# 中枢神経系における新規遺伝子PAL31の発現に関する研究

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平成7年度博士課程入学

獣医学専攻

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# General Introduction

## *General Introduction*

Highly developed central nervous system (CNS) is one of the properties of mammal animals [1]. Neuroepithelial cells in mammal embryo are highly proliferative [2]. They stop proliferation soon after birth with a slight difference dependent on animal species and brain region. Development of brain after birth mainly cause of enrichment of neural network. Although a few number of dividing cells are still observed in adult brain revealed by $^{3}$H-thymidine or BrdU incorporation experiments [3][4][5][6], almost all the neural cells are terminally differentiated and never go into mitotic cycle again.

Cells which have a property of self-renewal and contribute to neurons, astrocytes and oligodendrocytes are called neural stem cells. Those which still has self-renewal activity but their fates are restricted to several cell types are called progenitor cells [7]. For instance, O-2A progenitors can differentiate only into oligodendrocytes and type 2 astrocytes [8]. Maturation of central nervous system can be roughly divided into 4 level. 1: Pluripotent neural stem cells arise from ectoderm. 2: They determine the fate to neural progenitor cells and glial progenitor cells. 3: Immature progenitor cells stop mitosis and migrate into their final position. 4: Neurons extend axons to their targets and connect synapses to them. Glial cells surround, associate with and support axons dependent on their cell types [9].

Neuroepithelial growth are characterized as its elevator like movement in neural tube. During interphase, neuroepithelial cells attaches to the internal and external limiting membrane. Nuclei move toward the external membrane and start DNA replication. The late interphase and prophase nuclei form a series leading to mitosis as they approach the

lumen. During metaphase the cells lose their external attachment and round up toward the ventricle of the neural tube where cell division occurs. The daughter cells at early development both reenter the mitotic cycle [1]. In later stage of development, one daughter cells reenter the cycle but the other one does not and begins migration and differentiation [10]. There must be mechanisms to regulate the mitotic activity in the brain.

Periphery nervous system (PNS) are derived from neural crest cells. Neural crest cells are highly uncommitted and can differentiate into sensory neurons, Schwann cells, non-myelinating glial cells, melanocytes, adrenergic and cholinergic sympathetic neurons, cholinergic parasympathetic neurons, and enteric peptidergic and serotonergic neurons. Which cell type to differentiate are regulated by environmental factors of each cell [9][11]. One of the committed progenitors, sympathoadrenal precursor cells, can be isolated from the embryonic adrenal medulla or sympathetic ganglia. When the precursor cells are exposed to fibroblast growth factor and nerve growth factor (NGF), they can differentiate into adrenergic sympathetic neurons [12][13].

NGF enhances the sensory and sympathetic neuron to survive in vivo [14] and essential to its development and survival [15][16]. NGF also regulates survival, neurite outgrowth and neurotransmitter production in CNS [17][18]. Thus, neural crest cells is considered to be analogous to neural stem cells. Study of peripheral nervous system development is considered to refer to that of central nervous system as well [19][11]. Rat pheochromocytoma PC12 cells are originally from adrenal tumor [20], and sensitive to NGF and differentiate into sympathetic neuron like cells. Thus, PC12 cells are recognized to be a good model to study neural progenitor cells and its differentiation.

Blood brain barrier regulates the serum factors of high molecular weight to enter CNS in adult [21]. NGF and other neurotrophic factors are secreted from neuron itself [22][23][24][25][26][27] and astrocytes [28] in CNS, and they act and function as autocrine/ paracrine [29] and antero/ retrograde factors [17][30][31]. However, during ontogeny, Blood Brain Barrier is not matured [32][33] implicating that other factors from outside the brain can enter the brain directly.

This paper shows in chapter 1 that there is another possible facors from outside of embryo that influence CNS development. I will also describe in chapter 2 and 3 about a novel gene, PAL31, expressed in the embryonic brain cells and that PAL31 is suggested to relate with cell proliferation.

# Prolactin Receptor mRNA Expression in Fetal Rat Brain

## *Introduction*

Prolactin (PRL) is known to be able to induce maternal behaviour in rats [34], in which the two daily PRL surges from the anterior pituitary stop during mid-pregnancy when the placenta develops and secretes placental PRL-like molecules [35][36][37]. Therefore, one of the important targets for these PRL and PRL-like molecules seems to be the central nervous system (CNS). PRL receptors (PRL-Rs) are distributed in various CNS tissues, including the ovary, testis, pituitary, adrenal, kidney, intestine, mammary gland, liver, thymus, heart and others, of adult animals [38, 39][40]. PRL-Rs have also been found in various CNS namely the preoptic area, median eminence, olfactory bulb, arcuate nucleus, substantia nigra, and choroid plexus [40][39][41][42][43]. Recently, a direct effect of PRL on the proliferation of glial cells *in vitro* was reported [44].

The PRL-R belongs to the cytokine receptor superfamily [45] and there are two types of rat PRL-R, the short (S-PRL-R) and long form (L-PRL-R) [46][47], which are identical in the extracellular domain and differ in the cytoplasmic C-terminal region. PRL-Rs react with PRL and other PRL family members such as placental lactogens (PL)-I, II, Iv and Im, with high affinity [48, 49][50][51][52]. PRL-R mRNA is expressed in the late stage fetal rat adrenal gland, kidney, small intestine, liver and pancreas [53, 54][55] when maternal and fetal PRL secretion have already started. In the present study, I tried to detect PRL-R transcripts using semi-quantitative PCR, the Quant-Amp assay system, in rats during early fetal life when placental PRL family members are secreted.

## *Materials and Methods*

### Reagents

The Micro-FastTrack mRNA Isolation kit was purchased from Invitrogen (CA, USA), the cDNA synthesis kit, agarose and the AutoRead Sequencing kit were from Pharmacia LKB (Uppsala, Sweden) and Pfu DNA polymerase was purchased from Stratagene (CA, USA). All the PCR primers were systhesized using an Oligo 1000 DNA synthesizer (Beckman Instruments Inc., CA, USA). [Methyl 1', 2'-$^{3}$H] thymidine 5'-triphosphate ($^{3}$H-dTTP) ammonium salt (108 Ci/mmol) and the Quant-Amp assay system were from Amersham International plc (Buckinghamshire, England). All the other reagents used were purchased from Wako Pure Chemicals (Osaka, Japan).

### Animals

Adult Wistar-Imamichi rats were purchased from the Imamichi Institute for Animal Reproduction (Ibaraki, Japan). They were kept under lighting conditions of 14-h illumination and 10-h darkness (lights on from 05:00 to 19:00 h) and allowed free access to food and water. The day of insemination was designated embryonic day 0 (E0).

### Extraction of mRNA and PCR

The brains, livers, hearts, intestines and forelimbs were taken from embryos on E12, 14, 16, 18 and 20 after decapitation of the dams. The maternal liver on day 20 of pregnancy was also collected for use as a positive control.

mRNAs were extracted from each sample using the Micro-FastTrack kit according to the manufacturer's protocol and quantified by detecting

the 260nm optical density. cDNAs were synthesized using the method of Gubler [56] and used as templates for PCR. Every cDNA templates was subjected to PCR to amplify rat ribosomal protein L19 [57] to confirm that the cDNA synthesis was successful. Primers #359 (forward)/360 (reverse) and #359/361 were designed to amplify the S-PRL-R (putative PCR product: 330 bp) and L-PRL-R (420 bp), respectively (Fig. 1-1A) [46][47]. The PCR involved 30 cycles of denaturation (91°C for 1 min), annealing (45°C for 1 min) and extension (72°C for 2 min). A second PCR was carried out in the same manner using a portion of the reaction mixture as a template. After the second PCR, a portion of the reaction mixture was subjected to electrophoresis in 1% (w/v) agarose gel.

The PCR products were subcloned and sequenced as described previously [58] using an ALF DNA sequencer (Pharmacia LKB) [59].

**Semi-quantitative PCR**

The reaction mixture comprised 30 ng cDNA, 20 $\mu$M dNTP mixture, including 1 $\mu$Ci $^{3}$H-dTTP, 100 nM each 5'-biotin-labeled #359/non-biotinylated #360 primer and the PCR buffer supplemented with 1.25 U Pfu polymerase in a total volume of 55 $\mu$l. The reaction involved 50 cycles of denaturation (91°C for 1 min), annealing (51°C for 45 sec) and extension (72°C for 2 min), after which 25 $\mu$l each reaction mixture was added to 500 $\mu$l 0.5 mg/ml streptavidin-coated scintillator SPA beads [60] in phosphate-buffered saline, and the amounts of $^{3}$H-dTTP incorporated were determined using a liquid scintillation counter LSC-5100 (Aloka, Tokyo, Japan).

A standard curve was constructed using a cloned S-PRL-R fragment. Only biotin-labeled by the specific primer for the S-PRL-R and 3H-dTTP-incorporated PCR products produce light, the intensity of which is proportional to the amounts of amplified nucleotides.

## *Results*

### PRL-R mRNA expression in fetal rat brains

The mRNAs from fetal rat brain (from more than 3 littermates) were reverse transcribed and then subjected to PCR to amplify specific domains of the S- and L-PRL-R. The results are shown in Fig. 1-1B. The 330-bp S-PRL-R PCR band was seen from E12 to 20, and the nucleotide sequence of the band on each day was 100% identical to that of the S-PRL-R cDNAs. The 420-bp (S-PRL-R) PCR product was observed on E12, 14, 18, but the bands were too week to confirm. Therefore, at least S-PRL-R mRNAs are expressed in the fetal rat brain as early as E12 and E14.

### Semi-quantitation of S-PRL-R mRNA

When the primer #359/361 was used to amplify L-PRL-R, nonspecific longer PCR bands were occasionally seen, whereas only one specific band was observed when #359/360 was used. As the presence of nonspecific bands reduce the reliability of PCR product quantitation by this procedure, only expression of the S-PRL-R was evaluated. The PCR conditions were reconstructed to quantify the copy numbers precisely. The tritium incorporation in PCR products using the PRL-R template increased exponentially following amplification by 35-55 cycles of PCR. Then, the standard curve indicated that the $4.2 \times 10^3 \sim 4.2 \times 10^6$ copies of the templates were measurable during the 50 reaction cycles (Fig. 1-3A).

S-PRL-R mRNA expression levels were measured and the data were expressed as means ± SE per mg tissue ( n=3 Fig. 1-3B). The S-PRL-R mRNA was detected on E12 and 14, and its level increased on E18 and

20. Therefore, the expression of S-PRL-R mRNA occurs very early in the fetal rat brain and increased as fetal development progresses.

**PRL-Rs in other tissues**

Expression of PRL-R mRNAs in other fetal tissues was investigated using the PCR system (Fig. 1-4). S-PRL-R mRNA expression was detected in the liver on E20, the heart on E12 to 20, the intestine on E16 to 20 and the forelimb on E20. Specific bands corresponding to L-PRL-R were observed only in the heart on E20; no such bands were detected in the other 3 tissues.

## *Discussion*

In the present study, I found that mRNA of the S-PRL-R was detected on E12. Expression of PRL receptor mRNA at such an early stage has not been reported. Extensive mitosis, differentiation and migration occur in the brain during the fetal period [61] [62] when mid-pregnancy-specific placental PRL-like molecules are secreted. It is noteworthy that PRL-R mRNA was expressed at such an early developmental stage when the CNS stem cells exist [63] and its expression in the fetal brain showed a biphasic pattern. These data suggest that the developing brain is a target for the members of the PRL family.

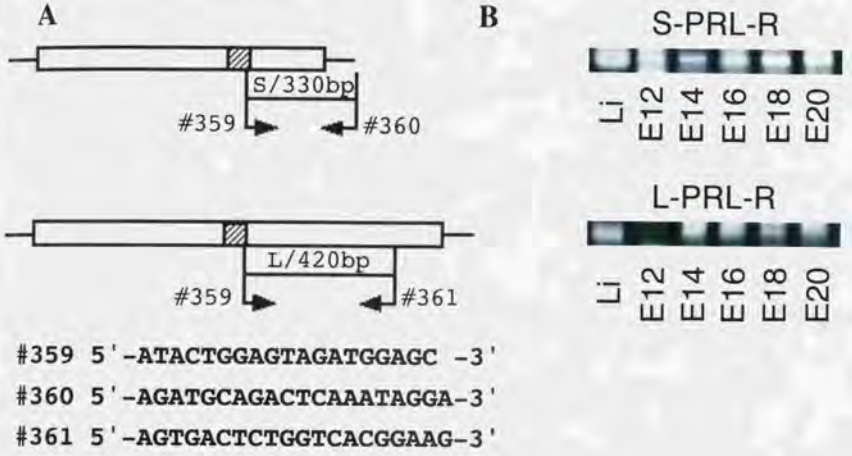
Freemark et al. [54] reported that the mRNA for S- and L-PRL-R were already expressed at E19 in the liver and intestine. However, the mRNAs for the S- and L-PRL-R were not detectable in the fetal heart in their study. This discrepancy may have been due to the experimental conditions employed, including the primer design and PCR conditions. However, it is clear that the PRL-R is expressed in a variety of tissues at a fetal stage earlier than has been generally assumed.

The low levels of mRNA expression observed may be due to a limited expressional area and/or number of brain cells. Taking the reports suggesting that PRL stimulates DA secretion [64, 65] and that DAergic neurons are first observed on E12 in the ventral prosencephalon [66] into consideration, expression of PRL-R mRNA in the fetal brain on E12 to 14 may be related to differentiated DAergic neurons. Some neurotransmitters have been suggested to play roles in regulating neuronal growth during the early fetal period [67][68][69].

The reason why expression level of S-PRL-R was reduced was presently unknown. In rats, type 1 astrocytes differentiate from the

subventricular zone on E16 [8][70] and PRL was found to enhance neonatal astrocytic proliferation [44]. The increased PRL-R mRNA expression during late pregnancy may depend partly on an increased number of astrocytes.

mRNA for S-PRL-R has been shown to be present in the fetal rat brain and heart by E12, whereas PRL surges from the maternal anterior pituitary cease on E10 [35]. Since no PRL synthesis was observed in the fetal anterior pituitary during this period [71], PRL-like molecules other than pituitary-derived PRL can be considered as candidates for the PRL-R ligands. Proteins which reacted with an antibody against PL were found in the amniotic fluid and serum of rat [51][72][53], and in humans, placental hormones and placenta-derived factors are present in amniotic fluid and plasma [73] [74].Therefore, the placental PRL family members are candidates for PRL-R ligands in fetal rat brains, because they are able to react with PRL-Rs and biosynthesis of large amounts of various PRL-like molecules begins during early to mid-pregnancy [75].




**#359 5'-ATACTGGAGTAGATGGAGC -3'**

**#360 5'-AGATGCAGACTCAAATAGGA-3'**

**#361 5'-AGTGACTCTGGTCACGGAAG-3'**

Fig. 1-1 (A) PCR primer sets were designed to amplify the short form PRL-R (S-PRL-R) (upper panel: #359/ 360) and long form PRL-R (L-PRL-R) (lower panel: #359/ 361). The predicted PCR product band lengths are shown (S-PRL-R, 330 bp; L-PRL-R, 420 bp). The hatched box indicates the transmembrane domain. (B) PRL-R mRNA expression in fetal rat brains from E12 to 20. Portions of the PCR reaction mixtures were analyzed by 1 % (w/v) agarose gel electrophoresis and stained with ethidium bromide. S-PRL-R PCR product was detected in fetal brains on E12 to 20. Positive controls for PRL-Rs (rat maternal liver on day 20 of pregnancy) are shown in lane Li.

