification, entorhinal cortical sections showing strong BAI1-AP4 hybridization signals (D) or β-galactosidase activity (E) in neurons of layers II–III and moderate signals in layers IV–VI. Pyramidal cells of all fields (CA1, CA2, CA3, and CA4) of Ammon's horn and granule cells of the DG show moderate BAI1-AP4 hybridization signals (F) or β-galactosidase activity (G). Cerebellar cortical sections show BAI1-AP4 hybridization signals (H) or β-galactosidase activity (I) in the molecular (M, very weak) and granular (G, moderate) layers. (J) In high-power fields of cerebral cortex, β-galactosidase was expressed in some neurons, but not all cortical neurons. β-Galactosidase-positive neurons have pale blue perikarya with one or two dark blue spots. (K) β-Galactosidase-expressing neurons in the cerebral cortex were also stained with anti-neuronal nuclei antibody, a neuron-specific marker. Scale bars D,E, 50 μm; F,G, 100 μm; H,I, 220 μm; and J,K, 25 μm.

cochlear, and vestibular nucleus, and also present in the brain stem reticular formation (Fig. 5B). These results match the distribution of endogenous BAI1-AP4 mRNA [5]. At higher magnification, BAI1-AP4 hybridization signals and β-galactosidase activity show strong signals in neurons of layers II–III and moderate signals in layers IV–VI of entorhinal cortical sections (Fig. 5D and E). Strong β-galactosidase activity (Fig. 5G) as well as BAI1-AP4 hybridization signals (Fig. 5F) were also noted in the pyramidal cells of all fields of the hippocampus proper and the granule cells of the DG. Cerebellar cortical sections showed BAI1-AP4 hybridization signals (Fig. 5H) or β-galactosidase activity (Fig. 5I) moderately in the granular layer, but very weakly in the molecular layer. These results indicated that the 5-kb BAI1-AP4 upstream sequence could drive lacZ expression in TG brain with major distributions to the cerebral cortex and hippocampal formation that are similar to those of endogenous BAI1-AP4.

However, endogenous BAI1-AP4 hybridization signals (Fig. 5H) but not exogenous β-galactosidase activity (Fig. 5I) were observed in most Purkinje cells. Thus, in contrast to cerebral cortex, expressions of β-galactosidase and BAI1-AP4 were not coincident in the cerebellum. This finding indicates that 5-kb of the BAI1-AP4 upstream sequence is still not enough to perfectly reproduce the endogenous BAI1-AP4 expression pattern in the brain, and suggests again that an additional regulatory region exists beyond this 5-kb upstream region. Recently, it was reported that the bacterial artificial chromosome (BAC) TG approach has several methodological advantages, including a high rate of success in reproducing endogenous expression patterns, because the carrying capacity of BACs is several hundred kilobases [15]. Thus, further extending of 5′ upstream sequence into present BAI1-AP4 promoter-transgene construct or using BAC TG vectors is needed to obtain perfect reproduction of the endogenous BAI1-AP4 expression pattern in the brain.

To understand the mechanisms of neuronal development, many researchers are interested in neuron-specific promoters and regulatory elements [16,17]. Many of the promoters that have been examined have failed to confer spatial or temporal specificity to the transgene [18–21]. In contrast, the promoter elements of NF-L [22], dopamine β-hydroxylase [23], and NSE [3] have been shown to exhibit neuron-specific targeting and approach the temporal expression patterns of the endogenous genes. These reports also indicated that the fidelity of spatial and temporal expression of transgenes driven by a neural-specific promoter was dependent on the size of promoter fragment and the site of transgene insertion. However, their targeting sites in the brain and the onset of their peak expression are different from that of BAI1-AP4.

Next, we determined which cell types within the nervous system expressed β-galactosidase. In high-power views, β-galactosidase was expressed in some neurons of the cerebral cortex. Some but not all of cortical neurons and most neurons positive for β-galactosidase had pale blue perikarya with one or two dark blue spots, usually found against the cell membrane (Fig. 5J). This result suggests that β-galactosidase is largely restricted to cell bodies of mature neurons. In addition, the hybridization signal of BAI1-AP4 was almost exclusively of neuronal origin (Fig. 5D), whereas neuroglia and neuraxis appeared to express low to undetectable levels [5]. Also, higher magnification of the cerebellum showed that granule cells and neurons of the deep cerebellar nuclei were stained (Fig. 5I). In contrast, the molecular layer showed sparse reaction, suggesting low-level expression of BAI1-AP4 or β-galactosidase (Fig. 5H and I). Glial cells in the white matter showed no reaction (Fig. 5H and I). To confirm the neural specificity of the lacZ-transgene expression pattern, we double-stained the BAI1-AP4/lacZ TG brain tissue with X-gal and anti-neuronal nuclei antibody, a neuron-specific marker. β-Galactosidase-expressed neurons in the cerebral cortex (Fig. 5K) and hippocampal formation (data not shown) were also stained with anti-neuronal nuclei antibody. These observations demonstrated again that endogenous BAI1-AP4 and lacZ-transgene expression were specific to mature neurons.