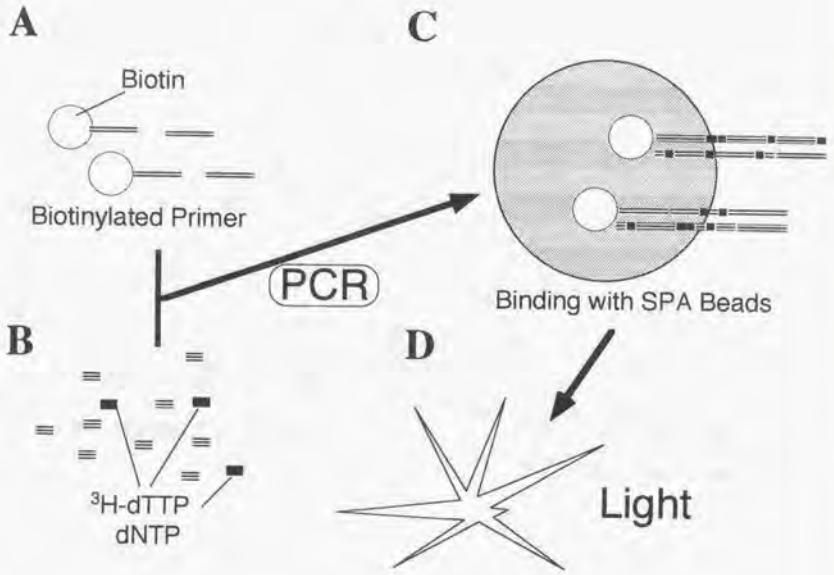



Fig. 1-2 Principle of semi-quantitative PCR. (A) One of primers is labeled with biotin at 5' terminus. (B) PCR is carried out with [methyl,1',2',-$^3$H] thymidine 5'-triphosphate( $^3$H-dTTP) in the reaction mixture. (C) Only the oligonucleotide fragment specifically amplified by biotinylated primer associates with streptavidin coated beads. (D) PCR fragment-associated beads can be excited by beta ray from $^3$H and emitt fluorescence.

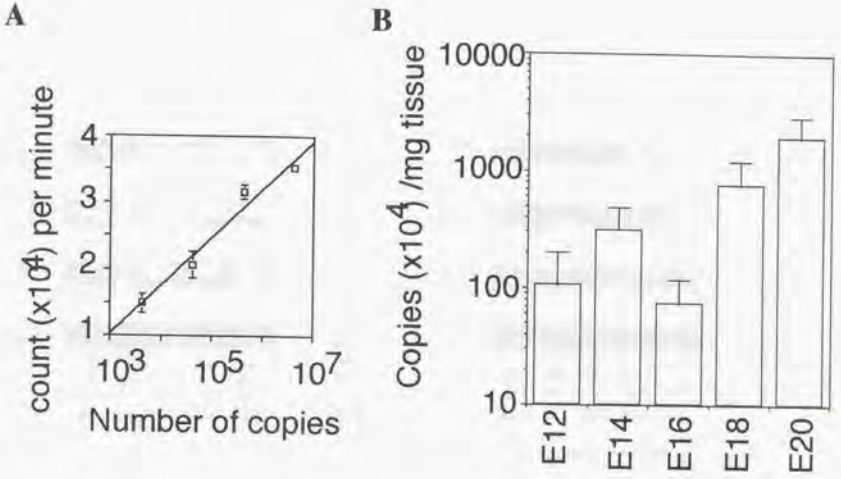
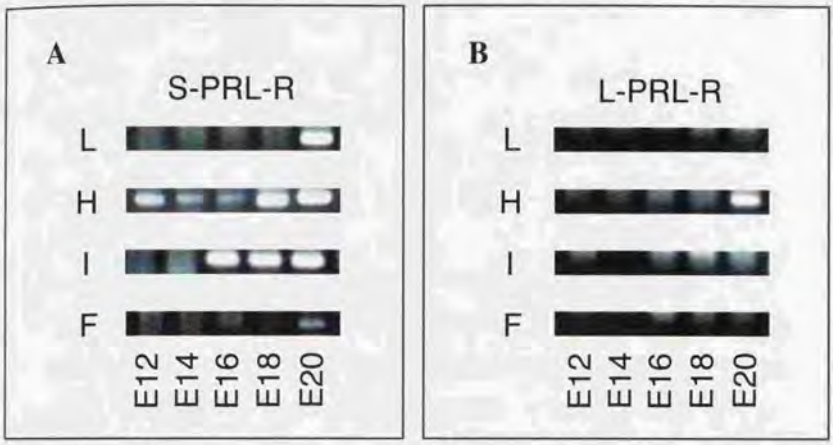



Fig. 1-3 Quantitation of S-PRL-R mRNA expression in rat embryos. (A) Standard curve was constructed by measuring $^{3}$H-dTTP incorporation into the PCR product of rat S-PRL-R. Horizontal line indicates the number of S-PRL-R fragment added in the PCR reaction. (B) Changes in S-PRL-R levels in the brain from E12 to 20. Vertical line indicates the copy number of S-PRL-R extimated from the standard curve. The levels were quantified by extrapolation against the standard curve. Values are means ± SE (n=3).




C

S-PRL-R

| | E12 | 14 | 16 | 18 | 20 |
|---|---|---|---|---|---|
| L | - | - | - | - | + |
| H | + | + | + | + | + |
| B | + | + | + | + | + |
| I | - | - | + | + | + |
| F | - | - | - | - | + |

L-PRL-R

| | E12 | 14 | 16 | 18 | 20 |
|---|---|---|---|---|---|
| L | - | - | - | - | - |
| H | + | ± | ± | + | + |
| B | ± | + | ± | + | + |
| I | - | - | - | - | - |
| F | - | - | - | - | - |

Fig. 1-4 PRL-Rs mRNA expression in various rat fetal tissues from E12 to 20. Primer set #359/ 360 was used to amplify S-PRL-R (A), and #359/ 361 for L-PRL-R (B). Protions of the PCR reaction mixtures were analyzed by 1% (w/v) agarose electrophoresis. L, liver; H, heart; I, intestine; F, forelimb. (C) Diagrams which show PRL-Rs mRNA expression in various rat fetal tissues.

# Chapter 2: Molecular Cloning of PAL31 (Proliferation Related Acidic Leucine Rich Protein with Molecular Size of 31 Kilo-Dalton) from Fetal Rat Brain

## *Introduction*

In early embryonic stage, neural stem cells are rapidly proliferating [2]. Number of dividing cells are dramatically decreased during development and growth rate are retarded during development (Fig. 2-1) [2]. There are several reports that the restricted area such as subventricular zone and dentate gyrus of hippocampus continue to replicate themselves even in the adult rodents [76][5][77][78][11] and adult human [6]), but the number of dividing cells are very few and exceptional. Taken these data, one of the major difference between embryonic and adult brain is its mitotic activity. Thus, genes that are involved in the cell proliferation must be downregulated during development.

The purpose of the experiments in this chapter is to identify the genes predominantly expressed in the developing CNS. For this purpose, differential display PCR was employed because of its high sensitivity and enability to monitor large number of genes at once. As a result, one novel gene, PAL31 was cloned from embryonic day 12 (E12) rat brain. In this chapter, I will describe a molecular cloning and characterization of this novel clone.

## *Materials and Methods*

### Reagents

The mRNA Differential Display system was purchased from GeneHunter (MA, USA). The 5'RACE system, Super Script II reverse transcriptase, Dulbecco's minimum essential medium (DMEM) and TRIZOL reagent were purchased from GIBCO BRL Life Technology Inc. ( NY, USA). The Random Primer DNA Labeling Kit was from Takara Shuzo (Kyoto, Japan).The pGEM-T Easy vector system was from Promega (WI, USA). The pET-32a expression vector was from Novagen (WI, USA). ProbeQuant Sephadex G-25 column, protein A fast flow sepharose, ECL fluorescence detection kit were from Amersham Pharmacia Biotech. [$\alpha$-$^{35}$S]-dATP (1175 Ci/mmol) and [$\alpha$-$^{32}$P]-dCTP (1200 Ci/mmol) were from NEN (Tokyo, Japan). Centricon-10 was from Amicon (MA, USA). HPLC gel filtration column TSK gel-SW3000XL was from Toso (Tokyo, Japan). Unless otherwise mentioned, other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

### Animal treatment

Adult Wistar Imamichi rats (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) were kept under lightening condition of 14 h lighting and 10 h darkness with free access to food and water as in chapter I. The day that the sperm was observed in a vaginal smear was designated as embryonic day 0 (E0). Balb/c mice were purchased from Nippon CREA (Tokyo, Japan). Embryonic whole brains, sagitally sectioned whole brain from adult male rat (approximately six months old) and adult male tissues were frozen quickly and stored at -80°C until required for experiments. E12 brain was removed under microscopy.

Mouse livers were obtained from 12 week-aged mice fasted for one night before sampling. Samples were removed and quickly used for genome extraction.

**Differential Display**

Total RNA was extracted from brains by TRIZOL reagent according to manufacturer's instructions. Total RNA (2 μg) was transcribed by using $T_{12}M$, (M means not T), A primer and Super Script II reverse transcriptase. Reaction mixture contained: 10 ng cDNA, 2 μM dNTP, 50 μM $\alpha$-$^{35}$S-dATP, 1 μM $T_{12}MA$, 0.2 μM 10-mer random primer, 50 mU/μl Tae polymerase. PCR conditions were; 94 °C for 6 min, 40 °C for 5 min, 72 °C for 5 min; and 29 cycles of 94 °C for 30 sec, 40 °C for 2 min, 72 °C for 30 sec. Amplified products were fractionated on 6 % polyacrylamide gel containing 7 M urea and 1x TBE (10 mM Tris, 10 mM boric acid, 2 mM EDTA) at 500 V in 0.8x TBE buffer for overnight. Gel was replaced on the filter membrane, dried out, and subjected to autoradiography. Bands of interest were cut out under the transparency of autoradiography, and bands were used as template to reamplify the fragments in the same condition.

**RNA dot blot analysis and Northern hybridization**

For screening, total RNAs (4 μg) from whole E12 brains and a half of the sagitally sectioned adult whole brain in sample buffer (50 % formamide, 1.75 % formaldehyde, 1x MOPS solution) were heat denatured and applied onto Hybond N+ nylon membrane (Amersham) through 96 well dot blotter (Millipore, MA, USA) by aspiration. RNAs were crosslinked by UV crosslinker (NipponGene, Tokyo, Japan). cDNA probes were labeled with $\alpha$-$^{32}$P-dCTP by random primer and hybridized at 42°C for overnight. Hybridization buffer contained 5x SSPE, 50 %

formamide, 5x Denhardt' solution, 100 μg/ml of salmon sperm DNA. Membranes were washed once by 1x SSC 0.1 % SDS buffer at room temperature for 5 min, and then by 0.2x SSC 0.1 % SDS buffer at 50°C till background level reached below 1,000 cpm. Membranes were wrapped and the signal intensities were detected by Fuji Bio Image Analyzer 2000 (Fuji Photo Film, Kanagawa, Japan). Data were normalized by g3pdh intensity rehybridized on the same membranes. For northern hybridization, cDNA fragment 4p16-r1 was cut from the pGEM-T cloning vector and used for the probe (Fig. 2-2). Control 18S rRNA probe is a cDNA fragment of 18S rRNA (1,432- 1,683 nucleotide (nt) [79]. Total RNA (3 μg) from each sample were fractionated on 1 % agarose gel and transferred to BIODYNE nylon membrane (Pall, NY, USA). Probe was labeled and hybridized as the same protocol as the RNA dot blot. Membranes were wrapped and subjected to autoradiography. Membranes were then washed again and rehybridized with 18S rRNA probe. Since g3pdh and 18s rRNA intensity made the same result, I used both probes for control. Northern blots were tested for more than three times, and similar results were always observed.

**5'-RACE**

Primers #671 [5'-TTT GTT GGG GCG AAG TCC CCC AGG CTT GGT-3'] and #678 [5'-TTA TCC TGG TCC TCT CGG TCA TAG CCA T-3'] were designed on the basis of the sequence information from 4p16 cDNA and 5' RACE cDNA fragment 4p16-r1 (Fig. 2-3). Procedure for 5' RACE was performed according to the manufacturer's instructions. Briefly, first-strand cDNA was prepared from total RNA extract from rat E12 brain using primer #671. Then, oligo-dC tail was added at the 3' terminal of the cDNA by terminal deoxytransferase. PCR was performed by using 0.2 μM anchor primer [5'- CUA CUA CUA CUA GGC CAC

GCG TCG ACT AG, ACG GGI IGG GII GGG IIG-3' (U, deoxyuridine; I, deoxyinosine)] 0.2 μM primer #671 , 200 μM dNTP, 1.39 mM $MgCl_2$, and 33 mU/μl Taq polymerase. PCR conditions were; 94°C for 5 min and maintained at 80°C for 5 min and polymerase was added to each sample tube (Hot-start); one cycle of 55°C for 5 min, 72°C for 5 min, 94°C for 1 min; 28 cycles of 55°C for 2 min, 72°C for 1 min, 94°C for 30 sec; and additional incubation at 55°C for 2 min and 72°C for 10 min. Specific amplification was confirmed by using the other primer inside #671 and anchor primer (nested PCR, data not shown). Second 5' RACE was performed in the same way, except using primer #678 instead of #671. Open reading frame (ORF) of PAL31 was amplified in the same condition except by a primer set of #679 [5'-CC ATG GAC ATG AAG AGG AGG ATC CA-3'] which contained a Nco I site at the 5'-terminal end and #680 [5'-GTC GAC TTA GTC GTC TTC TCC TTC GTC ATC T-3'] which contained a termination codon and a Sal I site.

**Similarity Search**

Isoelectrophoretic point was estimated by Expasy software (http://expasy.hcuge.ch/ch2d/pi_tool.html). Homologous genes were searched by FASTA and BLAST method available in DNA information and stock center (http://www.dna.affrc.go.jp/). Hydrophobicity, secondary structure prediction and homology was caliculated by using MacMollyTetra software (Softgene, Berlin, Germany). Conditions: minimal match length =3, number of mismatch =0, gap penalty =3, mismatch penalty =1. Homology of each protein against rPAL31 (Table 2-1A) and against rLANP (Table 2-1B) was caliculated between their total protein sequence, N-terminal sequence before starting of acidic region, and acidic region, respectively. Acidic region was defined as a sequence of continuous glutamate or asparatate residues for more than

five residues and all the C-terminal region following. For instance, homology of PHAP I/ mapmodulin against rPAL31 were 56.0, 64.8, 51.6 % ( in total 249 aa, N-terminal 167 aa, 82 aa of acidic region, respectively), while those agasint rLANP were 88.0, 88.6, 83.5 %.

**Cloning of mouse PAL31**

Primers #679 and #716 [5'-TAC AAT CGC AGT GTA ACT GGC AGC-3'] based on the EST tag AA009043 were used to amplify mouse PAL31 (mPAL31) cDNA. Total RNA was extracted from 12 week old adult mouse brain and reverse transcribed to cDNA. For PCR, Pfu polymerase which has 3'-endnuclease activity was used for precise amplification of mPAL31. The PCR reaction involved 30 cycles of denaturation (94°C for 30 sec), annealing (52°C for 1 min) and extension (72°C for 1 min) and another incubation at 94°C for 5 min and 42°C for 5 min and 72°C for 5 min). Fidelity of PCR was also confirmed by nested PCR. Primer sets #679/680, #715 ([5'-GGT CAC TAA CCG GAG TGA TTA CCG A-3'], which encodes 372-396 nt of mPAL31)/716 were used for second PCR by using amplified products as a template.

**Southern Blot Analysis**

Digoxigenin (DIG)-labeled mPAL31 RNA probe was generated by using DIG RNA labeling Kit (Boelinger Mannheim). Linearlized pBluescript SK(-) vector containing an 1,127 base pair fragment of mPAL31 was used as a template, and 981 bp long probe including 925 bp of PAL31 sequence was generated by T7 RNA polymerase.

For genome DNA extraction, Smple was homogenized in ice-cold buffer of 20 mM Tris-HCl (pH 7.6), 0.1 M NaCl, 1.5 mM $MgCl_2$ using Teflon-glass homogenizer at 1,000 rpm. The lysate was centrifuged at 700x g for 10 min. After removing supernatant, pellet was suspended in

10 mM Tris-HCl (pH 7.6), 0.1 M NaCl, 1 mM EDTA. 0.5 % SDS and 0.1 mg/ml Proteinase K were added to the sample and incubated overnight at 50°C. Then the sample was refined by phenol /chloroform (P/I) extraction by gentle rotation with several times of eschange of P/I to remove organic contamintants. RNA was removed by incubation of 5 μg/ml of RNase A at 37°C for 1 h. Genomic DNA was precipitated and washed with Ethanol with centrifugation at 1,600x g. Genomic DNA sample was digested by restriction enzymes EcoR I, BamH I, Hind III and Msp I, respectively. Each enzyme was added at the concentration of 10 U/μg DNA and incubated at 37°C for 12 h. On the next day, 2.5- 5 unit per 1 μg DNA of each enzyme was added again and incubation was continued for 2 h to complete DNA digestion. Digested genomic DNA (10 μg/lane) was subjected to 0.8 % agarose gel, transferred to nylon hybridization membrane, and hybridized at 65°C with DIG-labeled RNA probe. Filter was rinsed at 70°C with 2x SSC. Signal was detected with DIG Nucleic Acid Detection Kit (Boelinger Mannheim).

**Recombinant protein expression and antiserum production**

cDNA fragment that contains full open reading frame (ORF) of rPAL31 was subcloned into pET-32a vector between Nco I and Sal I site. The vector was transformed into E. coli AD494.pET-32a vector can add thioredoxin , $His_6$ and S-Tag to the N-terminal sequence of PAL31 to enhance the solubility of the prortein and for easy purification by $Ni^{2+}$-agarose affinity column. Bacteria were cultured in LB medium containing 50 μg/ml ampicilin and 20 μg/ml kanamycin till 600 nm optical density of the medium reaches 0.5. Protein was induced by adding 1 mM IPTG to the medium and following incubation at 37°C for 5 h. After induction, cells were recovered, resolved in the binding buffer (25 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM imidazole), sonicated, and centrifuged

(7,500x g, 4°C, 20 min). Supernatant was applied onto the $Ni^{2+}$-agarose column. The column was washed with Wash buffer (same as the binding buffer except 15 mM imidazole), and the associated protein was eluted by Elution buffer (same as the binding buffer except 40 mM imidazole). This fraction was electrophoresed, specific 48 kDa band was cut, dried out, and mixed with Freund's adjuvant. Antigen was used to prime and boost two New Zealand white rabbits by s.c. injection of 1 mg of Ag per animal every two weeks. Rabbits were sacrificed and antisera were recovered 1 week after second boost immunization, and supplied for the experiment. For antigen specificity, the antiserum was preabsorbed with excess amount of recPAL31 (12 μg) (Fig. 2-11A).

**Western blot analysis**

Tissue samples were directly homogenized in 1x sample buffer (62.5 mM Tris- HCl (pH 6.8), 2 % SDS, 10% sucrose). Homogenates were filtered through nylon mesh (100 μm pore size) and centrifuged at 7,500 rpm for 5 min. Supernatants were recovered and protein concentration were estimated by BCA kit (Pierce, IL, USA) by using bovine serum albumin as a standard. Then, dithiothreitol and bromo-phenol-blue dye were added in each sample and 25 μg/lane of total protein were fractionated onto 12 % polyacrylamide gel. Then, proteins were transferred to PVDF membrane by using semi-dry elecroblotter (BIO-RAD). Western blot was carried out according to Molecular Cloning [80] and signals were detected by ECL kit.

**Recombinant PAL31 digestion and gel filtration**

Eluate was concentrated by using centricon-10 and dialysed to the thrombin buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl), and incubated with 0.15 mU/μl of biotinylated thrombin (Novagen) at 20 °C

for 16 h. After removal of thrombin by streptavidin agarose column, sample was concentrated again and applied onto high performance liquid chromatography (HPLC) gel filtration. 122 to 183 μg protein in 60 to 150 ml running buffer (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1 mM EDTA) was injected on the TSK gel-SW3000XL column and run at flow rate of 0.5 ml/min. Molecular size was estimated by the retention time of bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and myoglobin (16.9 kDa). Gel filtration was tested for three times. Protein concentration was monitored by detecting the absorbance of 215 and 280 nm ultraviolet. Protein concentrations were finally determined by BCA kit by using bovine serum albumin as standard. In summary, 65.7 μg of recPAL31 was purified from 1 L bacterial culture.

## *Results*

### Screening of cDNA clone 4p16

Differential display was carried out to identify genes predominantly expressed in the fetal brain. Two clones were finally screened out by RNA blot. One was appeared to be identical to previously identified genes, thymosin beta 10 [81]. It is a 5 kDa polypeptide, and associates with monomer actin [82][83]. It is already known to be downregulated during development [84][85]. The other clone 4p16 was subjected further to obtain the full sequence because no other homologous genes were identified in DNA data bank at the time of screening. This fragment encoded approximately 1.5 kilobase (kb) gene, and it was expressed highly in E12 brain, compared with adult brain (Fig. 2-2).

### 5'-RACE and cloning of full sequence of PAL31

One end of 4p16 fragment contained putative polyadenylation site (AATAAA, 1180-85 base), thus this direction was assumed to be a 3'-terminus of the cDNA. Primer #671 for 5'-RACE was designed to obtain more 5' end sequence of 4p16, and 998 nucleotides (nt) of 5'-RACE PCR fragment, 4p16-r1. was amplified. Since 4p16-r1 contained a putative open reading frame (ORF) which lacks translation start codon (ATG), second 5'-RACE was carried out to elongate and obtain full ORF of the gene by using primer #678. An alignment of 4p16, first 5'-RACE fragment 4p16-r1 and second 5'-RACE fragment is shown in Fig. 2-3. By connecting sequence of these three fragments, 1,225 nt sequence for 4p16 cDNA was determined. It contained ORF of 816 nt, encoding a polypeptide of 272 amino acid residues (aa). Estimated molecular weight was 31,064 dalton (Da). Predominant amino acid residues found in 272 aa

were; 64 glutamate (23.53 % of total aa), 44 aspartate (16.18 %) and 31 leucine (11.40 %). Estimated pI was 3.87, implicating that it is highly acidic protein. All the leucine residues were contained in the N-terminal half region (1-145 aa) of the protein, thus this half region was rich in leucine (21.38 %). From these characteristics and the data shown in chapter 2 and 3, this gene was named PAL31, for proliferation related acidic leucine rich protein with MW of 31 kDa.

**Cloning of mouse PAL31**

Several highly homologous expression sequence tags (ESTs) cloned from mouse cDNA library were found in the DNA data bank (AA009043, AA066733, AA071604, AA105703, AA114610, AA125537, and AA177286). Almost identical gene to rat PAL31 (rPAL31) in mouse was predicted from the result of alignment of these ESTs (Fig. 2-5A). Then PCR fragment of 1,026 contained the ORF encoded 272 aa was successfully amplified from adult male mouse brain (Fig. 2-5A). It showed 98.9 % identity in protein sequence (Fig. 2-5B). Considering the similarity, this gene was concluded as mouse PAL31 (mPAL31).

**PAL31 homology search from DNA data bank**

All the homologous genes, their putative aa length (total, N-terminus, acidic region), and homology are shown in Table 2-1. Homologues are; rat cerebellar leucine rich acidic nuclear protein (rLANP) [86], human putative HLA DRα associated protein I (PHAP I) [87] or microtubule associated protein moeulater mapmodulin [88, 89]), mouse phosphoprotein 32 (pp32) [90] , nuclear silver stainable protein SSP29 [91], and acidic protein rich in leucine (APRIL)([92]. SSP29 and APRIL is likely to be the same gene because the protein sequence is different only in the predicted start codon. Predicted SSP29 sequence

starts as Met Asp Met..., while APRIL sequence is reported to start from $Met^3$ of SSP29. All of them contained LRRs in the N-terminal region, acidic region and putative NLS at the C-terminal region.

In comparison with the homology against rPAL31 (Table 2-1A) and against rLANP (Table 2-1B), homologues showed differential homology against each molecule. For instance, PHAP I/ mapmodulin was more homologous to rLANP, while SSP29 was rather homologous to rPAL31 in their total, N-terminal region, and C-terminal region. First 20 amino acid sequences at the N terminus (Fig. 2-6A) and the last 20 amino acid sequences at the C-terminus (Fig. 2-6B) were compared in PAL31 family proteins. SSP29 was again shown to be rPAL31 like protein, while PHAP I/ mapmodulin and mPP32 were close to rLANP and mLANP.

Although the consensus residues of LRRs were largely conserved between rPAL31 and rLANP (Fig. 2-7A), major difference was that rLANP has a single truncated form of EAPDSDG/VEVD (EA...VD) motif (Fig. 2-7A). Only rPAL31 and mPAL31 share the repeat of this motif. Truncated single motif was detected in all other molecules (Fig. 2-7B).

**mPAL31 genome analysis**

Southern hybridization was carried out against Balb/c mouse genome for the purpose of genomic analysis of PAL31. Digestion by restriction enzyme EcoR I, BamH I, Hind III and Msp I were carried out respectively. Various bands pattern depending on each enzyme were detected by using mouse PAL31 probe (981 bp) (Fig. 2-9), suggesting that PAL31 is coded separately into several exons. PAL31 genome was estimated to encode at least 47.8 kb from the result of BamH I digestion.

**rPAL31 mRNA expression in adult tissues.**

To investigate the gene expression pattern in another tissues, RNA were extracted from various adult rat tissue. Approximately 1.5 kb PAL31 mRNA band was detected predominantly in E12 brain (Fig. 2-10). The signal was dramatically reduced in adult brain as same as the data shown in Fig. 2-2. Northern blot data showed that PAL31 gene was low but distributed ubiquitously. Relatively high level of expression was observed in the spleen, testis, and ovary, all of which contain number of dividing cells. However, none of them express PAL31 mRNA higher than embryonic brain (Fig. 2-10).

**Production of anti PAL31 and western blotting**

To analyze the expression pattern of PAL31 protein, specific anti PAL31 antiserum was produced (Fig. 2-11A). PAL31 protein expression level was detected in the developmental rat brain (Fig. 2-11B). Western blot detected the strong signal of approximately 31 kDa in E12 brain. It was the same size as the prediction from deduced amino acid sequence of PAL31. Protein expression level was investigated in the other age of brain. This 31 kDa signal was gradually decreased from E12 to adult brain. The signal was under detectable level in the adult brain. Interestingly, additional lower molecular band was observed by western blot. The two bands were downregulated through brain development simultaneously.

**recPAL31 Purification**

Tag-digested recPAL31 was purified by gel filtration (Fig. 2-12). Thioredoxin and $His_6$ tag was deleted by thrombin, and fragments and another contaminants were filtrated by using HPLC. The peak of recPAL31 (approximately 16 min) was observed at the retention time corresponding to approximately 91.2 kDa. Further analysis by SDS-

PAGE followed by coomassie staining revealed that there was only 36 kDa protein band (same as the predicted molecular size of recPAL31) in this fraction. No other bands were observed in 12 % polyacrylamide gel. Anti PAL31 antiserum reacted only to this band in this fraction, suggesting that recPAL31 was eluted as trimer.

## *Discussion*

In this study, a novel gene PAL31 was identified from rat embryonic brain. PAL31 mRNA and protein were predominantly expressed in the embryonic brain. PAL31 appeared to be a member of protein family molecules. PAL31 has several unique motifs. Some of which are suggested to be concerned with the protein- protein interaction or subcellular localization.

### Protein structure of PAL31

There were five unique characteristics in the deduced protein sequence. First, there are three complete (18-42, 65-88, 89-111 aa), one incomplete (44-64 aa) and one half (114-125 aa) putative leucine rich repeats (LRRs) at the N terminal residues (Fig. 2-4D). Second, acidic amino acid residue are predominant in the C terminal region. 181-272 aa contained 70 acidic aa (76.09 %) and showed highly hydrophilic region (Fig. 2-4B).Third, this protein has putative nuclear localization signal (260-263 aa, KRKR) at the proximal to the stop codon. Thus the intracellular localization will define the function of this molecule. NLS in PAL31 and several NLS previously reported are shown in Fig. 2-4E.

Fourth, there is a repeat of EAPDSDG/VEVD at the proximal to the N terminus of acidic region (154-163 and 167-176 aa). Fifth, there are also found many putative phosphorylation target sites by casein kinase II ($Ser^{56}$, $Ser^{104}$, $Ser^{143}$, $Ser^{158}$, $Ser^{164}$, $Ser^{171}$, $Ser^{225}$), protein kinase C ($Thr^{126}$, $Ser^{255}$), and protein kinase A ($Thr^{265}$) in its sequence. PAL31 homologues, mapmodulin [89] and PP32, are reported to be a phosphoprotein, and PP32 is a substrate at least for casein kinase II ([90]).

Analysis of recombinant PAL31 by gel filtration suggests that recPAL31 forms homotrimer complex in vitro. The difference of estimated MW and from the prediction of that of trimer (36x 3 =108 kDa) may depend on the three dimensional structure of the protein complex. Formation of trimer complex in vitro was reported in the case of mapmodulin [88, 89]. LRR is a continual repeat of leucine in every two or three residue, and this region is strongly suggested to participate in protein-protein interaction [93-95]. It consists tandem repeat of alpha helix and beta sheet approximately parallel to each other. For example, it can be observed in the ligand binding domain of ribonuclease inhibitor [94] or follicle stimulating hormone receptor [96].

PHAP I/mapmoduolin can construct homodimer and trimer, and also bind to microtubule associated protein MAP2, MAP4, tau, in vitro and vivo, and also with golgi apparatus membrane [88, 89]. Phosphorylation of mapmodulin is reported to be important to associate with MAP4 [89]. PHAP I/mapmoduolin is re-identified as specific protein phosphatase 2A inhibitor [97], though there is now a contradictory report [89]. C-terminal acidic part of mapmodulin is reported to be important for association with MAP4 [89]. Purified tag deleted recPAL31 does not have any phosphatase inhibitory activities (data not shown). LANP is also found to associate with a responsible gene of spinocerebellar ataxia type 1, ataxin-1 [98]. Another PAL31 homologue PP32 is originally identified as sharing epitope with human erythrocyte protein 4.1 [99], and also associate with myosin in vitro. There is also an cytoskeleton binding protein in nucleus [100]. SSP29 associates specifically with RNA polymerase I [91]. RNA polymerase I transcribes ribosome RNA and it is accumulated in nucleolus. Gel filtration data, presence of LRR in PAL31 sequence and previous reports of homologous proteins assume that

PAL31 associates with itself, other PAL31 family proteins, and/ or other unknown molecules in vivo.

Acidic region is often seen in the nuclear protein [101], centromere protein B [102], spermine-binding protein [103], HMG-1 [104]. The significance of this region is unknown. There are at least two types of NLS reported. One is single NLS found in, for instance, immediate early gene and transcription factor v-jun [105], transcription factor NF-κB [106], SV40-T antigen [107] (Fig. 2-4E). The other one is bipartite basic type in p53 [108] and nucleoplasmin [109], which has two basic amino acid regions separated by approximately 20 aa residues. Functional difference between them is not known. PAL31 has one typical single type NLS. Presence of acidic region and NLS in its sequence raise the possibility that PAL31 is a nuclear protein.

Western blot analysis revealed that there are two types of proteins that anti PAL31 antiserum reacts. Phosphorylational modulation by kinases and phosphatases is one of the major mechanisms of gene expression. For example, protein phosphorylation changes activity of transcription factor CREB [110][111], activates and deactivates signal cascades [112][113][114], enhances cells to proliferate [115][116][117, 118], differentiate [119][120][121][122]. There are several possible phoshporylation sites ($Ser^{56}$, $Ser^{104}$, $Ser^{143}$, $Ser^{158}$, $Ser^{164}$, $Ser^{171}$, $Ser^{225}$, $Thr^{126}$, $Ser^{255}$ and $Thr^{265}$) in the PAL31, and therefore, the two distinct forms may reflect the extent of protein phosphorylation of PAL31.