3.7. Developmental expression of β-galactosidase in cerebral cortex and hippocampus

Since, in the Northern blot results, the *lacZ*-transgene expression from the BAI1-AP4 promoter was developmentally regulated (Fig. 4A), we examined whether β-galactosidase was developmentally expressed in cerebral cortex and hippocampal formation of the BAI1-AP4/lacZ TG mouse brain. The brain tissues from one litter were used at sequential time points after birth. A very low level of the β-galactosidase was expressed throughout the whole cerebral cortex (Fig. 6A) and

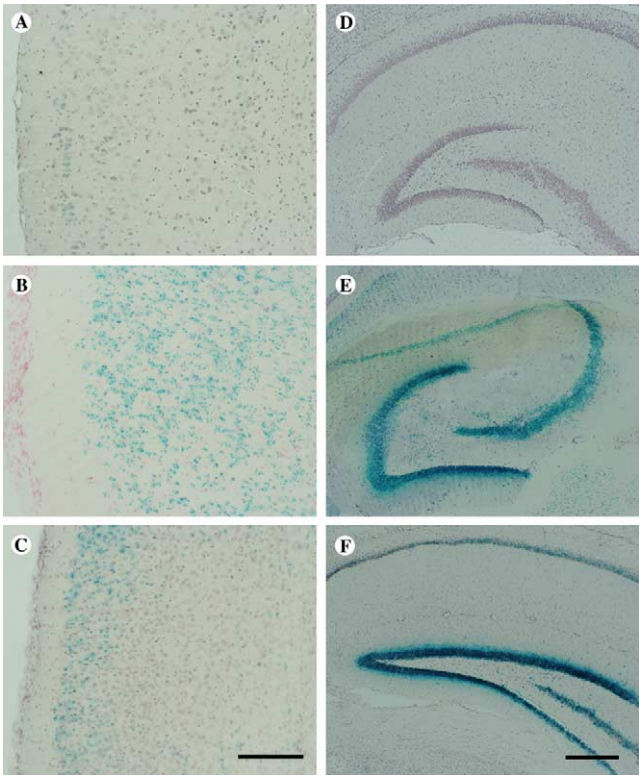

Fig. 6. Developmental expression of β-galactosidase in cerebral cortex and hippocampal formation of the BAI1-AP4/lacZ TG mouse brain. Cerebral cortex (A–C) and hippocampus (D–F) of the BAI1-AP4/lacZ TG mouse brain were stained with X-gal at time points after birth (5th day, 1 month, and 6 months). At day 5, a very low activity of the β-galactosidase was detected throughout the whole cerebral cortex (A) and hippocampus (D). At 4 weeks, the expression level of the β-galactosidase increased highly in the whole cerebral cortex (B) and hippocampus (E). In adulthood (6 months), β-galactosidase activity decreased slightly in the cerebral cortex (C), but it was maintained in the hippocampus (F). Scale bars, 100 μm.

hippocampal formation (Fig. 6D) at the 5th day after birth. At 4 weeks after birth, the expression level of β-galactosidase increased in the whole cerebral cortex (Fig. 6B) and hippocampal formation (Fig. 6E). However, at 6 months after birth, β-galactosidase expression decreased slightly in the cerebral cortex (Fig. 6C), but it was maintained in the hippocampal formation (Fig. 6F). Thus, the expression levels of β-galactosidase in cerebral cortex and hippocampal formation, determined by X-gal staining during development, were just following the lacZ-transgene mRNA expression pattern. These results demonstrated that the lacZ-transgene expression by BAI1-AP4 promoter was developmentally regulated in the TG brain. Thus, spatial and temporal expression of β-galactosidase was roughly the same as BAI1-AP4 in most neurons of the cerebral cortex and hippocampal formation.

We also made a TG mouse expressing herpes simplex virus-thymidine kinase (HSV-TK) gene under the control of the BAI1-AP4 promoter. We observed that some mature neurons expressing the HSV-TK transgene in the cerebral cortex and hippocampus proper exhibited a mild degree of necrosis, such as a shrunken cell body and pyknotic dark nucleus following ganciclovir administration (data not shown). The functional efficacy of these morphological changes was demonstrated by abnormal behavior, such as circling or tumbling. Thus, generation of HSV-TK TG mice shows that the BAI1-AP4 promoter functionally drives transgene expression in specific brain regions of the adult mouse.

Collectively, our present results show that the 5-kb BAI1-AP4 upstream sequence expresses a reporter gene in TG brain with major distributions to the cerebral cortex and hippocampal formation with a developmentally upregulated pattern. Various valuable region-specific brain disease models, such as Alzheimer's disease, might be developed by overexpressing pathological mutant proteins in restricted brain regions under control of the BAI1-AP4 promoter. Moreover, this promoter could be used for resolving the mechanisms by which activation of gene expression occurs in the adult brain.

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