EAPDSDG/VEVD motif is an unknown motif at the middle part of PAL31. Hydrophilicity is decreased in this repeat. It may be important to define the three dimensional structure of PAL31.

**PAL31 genome analysis**

Southern blot data suggests that PAL31 gene is coded on the over 48 kbp genomic region, and it is separated into several exons. mPAL31 gene has one BamH I site (17-22 bp), one Hind III site (202-207 bp) and three Msp I sites (39-43, 46-49, 381-384 bp) in its probe cDNA sequence. If mPAL31 does not have any introns in its genome sequence, two bands will be detected by southern hybridization in the samples of Bam HI and Hind III digestive. The result that number of digested bands were detected in each enzyme implicates that PAL31 gene is encoded on several exons spaced by introns. Obtaining of genome sequence and chromosomal localization analysis are required for further analysis of PAL31 genome analysis.

**PAL31 family proteins and subclassification**

Computer homology search indicates that N-terminal portion of each protein possesses higher homology than acidic part (for instance, rLANP has 63.1 % identity to rPAL31 in N-terminal region, while it has 36.6 % identity in acidic part). Computer homology analysis and direct sequence comparisons were compareble to the result of that SSP29 is more resembles to rPAL31, and PHAP I/ mapmodulin resembles to rLANP. Thus, these molecules are at first divided into two subfamily, that is PAL31 subfamilies (rPAL31, mPAL31, SSP29/ APRIL) and LANP subfamilies (rLANP, mLANP, mPP32, PHAP I/ mapmodulin).

According to the size of molecules, PAL31 subfamily is further classified into two subfamilies by following reasons. First, rPAL31 and mPAL31 appeared to be the largest molecules among all the homologous proteins. rPAL31 and mPAL31 have the longest acidic region (92 aa), but SSP29 and APRIL have shorter length of acidic region (86 aa). rLANP , mLANP, mPP32 and PHAP I/ mapmodulin have the shortest

one (82-83 aa). Second, the repeat of EA...VD distinguishes rPAL31 and mPAL31 from any other PAL31 homologous proteins.

Taking all this, PAL31 homologues can be classified into three subfamilies (Fig. 2-8B): PAL31 subfamily which has long acidic region and a repeat of EA...VD; SSP29 subfamily which is more homologous to PAL31 than LANP, has middle length acidic region and single EA...VD; LANP/ pp32 subfamily which has shorter acidic region and single EA...VD. In this context, PHAP I/ mapmodulin can be adopted to be a LANP/ pp32 subfamily molecule. PHAP I2b belongs to SSP29, since it is an alternative splicing form of SSP29.

PP35 and PP42, human erythrocyte cytoskeletal protein 4.1 [123] which share epitope with PP32 in mouse $A_{20}$ B lymphoblast [90]. Moreover, mouse has two LANP subfamily proteins (mLANP and mPP32) and human has at least two SSP29 subfamily proteins (SSP29/ APRIL and PHAP I2b). It also raises the possibility that there are plural unknown PAL31 family molecules in rat and human.

In summary, PAL31 gene was cloned from fetal mitotic brain, and it is a component of a protein family in mammal species.

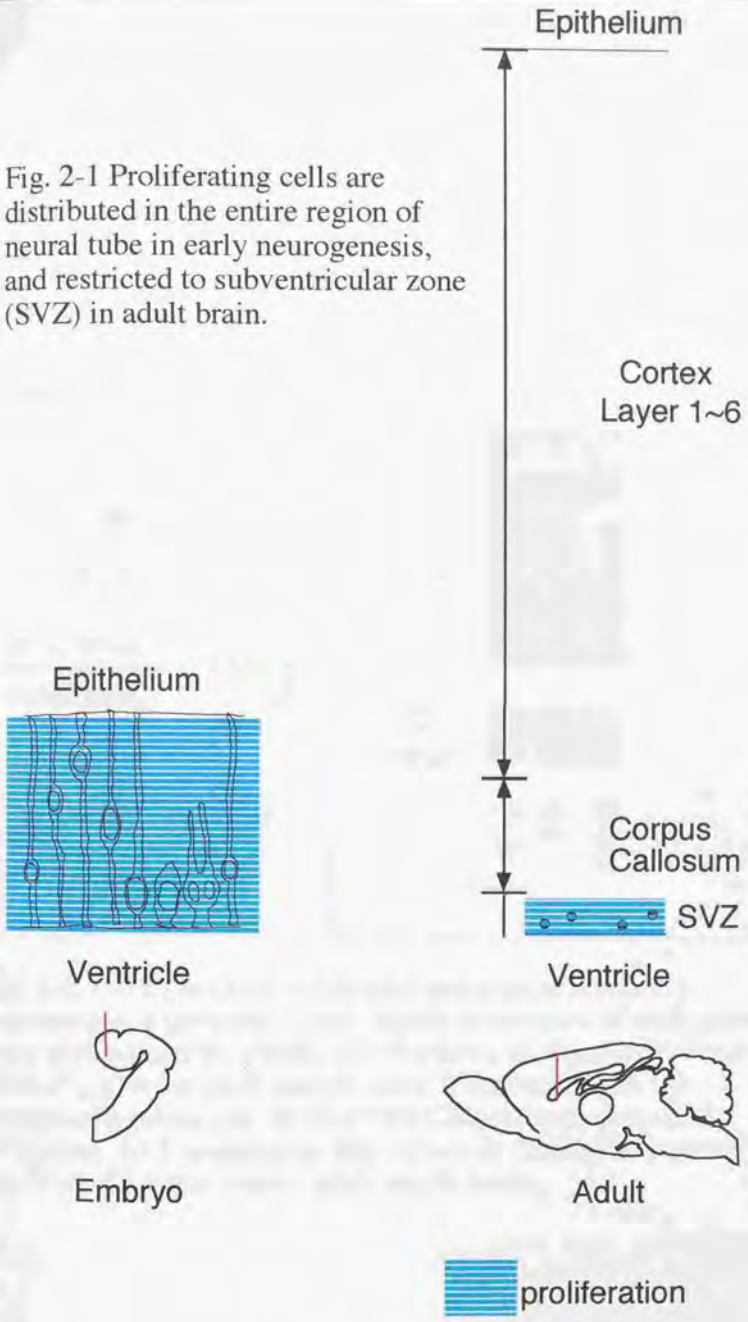



Fig. 2-1 Proliferating cells are distributed in the entire region of neural tube in early neurogenesis, and restricted to subventricular zone (SVZ) in adult brain.

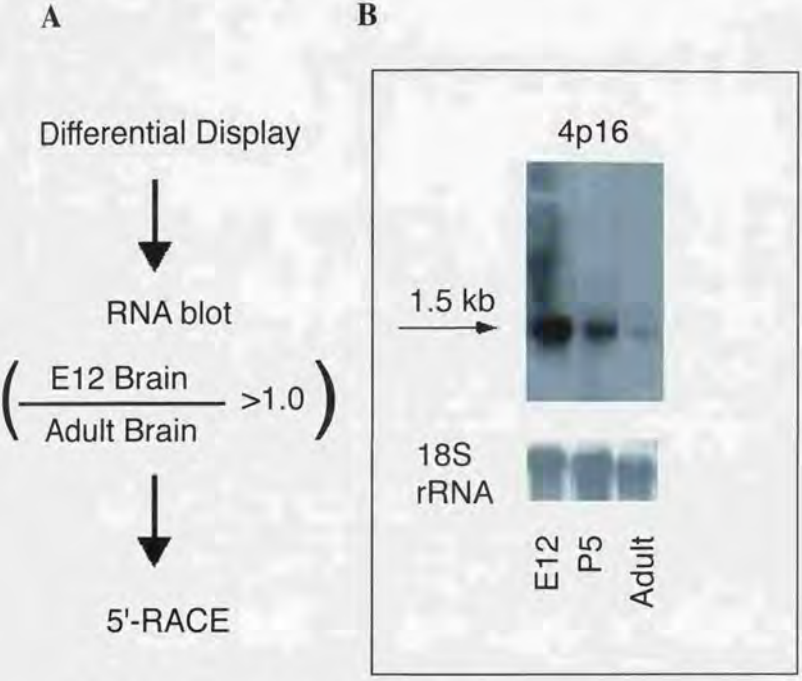



Fig. 2-2. (A) Flowchart to identify genes predominantly expressed in embryonic brain. Signal intensities of each gene were normalized by g3pdh. (B) Northern blot analysis. Total RNA (3 μg) from each sample were fractionated on 1% denatured agarose gel. 4p16-r1 RACE fragment was used as a probe. E12, embryonic day 12 whole brain; P5, potnatal day 5 whole brain; Adult, adult whole brain.

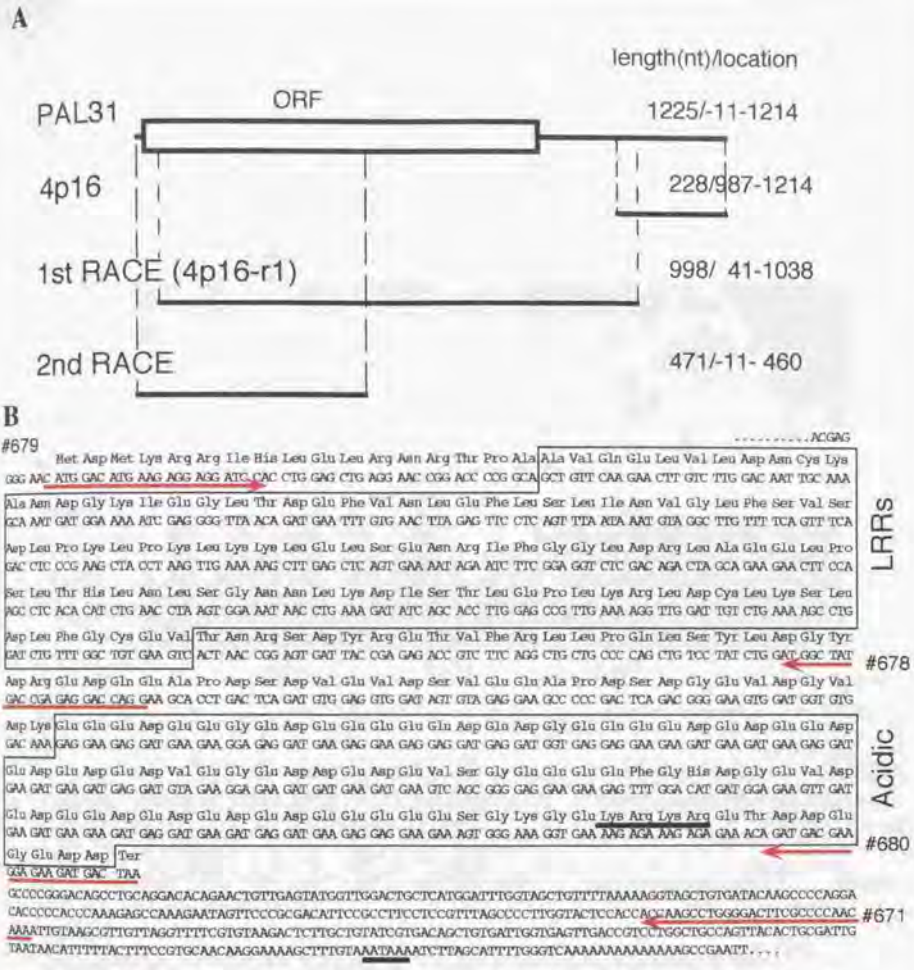



Fig. 2-3 Nucleotide and deduced amino acid sequence of PAL31. (A) Length and location of differential display PCR fragment (4p16), first 5'-RACE fragment (4p16-r1) and second 5'-RACE fragment are shown. 4p16-r1 was used as a probe for Northern hybridization. (B) Full nucleotide sequence of rat PAL31 and its deduced a.a. sequence. Primers for 5'-RACE and to amplify ORF of PAL31 are indicated by arrows. Leucine rich repeats (LRRs), acidic region (Acidic) are in the boxes. Putative nuclear localization signal and poly (A) signal is underlined.

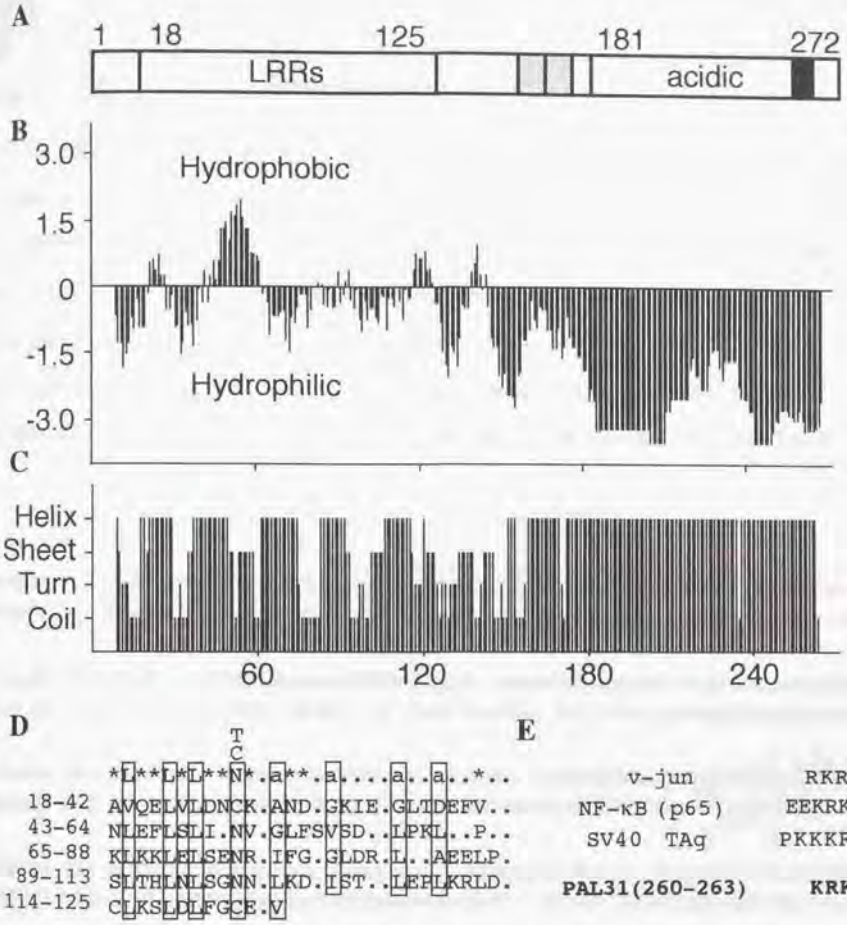
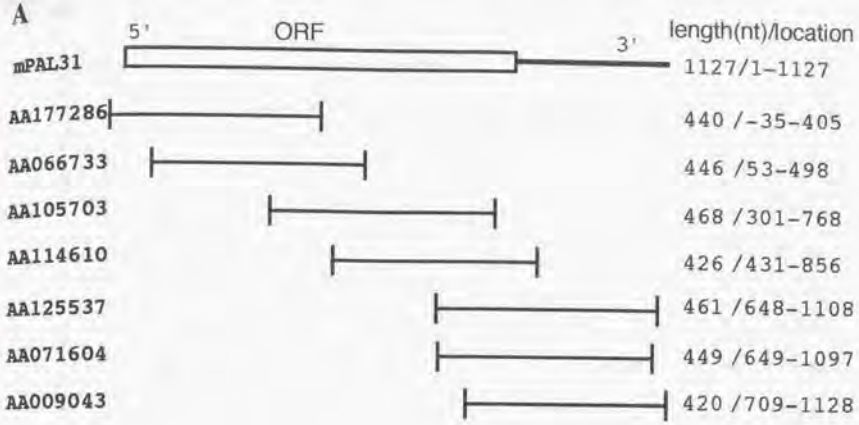



Fig. 2-4 (A) Putative motifs in PAL31 protein. Residues 18-125, leucine rich repeats (LRRs); 181-272, acidic region; 260-263, NLS (black box); 154-176, EAPDSDG/VEVD motifs (dotted box). (B) Hydropathy and (C) secondary structure of PAL31 calculated by Robson-Garnier folding prediction. (D) LRRs in PAL31 sequence. Consensus residues are at the top line. Asterisc indicates any amino acid, "a" denotes aliphatic amino acid, and dot indicates possible deletion. Conserved residues are in boxes. (E) Sequence of nuclear localization signal (NLS). Several NLS previously reported are also shown.

A




B

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rPAL31     1-60 MDMKRRIHLELRNRTPAAVQELVLDNCKANDGKIEGLTDEFVNLEFLSLINVGLFSVSDL
mPAL31     1-60 MDMKRRIHLELRNRTPAAVRELVLDNCKAMDGKIEGLTDEFVNLEFLSLISVGLFSVSDL

rPAL31    61-120 PKLPKLKKLELSENRIFGGLDRLAEELPSLTHLNLSGNNLKDISTLEPLKRLDCLKSLDL
mPAL31    61-120 PKLPKLKKLELSENRIFGGLDRLAEELPSLTHLNLSGNNLKDISTLEPLKRLDCLKSLDL

rPAL31  121-180 FGCEVTNRSDYRETVFRLLPQLSYLDGYDREDQEAPDSDVEVDSVEEAPDSDGEVDGVDK
mPAL31  121-180 FGCEVTNRSDYRETVFRLLPQLSYLDGYDREDQEAPDSDVEVDSVEEAPDSDGEVDGVDK

rPAL31  181-240 EEEDEEGEDEEEEEDEDGEEEEDEDEEDEDEDEDVEGEDDEDEVSGEEEEFGHDGEVDED
mPAL31  181-240 EEEDEEGEDEEEEEDEDGEEEEDEDEEDEDEDEDVEGEDDEDEVSGEEEEFGHDGEVDED

rPAL31  241-272 EEDEDEDEDEEEEESGKGEKRKRETDDEGEDD
mPAL31  241-272 EEDEDEDEDEEEEESGKGEKRKRETDDEGEDD
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Fig. 2-5 Cloning of mouse PAL31 (mPAL31). (A) Nucleotide structure of mPAL31 is shown at top. Putative ORF is in the box. Expression sequence tags (ESTs) are aligned and shown below. Length and location of each EST are also indicated. (B) Comparison of rat PAL31 (rPAL31) and mPAL31 protein sequences. Amino acid residues not identical are in boxes.

**A**

Amino acid residues and homology against rPAL31

| Animal | Name | total/*homology* | | N-term/*homology* | | acidic/*homology* | |
|---|---|---|---|---|---|---|---|
| Rat | rPAL31 | 272 | *100.0%* | 180 | *100.0%* | 92 | *100.0%* |
| | rLANP | 247 | *56.3* | 164 | *63.1* | 83 | *36.6* |
| Mouse | mPAL31 | 272 | *98.9* | 180 | *98.3* | 92 | *100.0* |
| | mLANP | 247 | *55.6* | 164 | *63.6* | 83 | *31.6* |
| | mPP32 | 247 | *55.2* | 164 | *63.6* | 83 | *30.8* |
| Human | PHAP I* | 249 | *56.0* | 167 | *64.8* | 82 | *51.6* |
| | SSP29 | 251 | *71.4* | 165 | *80.6* | 86 | *69.8* |
| | APRIL | 249 | *70.7* | 163 | *79.5* | 86 | *69.8* |
| | PHAPI2b | 195 | *57.7* | 165 | *80.6* | 30 | *20.1* |

*=mapmodulin

**B**

Amino acid residues and homology against rLANP

| Animal | Name | total/*homology* | | N-term/*homology* | | acidic/*homology* | |
|---|---|---|---|---|---|---|---|
| Rat | rPAL31 | 272 | *56.3%* | 180 | *63.1%* | 92 | *42.6%* |
| | rLANP | 247 | *100.0* | 164 | *100.0* | 83 | *100.0* |
| Mouse | mPAL31 | 272 | *55.9* | 180 | *62.5* | 92 | *42.6* |
| | mLANP | 247 | *98.4* | 164 | *99.4* | 83 | *96.4* |
| | mPP32 | 247 | *98.0* | 164 | *99.4* | 83 | *95.2* |
| Human | PHAP I* | 249 | *88.0* | 167 | *88.6* | 82 | *83.5* |
| | SSP29 | 251 | *68.0* | 165 | *73.7* | 86 | *42.4* |
| | APRIL | 249 | *68.0* | 163 | *73.7* | 86 | *42.4* |
| | PHAPI2b | 195 | *56.8* | 165 | *73.7* | 30 | *11.4* |

*=mapmodulin

Table. 2-1(A) Proteins which show high homology to rPAL31 (number of amino acid residues, homology in total protein sequence, N-terminal, acidic region, respectively) are listed. (B) The same homology search against rLANP. Note that SSP29 and APRIL show higher homology to rPAL31 than LANP, while PHAP I shows higher homology to rLANP than rPAL31.

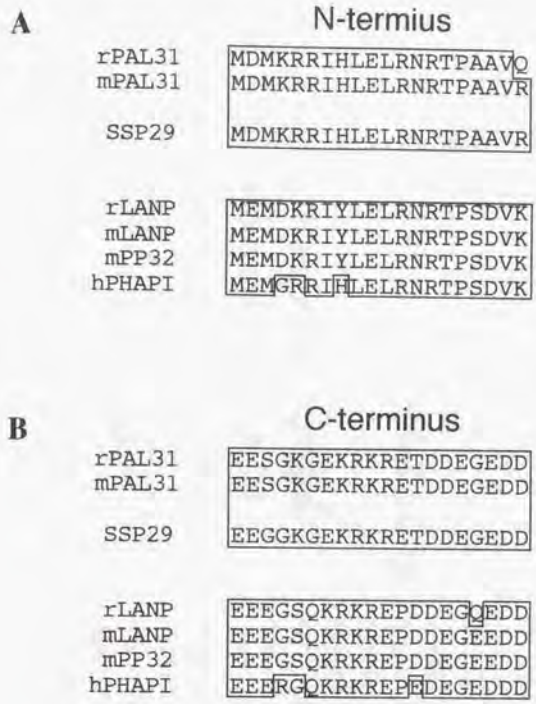



Fig. 2-6 Sequence comparison of rPAL31, mPAL31 and their homologues. (A) Amino acids residues ( 1-20 ) are aligned. Identical residues among rPAL31, mPAL31, SSP29 or among rLANP, mLANP, mPP32, hPHAPI are in the boxes. (B) Amino acids of last 20 amino acids are aligned. Identical residues are in the boxes as (A).

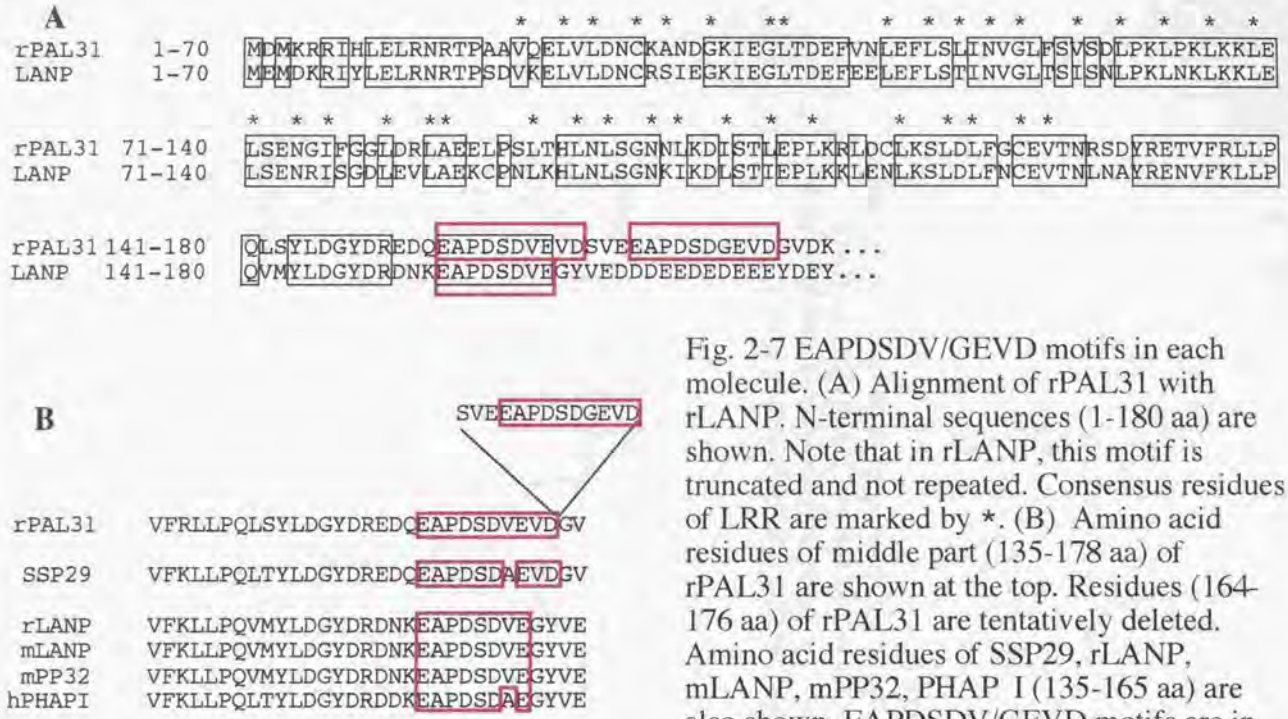



Fig. 2-7 EAPDSDV/GEVD motifs in each molecule. (A) Alignment of rPAL31 with rLANP. N-terminal sequences (1-180 aa) are shown. Note that in rLANP, this motif is truncated and not repeated. Consensus residues of LRR are marked by *. (B) Amino acid residues of middle part (135-178 aa) of rPAL31 are shown at the top. Residues (164-176 aa) of rPAL31 are tentatively deleted. Amino acid residues of SSP29, rLANP, mLANP, mPP32, PHAP I (135-165 aa) are also shown. EAPDSDV/GEVD motifs are in the red box.

| | PAL31 subfamily | SSP29 subfamily | LANP subfamily |
|---|---|---|---|
| acidic region | long | medium | short |
| EAPDSDG/V EVD motif | 2 | 1 | 1 |
| rat | rPAL31 | ? | rLANP |
| mouse | mPAL31 | ? | mLANP mPP32 |
| human | ? | SSP29 PHAPI2b | PHAPI |

Fig. 2-8 Putative classification of PAL31 homologous proteins. Their properties (length of acidic region, number of EAPDSDG/VEVD motif) are indicated. Proteins in each species are also indicated.

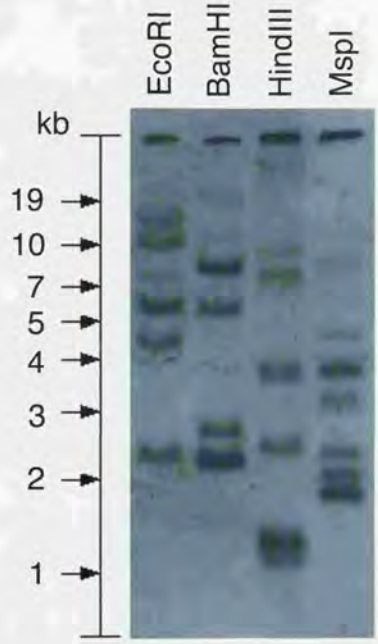



Fig. 2-9 Southern blot analysis of mPAL31. Genome DNA from Balb/c mouse was digested by EcoR I, Bam H I, Hind III, and Msp I, respectively. Digested DNA (10 μg/lane) were fractionated onto 0.8 % agarose gel. DIG labeled cDNA which encodes entire ORF of mPAL31 was used as probe.


Fig. 2-10 rPAL31 mRNA expression in various adult tissue. Total RNA (3 μg) from each sample were fractionated onto 1% denatured agarose gel. E12, embryonic day 12 brain; B, adult brain; Lu, lung; H, heart; S, spleen; Li, liver; I, small intestine; K, kidney; T, testis; O, ovary; A, adrenal gland.

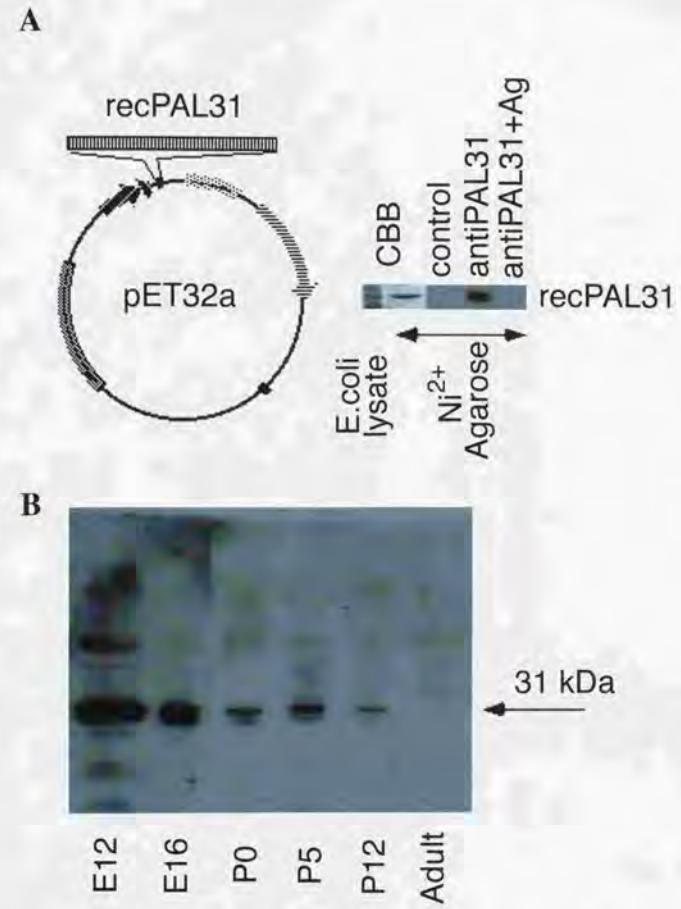



Fig. 2-11 Production of anti PAL31 antiserum and detection of PAL31 in the rat brain. (A) Structure of recombinant PAL31 expression vector, and specificity of anti PAL31 antiserum. Purified recPAL31 were subjected to SDS-PAGE, transfered, and incubated with normal rabbit serum (control), anti PAL31 antiserum (anti PAL31), antiserum coincubated with excess amount (12 μg) of recPAL31 (anti PAL31+Ag). (B) Western blot analysis to detect PAL31 expression in the brains. Total proteins (25 μg) extracted from each brain were fractionated onto SDS-PAGE (12 % polyacrylamide gel), transfered, and blotted with anti PAL31 antiserum.

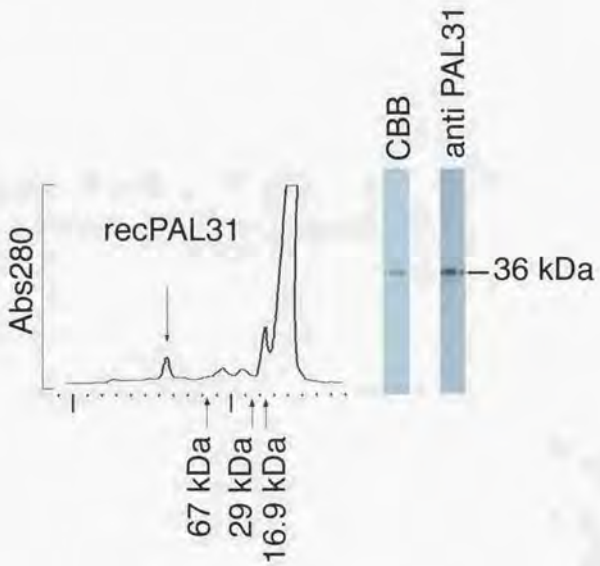



Fig. 2-12 Purification of Tag digested recPAL31. Trx and His tag was digested by thrombin and filtrated by HPLC. Arrows show the peak of recPAL31 and peak of MW markers. MW of recPAL31 (36 kDa) estimated by markers was approximately 92.6 kDa. Portions of fraction (16-17 min) were subjected to SDS-PAGE, stained by coomassie brilliant blue (CBB) or blotted with anti PAL31 antiserum.

# Chapter 3: PAL31 Expession in the Brain ~Immunohistochemical Study

## *Introduction*

PAL31 is expressed in the embryonic brain, and both the mRNA and protein level is downregulated during development. To elucidate the function of PAL31, tissue distribution and intracellular localization of the PAL31 protein will be necessary.

In embryonic brain, stem cells and neural/ glial progenitor cells are rapidly proliferating in entire region of the brain [3][1]. In adult, most of the cells are terminally differentiated, but very few cells in the subventricular zone (SVZ) are reported to continue division even in the adulthood [11, 78][6]. It is important to study where in the brain PAL31 is expressed.

PAL31 has putative NLS and acidic region in its protein sequence. However, not all the nuclear proteins have NLS [124][125]. Recently, there is a report of third translocational signal sequence in the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 [126]. A transcription factor NF-κB has NLS but usually exists in the cytosol. It translocates to nucleus when activated [127][128]. In addition, one of the PAL31 family protein PHAP I/ mapmoduolin is present in cytosol [87][88, 89]. PHAP II or NAP 1 is reported concomitantly with PHAP I with its affinity to HLA-DR cytoplasmic domain, and it is reported to translocated into the nucleus in S-phase, and predicted to regulate chromatin assembly [129].

Proliferating cell nuclear antigen (PCNA) is established to be a marker for cell proliferation, and also S-phase specific marker [130]. PCNA is originally identified as an antigen in proliferating cell nucleus, which reacts with a certain serum from a patient suffering from systemic lupus erythematosus [131]. PCNA is a multi functional gene, which

enhances DNA replication [132-134][132] and regulates cell cycle [135] and also DNA repairment [136]. Nestin was originally identified as a neural progenitor cell specific marker [63]. It is an interfilamentous protein specifically expressed in neural progenitor cells.

Rat pheochrome cytoma PC12 cell is one of the models of neural progenitor cells. It stops mitosis by 7 days of NGF exposure and extends neurite like dendrites [20], and expresses several neural specific marker [137]. Since NGF is secreted in central nervous system [23][24], PC12 is also considered as a model of central nervous system. There are many reports about signal transduction system after NGF treatment [120][122][121][138][139][140][137].

In this chapter, I investigated the cellular and subcellular localization of PAL31 in the embryonic brain, adult brain, and PC12.

## *Materials and Methods*

### Reagents

Anti PCNA monoclonal antibody PC10 was from Oncogene Research Products (MA, USA). anti nestin antiserum was a gift from Dr. Tomooka (Tokyo Science University). Texas Red conjugated goat anti rabbit IgG was from ICN Pharmaceuticals (OH, USA). FITC conjugated goat anti mouse IgG was from Tago Immunologicals (CA, USA). Unless otherwise mentioned, other reagensts were purchased from Wako Pure Chemicals (Osaka, Japan).

### Immunohistochemistry

Anti PAL31 antiserum was IgG purified by Protein A sepharose column. rProtein A Sepharose Fast Flow column (0.5 ml) (Pharmacia) was washed by 10 ml $H_2O$, 10 ml elution bufer (50 mM Glycine-HCl, pH 2.6), again by 10 ml $H_2O$ and pH was adjusted to 8.5 by binding buffer (25 mM Tris- HCl pH 8.5). Antiserum (2 ml) through 0.45 μM filter was added onto the column, capped, and gently rotated at 4 °C for one night. Column was washed by 20x vol of binding buffer, and IgG was eluted by elution buffer. Eluate was recovered every 1 ml in 0.2 ml of 1M Tris-HCl (pH 8.5) immediately. Approximately 10 % of IgG protein from total serum protein was constantly recovered in this procedure.

Tissue samples were Bouin fixed and paraffin embedded. Sections of 4 μm thickness were mounted to vecstatin-coated slides, and incubated with IgG purified anti PAL31 antiserum (0.1 mg/ml) or another antibodies (dilution was according to manufacturer's protocol). Signals were visualized by using ABC kit (Wako). IgG-purified PAL31 rabbit antiserum was and as 0.1 mg/ml of concentration in 1 % BSA/PBS. Same

concentration of IgG-purified normal rabbit serum was used as control. Tissue samples were deparaffined, soaked in 2N HCl for 20 min, rinsed in PBS containing 0.05 % Tween-20 (PBS-T) twice for 5 min. Samples were circled with DAKO-PEN, preincubated with 15 ml of normal goat serum for blocking for 15 min. Then antiserum was added and incubated at 4 °C for overnight. Samples were rinsed in PBS-T twice for 5 min, and then incubated with biotinylated goat anti rabbit IgG antiserum for 30 min. Samples were rinsed in PBS-T twice for 5 min, and incubated with streptavidin-biotinylated horseradish peroxidase for 5 min and rinsed by PBS-T twice for 5 min. For the detection, DAB was added as 0.25 mg/ml in 0.5 M Tris-HCl (pH 7.5), 0.05 % $H_2O_2$ .

In case of PCNA, signals were revealed by using alkaline phosphatase conjugated secondary antibody with Vector Black as a substrate (Vector lab., CA, USA).

**Immunofluorescence**

For double staining, PAL31 antiserum was at first revealed following Texas Red conjugated goat anti rabbit IgG. Then, anti PCNA mAb was revealed by FITC conjugated goat anti mouse IgG. Both signals were scanned by confocal laser scanning microscopy (CLSM) LSM510 (Zeiss, Berlin, Germany). Texas Red was excited by 543 nm Helium/Neon laser and emitted light was detected through LP 585 nm filter. FITC was excited by 488 nm Argon laser and detected through BP 505-530 filter. FITC conjugated mouse second antibody showed small extent of cross reactivity against rabbit IgG, but it was ignorable since the extent of cross reaction was much lower than the signal against mouse IgG (comparison with Fig. 3-3a and c). Cross reactivity of anti rabbit antibody against anti mouse IgG was not tested, since PAL31 detection was completed before the PCNA detection.

Scanning conditions were exact the same in respective figures except Fig. 3-3. In Fig. 3-3, condition was exact the same in E12 sample (Fig. 3-3a, b and c). In adult brain SVZ, result was quite difficult to analyze because of the high background thus contrast and brightness were adjusted. Many self-fluorescent cells were detected in the tissue. They were considered as blood cells from their morphologies (Fig. 3-3d #). Self-fluorescent cells were both highly PAL31 and PCNA positive in the entire region of the cells, thus they were detected as strong yellow signal. Number of self-fluorescent cells were also observed in the endothelium.

To analyze intracellular localization of PAL31, fluorescent dye SYBR GREEN I (Molecular Probes, OR, USA) $10^{-7}$ fold diluted in PBS were added in the sample to stain DNA. Signals were scanned by CLSM TSC-SC (Leica, Berlin, Germany). Texas Red was detected through 600-700 nm filter, and SYBR GREEN I was detected through 505-550 nm filter. 20 slices along z axis in every 0.28 μm were scanned and projected to one image.

**PC12 cell culture**

PC12 was cultured in DMEM including 10 % horse serum, 5 % fetal calf serum, 5 U/ml penicillin/ streptomycin in 5% $CO_2$ at 37 °C on collagen coated coverslips. No trypan-blue stainable cells were observed during cell culture. For differentiation, medium was exchanged to DMEM containing 1 % horse serum and 50 ng/ml of NGF and incubated for 7 days on collagen coated dishes [141]. Medium was changed every 3 days. for immunohistochemistry, cells were fixed by methanol (-20 °C) for 5 min, dried out, and kept in -20 °C prior to use. For northern hybridization, cells were recovered, and extracted RNA were subjected to northern hybridization as described in chapter 2. PAL31 intensities were normalized by g3pdh intensities rehybridized on the same membrane.

## *Results*

### Distribution of PAL31 in the brain

PAL31 distribution in fetal rat brain (E12) was tested. PAL31 signal was detected in entire region of CNS (Fig. 3-1a). Observation in higher magnification (Fig. 3-2a) revealed that PAL31 was expressed in the same extent from the basal membrane of ventricle through the epithelial membrane of neural tube. PAL31 signal was detected in the nuclei, but the signal was detected in the cytosol as well. Adjacent section was also stained with anti PCNA antibody [130, 142]. PCNA signal was also observed in the nuclei of entire region of the CNS of embryo (Fig. 3-1b, 3-2b). Expression pattern of nestin in E12 embryo was also tested in another adjacent section. The signal was detected specifically in the neural tube. Strong nestin signal was observed in the epithelial side of the CNS (Fig. 3-2c).

In sagittal section of P5 brain, small number of the cells around ventricle appeared to be PAL31 positive (Fig. 3-2e). In the coronal section of adult brain, small number of cells only in the SVZ were PAL31 positive (Fig. 3-2i). PAL31 signal was not detected in the cerebral cortex. Number of PCNA positive cells were decreased, and found to be restricted around the ventricle in the P5 brain (Fig. 3-2f). A few number of PCNA positive cells were also detected only in SVZ (Fig. 3-2j). A few nestin positive cells were detected in the P5 SVZ (Fig. 3-2g), but the signal was undetectable in the adult SVZ (Fig. 3-2k).

Tissue samples were subjected to immunofluorescent study by CLSM for further analysis of PAL31 distribution. PAL31 was expressed in the entire region of the E12 neural tube (Fig. 3-3a and b) and so was PCNA. Cells in neural tube were apparently expressing PAL31 in the nucleus and

also PCNA at the same time, because the red signal which indicates PAL31 signal turned into yellow green color because of the double staining with PCNA (Fig. 3-3b). PAL31 signal was observed not only in the cell nucleus but also in the entire cell body. PAL31 was detected in the entire region of neural tube from basal ventricular region through epithelial region (Fig. 3-3a). PCNA signal was in the nuclei, and the signal was rather weak in the ventricular zone (Fig. 3-3b and c).

In adult SVZ, PCNA positive cells were detected in SVZ, and several cells appeared to be PAL31 negative (Fig. 3-3d +). However, very few number of cells were detected to be PAL31 positive in the cytosol and PCNA in the nucleus (Fig.3-3d * and its higher magnification, 3-3e). The cells which have this signal pattern were detected only in SVZ.

**PAL31 expression in PC12 cells with or without NGF treatment**

PAL31 mRNA expression in PC12 cells was decreased to 71.04 ± 7.06% by NGF treatment for 7 days ($p<0.05$, $n=3$) (Fig. 3-4). Strong PAL31 signal was detected in the dividing PC12 cells, and the signal was also downregulated by NGF treatment for 7 days (Fig. 3-4).

**PAL31 expression pattern and subcellular localization**

Then, mitotic PC12 was doublestained by PAL31 and PCNA. Immunofluorescence data in Fig. 3-5a shows that PAL31 was accumulated in certain particles in the nucleus. The number, size and intensity of the subnuclear compartment visualized by PAL31 were variable among cells. Occasionally, PAL31 signal was co-localized with PCNA. PAL31 was expressed in the cytosol as well. PAL31 was expressed in all the PC12 cells even if PCNA was not expressed (Fig. 3-5a). PAL31 signal intensity in cytosol was approximately the same level

in all the cells. The PAL31 signal in PC12 cells was disappeared by incubation with antiserum and excess amount of tag-digested recPAL31 purified in Fig.2-12 (comparison of Fig. 3-5c and d). For further analysis of intracellular localization of PAL31, PC12 was immunostained and observed in high resolution CLSM (Fig. 3-6). PAL31 signal in the subcompartments of each nucleus was not detected concomitantly with SYBR GREEN. In addition, PAL31 in the cytosol was not diffusible in itself, rather enclosed in certain structures.

## *Discussion*

Immunohistochemical study revealed that PAL31 is predominantly expressed in developing embryonic brain. Its intracellular localization of PAL31 is nuclei and cytosol, suggesting that localization of PAL31 in to nucleus is regulated

### PAL31 distribution

Northern hybridization and western blot data in Chapter 2 and PC12 in this chapter show that PAL31 expession was downregulated during brain development and cell differentiation. Immunostaining and Immunofluorescent data of E12 brain shows that PAL31 expressing cells are distributed in the entire CNS, and they are in mitotic phase. The co-distribution of PAL31 positive cells and neuroprogenitor cells in embryonic brain suggests that PAL31 influences neural development. Distribution of PAL31 positive cells were restricted in SVZ in P5 and adult brain. In adult SVZ, small number of PCNA positive [11, 78][6](in the nucleus) PAL31 positive (in the cytosol) cells can be found. They are probably distinct from terminal differentiated neuronal or glial cells, because they are roughly round shaped and PCNA positive (thus have mitotic activity).

Series of immunohistochemical study of tissue sections and PC12 cells indicate that the change of PAL31 expression reflect both the decrease of number of PAL31 expression cells, and the downregulation of PAL31 expression level of each cell. This PAL31 expression pattern corresponds well to the downregulation of distribution of mitotic cells in the brain [11, 78][6], and downregulation of mitotic activity of brain cells [2]. Thus, PAL31 is suggested to relate with proliferation.

**Intracellular localization and functions of PAL31**

It is now apparent that PAL31 is a nuclear protein. In PC12 cells, PAL31 is not in the nucleoplasm: it is restricted to certain nuclear particles. The one property of the particles is that DNA is not contained or in its level scarce, suggesting that this particle is nucleolus, which is known as the region of ribosomal construction [143]. In E12 brain, cell nuclei in neural tube are also PAL31 and PCNA double positive. Thus, PAL31 is localized in nucleus both in vivo and in cultured cell. PAL31 is also expressed in cytosol, and it is not diffusible, but enclosed in certain structure. PC12 cells contain not only mitochondria, Golgi apparatus, endoplasmic reticulum but also round and irregular size of dense core granules, which are similar to the structure often observed in sympathetic neurons [20]. Thus, endogenous PAL31 is localized in nucleus as well as cytosolic compartments, suggesting that the protein translocates between them depending on the cell cycle or physiological status.

When PAL31 is transiently expressed in COS-7 cells, it is expressed in nucleoplasm [144]. LANP is expressed predominantly in the whole nucleus in cerebellar Purkinje cell, but also in cytosol observed under light microscope [86][145]. When it is transiently expressed in COS-7, it accumulates in whole nucleus [145]. PP32 signal is detected in entire cell body including nucleus under light microscopic study [90]. SSP29 is predominantly in nucleoplasm and translocated into nucleolous by heat shock treatment [91]. Mapmodulin [89] are mainly in cytosol, both diffusible or localized in endoplasmic reticulum (ER) and Golgi membrane. However, electron microscopic analysis of PHAP I [87] describes about nuclear localization of the molecule. Thus, PAL31 family proteins generally have potentials to target nucleus, but the localization of endogenous form is regulated in the cell.

NLS is reported to associate with importin proteins [146, 147][148] and transported into nucleus by the aid of small guanidine phosphate protein, Ran [146]. Translocation of PAL31 into nucleus requires C-terminal region of its sequence [144]. PAL31 does not have putative O-GlcNAc site [125], nor M9 motif [124], the other modulation sites for nuclear targeting. Thus, it is highly probable that PAL31 associates with importin family with its NLS when targetted into nucleus.

Although total amount of PAL31 is constant in PC12 cells, intensity in the nucleus are variable depending on the cell cycle among cells. There are several reports that NLS is regulated via phosphorylation in the proximal to the NLS [149]. v-Jun is translocated into nucleus in cell cycle dependent manner [105][150]. It is accumulated in nucleus specifically in G2 phase, and dephosphorylation of $Ser^{248}$ adjacent to the NLS ($R^{249}$KRKL, Fig. 2-4D) is essential for nuclear targeting. NLS of a transcription factor NFκB component p65 is masked by IκB and it is retained in cytosol. Release from retention is effected by proteolytic degradation of IκB, which is triggered by IκB phosphorylation [127]. It results in unmasking of the p65-NLS, and $Ser^{258}$ that is 22 amino acid N terminal of NLS (Fig. 2-4D) enhance migration of NFκB into nucleus [106]. Moreover, phosphorylation of $Ser^{111}$ and $Ser^{112}$ which are casein kinase II targets increases binding affinity for NLS binding protein importin of SV-40 T antigen [151], while $Thr^{124}$ phosphorylation by cdk/cdc2 inhibits its import to the nucleus [107]. PAL31 has $Ser^{225}$, $Ser^{255}$ and $Thr^{265}$ putative phosphorylation sites in the proximal region of the NLS. It is possible that PAL31 nuclear localization is regulated by phosphorylation or dephosphorylation of these residues. Nuclear export signals contains leucine rich peptide [152]. Whether PAL31 also exports from nucleus to cytosol is not known.

In summary, PAL31 signal correspond to the cell proliferation marker PCNA during brain development. PAL31 is appeared to be a nuclear protein. Subcellular localization pattern is unique, and it suggests that PAL31 is regulated when it translocates to nucleus.

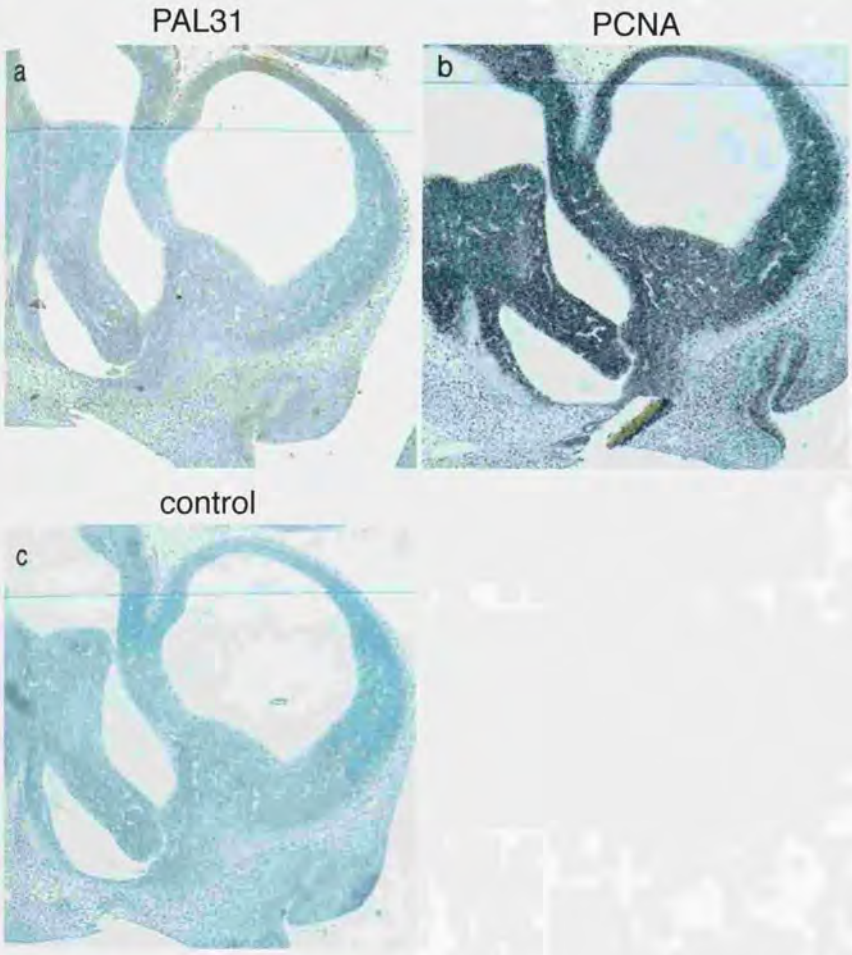



Fig. 3-1 PAL31 expression in the developing brain (x5). This figure shows that entire E12 head reagion cells including brain were PAL31 positive. a, anti PAL31 visualized by DAB; b, anti PCNA visualized by Vector Black; c, normal rabbit serum visualized by DAB.

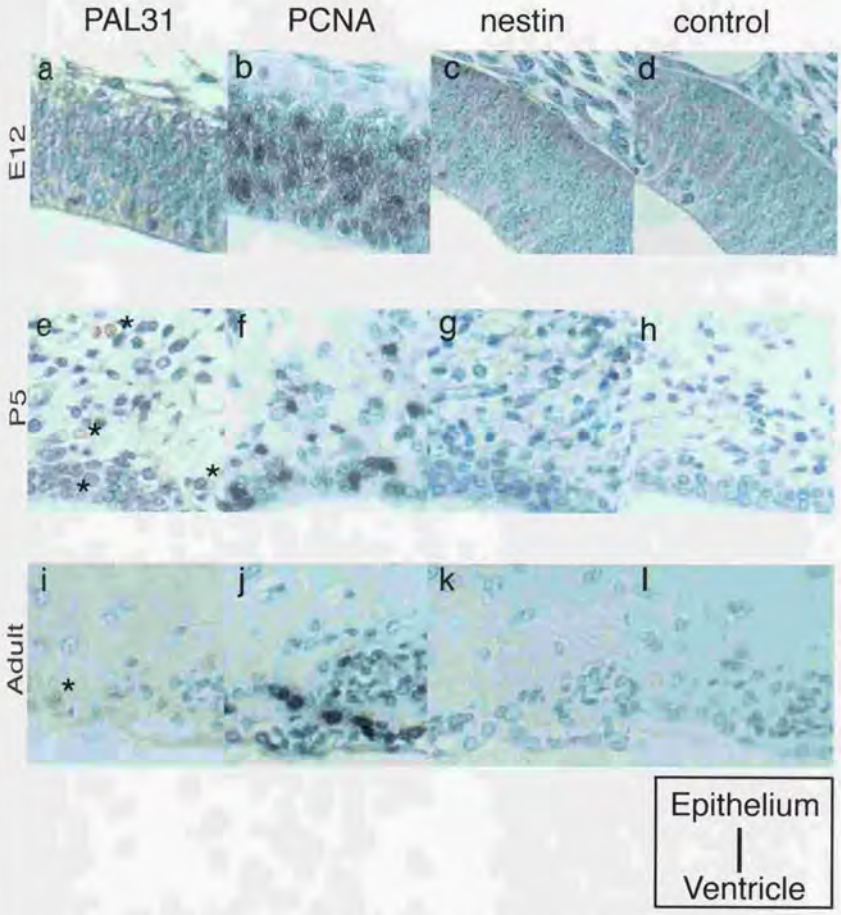
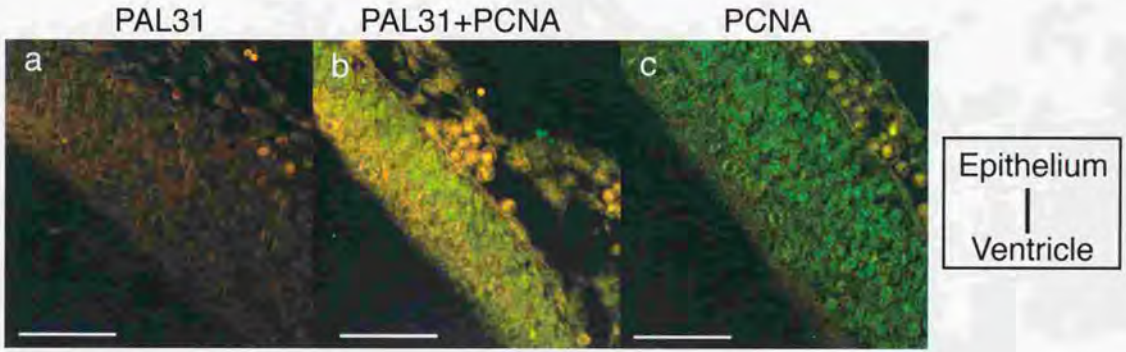



Fig. 3-2 PAL31 expression in the developing brain (x40). E12 (a- d), P5 (e- h) and adult brain (i- l) were immunostained as Fig.3-3. Small number of PAL31 positive cells were observed in SVZ (*), where proliferating cells exist. a, e, i, anti PAL31 visualized by DAB; b, f, j, anti PCNA visualized by Vector Black; c, g, k, anti nestin visualized by DAB; d, h, l, normal rabbit serum visualized by DAB.



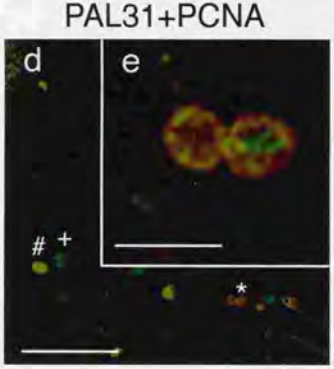




Fig. 3-3 PAL31 expression in the developing brain (immunofluorescence). E12 (a- c) and adult brain (d) were incubated with anti PAL31 antiserum (a, b, d) or with normal rabbit serum (c). Then, samples were incubated with anti PCNA mAb (b- d) antibody (a- d). Bar, 50 μm (a- d), 10 μm (e, higher magnification of * in d). +, PCNA positive cells. #, self-fluorescent cells. PAL31, red; PCNA, green.

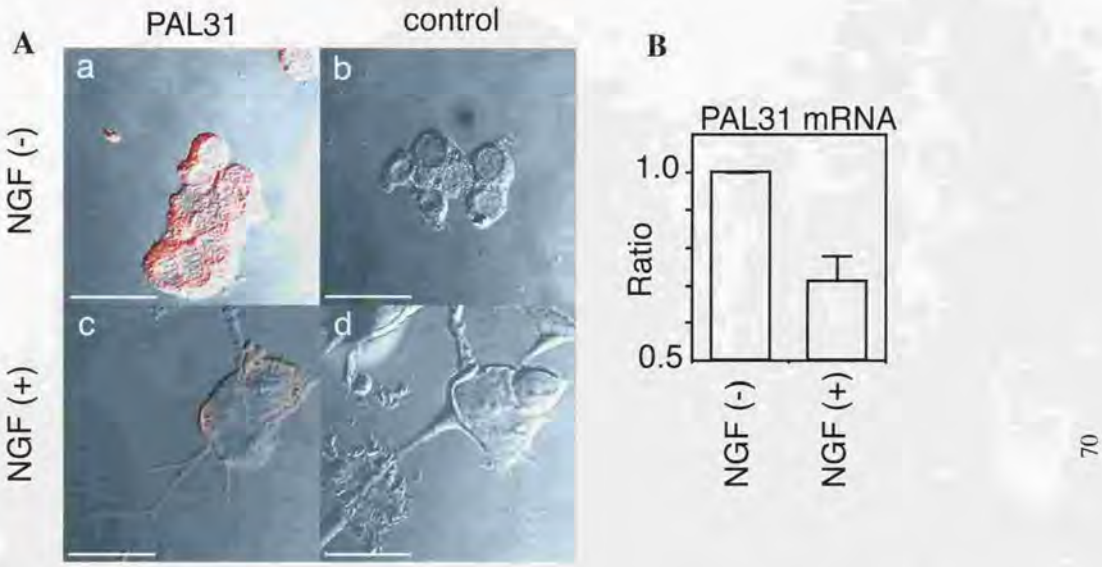



Fig. 3-4 PAL31 expression in PC12 cell was downregulated by NGF treatment. (A) PC12 cells were either treated without (a, b) or with (c, d) 50 ng/ml NGF for 7 days. Cells were fixed by methanol. a, c, anti PAL31; b, d, normal rabbit serum. PAL31 signal (red) is visualized with transmissional view. Bar, 50 μm. (B) PAL31 mRNA was downregulated to 71.04 ±7.06 (SE) % after NGF treatment for 7 days. n= 3, $p<0.01$ (Student's t-test).

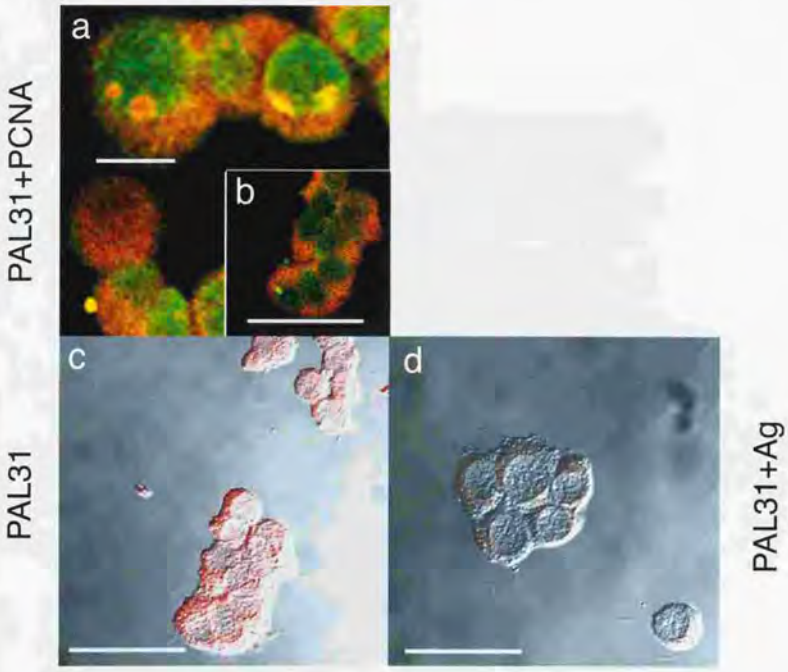



Fig. 3-5 PAL31 expression in PC12 cells compared with PCNA. b and c, PAL31 and PCNA; d, anti PAL31 antiserum was absorbed by 1.2 μg of recPAL31. c and d, PAL31 signal with transmissional view. Bar, 50 μm (b- d), 10 μm (a, higher magnification of different field of b). PAL31, red; PCNA, green.

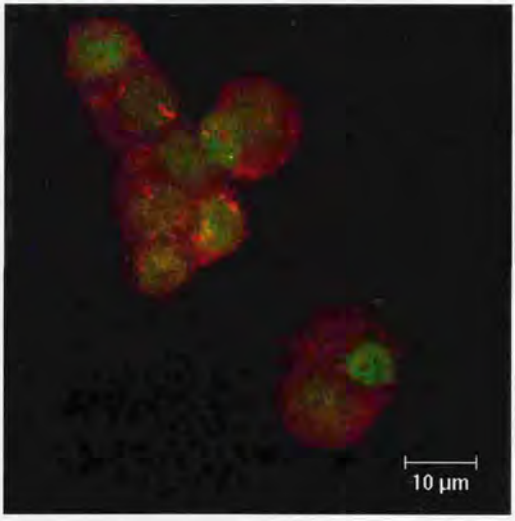



Fig. 3-6 PAL31 expression in PC12 cells.
PAL31 signal is visualized by Texas Red (red). Nucleus is visualized by SYBR GREEN I (green). PAL31 was expressed not only in the nucleus but also in cytosol.

# General Discussion

## *General Discussion*

It is assumed that placental hormone is a candidate factor to influence brain development. Viviparity is one of the properties that define and classify eutheria (placental mammals) from other mammal species. Placenta is an essential organ to maintain the pregnancy and embryonic development [153][154]. Placenta exchanges O2 and CO2, nutrients and metabolites between mother and pups and also expresses number of peptide and steroid hormones to maintain homeostasis of both mother and embryos [155][156]. It also guarantees embryos to grow in the another individual without any immunorejection [157]. Placental factors are detected in fetal plasma [73, 74], and PL antigenic proteins are found in amniotic fluid [158]. Blood brain barrier are undeveloped [32][33] in embryonic stage,and large molecules can target the brain as any other tissue during development. The result indicating that PRL-R mRNA is expressed in the early embryonic brain suggests that PL influences the development of the tissue. Recently, NGF was found to be secreted from lymphocyte and anterior pituitary [159], and secretion of NGF is altered by several cytokines [159]. Evidence of PRL-R expression in early embryonic brain and resent reports suggest the interaction between endocrine system and nervous system.

Northern blot data clearly shows that PAL31 gene is highly expressed in embryonic stage and low level in adult brain. PAL31 expression was restricted at SVZ in adult brain, where the small number of cells are in mitotic cycle [11, 78][6]. PAL31 expression in PC12 cells was repressed by inhibitor of mitosis and inducer of differentiation, NGF. PAL31 is also highly expressed in the rat lymphoma cells, Nb2. When Nb2 cells stop mitosis by serum starvation, PAL31 mRNA is

downregulated. When the cell cycle is recovered by serum administration, PAL31 mRNA level returns to the normal level of dividing cells. PRL stimulates cell proliferation in Nb2 cells and PAL31 is also induced at the start of S-phase in response to PRL [160]. Cell growth is retarded by antisense oligonucleotide for PAL31 [160]. These data suggest that PAL31 is necessary for proliferation of the cells.

PAL31 family members are reported to be highly expressed in neoplastic or dividing cells. LANP expression is at its peak in developing cerebellum [86]. SSPs are members of the malignant tumor markers [161], and SSP29 itself is highly expressed in cell line or neoplastic tissue [91]. pp32 is highly expressed in the neoplastic prostate [162], and also induced in B cells by lipopolysaccharide treatment [90], although it is reported to inhibit oncogene-induced focal formation [163]. Thus, functions of PAL31 family genes presently known are presumed to regulate (positively or negatively) cell mitosis. Among all, PAL31 is appeared to be important for neural development.

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## *Acknowledgements*

First of all, I would like to represent my heartfelt thanks to Dr. Kunio Shiota, my present supervisor and Dr. Tomoya Ogawa, my former supervisor and presently an executive at RIKEN. They encouraged me and gave essential advises whenever I had trouble in my experiments or writing a paper. It was a precious time to discuss with these excellent scientists. Without a doubt, it is my life-long treasure to have learned the essential part of scientific attitude from them in my first days as a scientist.

I would also like to thank Dr. Naka Hattori, Dr. Jun-ichi Aikawa, Dr. Takayuki Kamei, who collaborated and supported to publish my first paper. What I learned from them were not only numerous practical techniques but also how to design the series of experiments. Their ideas, life styles dedicated to science and black jokes were always fascinating.

I am deeply indebted to Dr. Yasushi Toyoshima, Mrs Junli Yan, Miss Hisako Takigawa, Mr. Weiyong Sun and Miss Mayumi Oda for collaboratiions and discussions all about PAL31 study. Although sometimes it might have been a hectic work, their skills and endurance were really great. I really appreciate them. I am also grateful to all the former and present members in the laboratory of cellular biochemistry, especially Dr. Satoshi Tanaka. I could not have done this work without their helps and cheers.

Finally, I thank my parents and my brother for all the victims and patience, and encouragement.

# 論文の内容の要旨

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論文題目「中枢神経系における新規遺伝子PAL31の発現に関する研究」

## 緒言

胎仔期の哺乳類中枢神経系(CNS)の特徴として、全ての細胞の増殖が盛んであることが挙げられる。一方、成熟個体(adult)では、増殖性神経前駆細胞は、脳室下層(SVZ)などの極めて一部に限られる。胎仔CNSでの細胞増殖活性を誘導・維持する機構に興味が持たれた。筆者は、ラットCNSの発生段階で、胎仔外の組織から分泌される因子が神経発生に関わる可能性を探り、また胎仔脳内で主に発現する遺伝子の探索をおこなった。その結果、胎盤性プロラクチン様ホルモン(PL)が神経発生に関与する可能性を得、また細胞増殖に関連すると示唆される新規遺伝子PAL31のクローニングに成功した。

## 第一章: Prolactin Receptor mRNA Expression in Fetal Rat Brain

CNSは脳血液関門によって、血中高分子の脳内への輸送を調節している。胎仔期には脳血液関門は未発達であり、内分泌物質が脳へ直接作用することが可能である。哺乳類では、妊娠中、胎仔の正常な発育と母体の恒常性を保つため、胎盤は多様な物質を分泌する内分泌器官として発達する。下垂体性ホルモンプロラクチン（PRL）及びPLは受容体(PRL-R）を共有し、母性行動の誘起、性行動や摂食行動の調節に関わることが報告され、CNSに影響をあたえている。本研究では、胎生12日(E12)の脳において二種類のPRL-Rs mRNAが発現していることを発見した。この時期は母体PRL、胎仔下垂体性PRLがどちらも発現しておらず、PRL-Rは生理的にはPL受容体として機能していると考えられた。short form PRL-R mRNAは胎仔脳の成長に伴い発現量が増大していた。

また、各受容体は肝臓、心臓、腸管、前肢において発現開始時期が異なり、臓器特異的なPRL-Rの発現調節が予想された。以上、PLが胎仔へ移行し、母体と共に、胎仔のCNSをはじめとする各組織の発達に影響を及ぼす可能性が得られた。

第二章: Molecular Cloning of PAL31 (Proliferation Related Acidic Leucine Rich Protein with Molecular Size of 31 Kilo-Dalton) from Fetal Rat Brain

特に胎仔発生時に重要な遺伝子を同定する目的で、ラットE12とadultの脳における遺伝子発現の変化を、Differential Display法を用いて検索した。E12で高度に発現する1,225塩基の新規遺伝子のクローニングに成功した。この遺伝子は全長272アミノ酸残基、分子量31,064の蛋白質をコードしていた。全残基中の64残基がglutamate(23.53%)、44残基がaspartate(16.18%)、31残基がleucine(11.40%)であり、さらにleucineはN末側145残基に遍在していた。この蛋白は酸性アミノ酸とleucine残基を豊富に含み、予想pIは3.87と酸性であった。後に述べるように、本遺伝子が増殖に関連していることが示唆されたため、この遺伝子をラットproliferation related acidic leucine rich protein with moleculear size of 31 kDa (rPAL31)と名付けた。予想配列のN末側には蛋白質間の相互作用が予想されるleucine rich repeats (LRRs)、C末側に酸性アミノ酸領域(Acidic Region)、予想核移行シグナル(NLS)、LRRsとAcidic Region間にEAPDSDG/VEVD(EA...VD)の繰り返し配列が認められ、また予想リン酸化部位も豊富に存在した。複数のExpression Sequence Tags (ESTs)より、マウスPAL31(mPAL31)の存在が予測され、アミノ酸配列が98.9%一致する同遺伝子がクローニングされた。mPAL31は複数のエクソンをもち、48kb以上のゲノム上にコードされると予想された。

PAL31には複数の相同遺伝子が報告されている。上記の配列の特徴は蛋白質間、動物間で非常によく保存されていた。これらはPAL31ファミリーを形成すると考えられ、詳細な解析の結果、さらに3種類に分類できた。すなわちAcidic Regionが最長(92残基)、EA...VDが二回存在するPAL31サブファミリー、AcidicRegionが中間(86残基)、EA...VDが単独のSSP29サブファミリー、Acidic Regionが最短(82-83残基)、EA...VDが単独のLANPサブファミリーである。PAL31/SSP29サブファミリーは互いの相同性がより高い。ラット、ヒトでも未知の遺伝子の存在が予想された。

rPAL31 mRNAは全長約1.5kbであり、胎仔期に高発現がみられ、adultでは著明に減少していた。蛋白質発現量もE12脳で高く、その後減少し、adultでは検出限界以下であった。またadultでは、rPAL31mRNAは調べられた全ての臓器(脳、肺、心臓、脾臓、肝臓、小腸、腎臓、精巣、卵巣、副腎)で発現し、脾臓、精巣、卵巣など増殖細胞が多く含まれる組織で発現量が高かった。以上、PAL31のクローニングに成功し、この遺伝子が高度に種間で保存された遺伝子群の一つであることが明らかになった。

ゲル濾過によって、PAL31の多量体形成の可能性を検索した。単量体で36kDaの組換えPAL31は、92kDaの画分に溶出され、PAL31がin vitroにおいて三量体構造を取ることが示された。PAL31が分子内にLRRsを持つことと考え合わせると、PAL31は自己、あるいは他の蛋白質との複合体として細胞内に存在すると予想された。

第三章: PAL31 Expession in the Brain~Immunohistochemical Study

胎仔期の神経管構成細胞は増殖が盛んであるが、その数は発達に伴い減少し、adultではSVZなどにごく少数存在する。免疫組織学的解析により、PAL31の組織分布と細胞内局在を調べた。E12においてはPAL31はCNS全体で発現が見られた。PAL31は細胞核も含めた細胞体全体で存在していた。PAL31陽性部位は、細胞増殖マーカーproliferating cell nuclear antigen (PCNA)、神経前駆細胞マーカーnestinとも発現部位が重複した。E12において、PAL31陽性細胞には神経前駆細胞も含まれ、またそれらは増殖性であった。生後5日(P5)においては、PAL31陽性細胞は脳室周囲に限局し、またadultではSVZでごく少数の細胞がPAL31を発現していた。増殖性細胞も、P5においては脳室周囲等、adultではSVZで少数存在していた。すなわち、CNSの発達に従ってPAL31陽性細胞、PCNA陽性細胞はどちらもその数が減少し、脳内分布が限局し、両者の存在部位は一致していた。さらに免疫蛍光染色により、PAL31陽性細胞はE12においてPCNAと二重陽性であり、adult SVZにおいても、PAL31・PCNA二重陽性の細胞が観察された。

神経栄養因子(NGF)によって増殖を停止し、神経様に分化するPC12細胞を用い、細胞内のPAL31発現量変化を調べた。7日間のNGF刺激により、PAL31 mRNA、また単一細胞中PAL31発現量が顕著に減少した。これにより、PAL31発現が細胞増殖と関連していることが示唆された。また、PAL31は核蛋白であることが判明し、分子内NLSをその移行シグナルとして実際に用いていることが示唆された。また、細胞質にもPAL31が観察され、核内移行が調節性であると考えられた。各細胞の細胞質でほぼ同程度にPAL31が染色される一方、核内シグナル強度にはばらつきがみられた。細胞質中でもPAL31は小器官様構造に集積しており、PAL31の、分子や小器官との相互作用とその調節がPAL31の機能に重要と考えられた。

結論

第一章では、妊娠特異的組織の胎盤から分泌されるPLが、神経発生に影響を及ぼす可能性を得た。第二章では、胎仔脳で高度に発現する新規遺伝子PAL31を同定した。第三章では、免疫組織化学解析により、PAL31は調節性核蛋白であり、PAL31が細胞増殖に関連していることが示唆された。哺乳類の胎仔期CNSの、構成細胞の高い増殖活性という特徴は、胎盤性因子の発達中CNSへの関与や、脳内PAL31遺伝子の発現調節により誘導・維持されている可能性がある。

## *Abstract*

Introduction

In embryonic central nervous system (CNS), neural stem cells or neural progenitor cells are in their mitotic cycle. However, in adult CNS, most of the neurons are terminally differentiated and never proliferate again. The cells which are still in mitotic phase are only restricted in several region such as subventricular zone (SVZ). Thus, it is interesting to know the mechanisms which induce and regulate the mitotic property of fetal CNS. I addressed to find out molecules that have roles in developing brain by two speculations; Factors from outside of the mammal embryo might target the embryonic brain; There are probably genes specifically expressed from the embryonic brain.

This report raised the possibility that placental hormone Placental Lactogen (PL) influences fetal brain development. It also described about molecular cloning of a novel gene, PAL 31, which is predominantly expressed in embryonic brain and suggested to relate with proliferation.

Chapter 1: Prolactin Receptor mRNA Expression in Fetal Rat Brain

Blood brain barrier is undeveloped in embryonic stage, thus large molecules that are resisted in adulthood can target the brain as any other tissue during development. Placenta develops during pregnancy as an endocrine organ. Placenta produces various types of hormones to maintain the homeostasis and normal growth of mother and fetuses. PLs share the receptor with pituitary hormone, prolactin (PRL). They are reported to target adult CNS and alter maternal behavior, mating behavior and food intake. In this experiment, PRL-R mRNAs were amplified from embryonic day 12 (E12) rat brain. There is no expression of PRL from mother nor from fetal pituitary at this early embryonic stage. Thus, the receptors are expected to function as PL-R. Short form PRL-R expression was increased during fetal development. PRL-R transcripts were amplified in the different period in each tissue. Thus, PRL-R mRNA is expressed in rat embryo in early stage, and a placental hormone, PL, is a candidate to bind to this receptor and influence the brain development.

Chapter 2: Molecular Cloning of PAL31 (Proliferation Related Acidic Leucine Rich Protein with Molecular Size of 31 Kilo-Dalton) from Fetal Rat Brain

By using differential display method, genes that are specifically expressed between E12 and adult brain were searched. One of the clones showed higher expression in the fetal brain than adult brain. Finally, a novel gene of 1,225 base pair was obtained. It encoded 272 aa protein. Estimated molecular size was 31,064. It contained 64 residues of glutamate (23.53 % of total), 44 aspartates (16.18 %) and 31 leucine (11.40 %). Leucine residues were only found at N-terminal 145 residues. Estimated pI is 3.87. This gene was also suggested to relate with cell proliferation as described later, it was named proliferation related acidic leucine rich protein with molecular size of 31 kDa (PAL31). Deduced amino acid sequence of PAL31 contained leucine rich repeats (LRRs) at the N-terminus. LRRs are predicted to be an association site with protein. There were also acidic region at the C-terminus, putative nuclear localization signal (NLS), a repeat of EAPDSDG/VEVD (EA...VD) motifs between LRRs and acidic region, and also several phosphorylation sites. Alignment of seven expression sequence tags (ESTs) resulted in identification of highly homologous (98.9 % in its ORF) mouse PAL31 (mPAL31). mPAL31 was predicted to be divided into several exons and encoded on genomic region of longer than 48 kb.

Several homologous genes were found out from DNA databank. The motifs and other properties were highly conserved among proteins and animal species. These were considered as PAL31 family proteins. From the more precise analysis, they were classified as three subfamilies. PAL31 subfamily which have long acidic region (92 aa) and a repeat of EA...VD; SSP29 subfamily which have middle length acidic region (86 aa) and single EA...VD motif; LANP subfamily which have shorter acidic region (82-83 aa) and single EA...VD motif. Other unknown homologous genes are predicted to be expressed both in rat and human.

PAL31 mRNA (1.5 kb) and protein levels were both predominant in E12 brain, and gradually decreased during development. PAL31 protein was under detectable level in adult brain. PAL31 transcript was also detected in all the tissues examined (brain, lung, heart, spleen, liver, intestine, kidney , testis, ovary, and adrenal gland). The level was higher in spleen, testis, ovary, those which contain large number of proliferating cells. Thus, PAL31 was cloned from rat embryonic brain and it was

found to be one of the family proteins which are highly conserved each other.

Gel filtration of recombinant PAL31 (recPAL31 of 36 kDa) resulted in elution of the protein as a molecular size of approximately 92 kDa. PAL31 is suggested to form homotrimer complex in vitro. This result and LRRs in its sequence predict that PAL31 exists as complex form by itself or through associating with other proteins in vivo.

Chapter 3: PAL31 Expression in the Brain~Immunohistochemical study

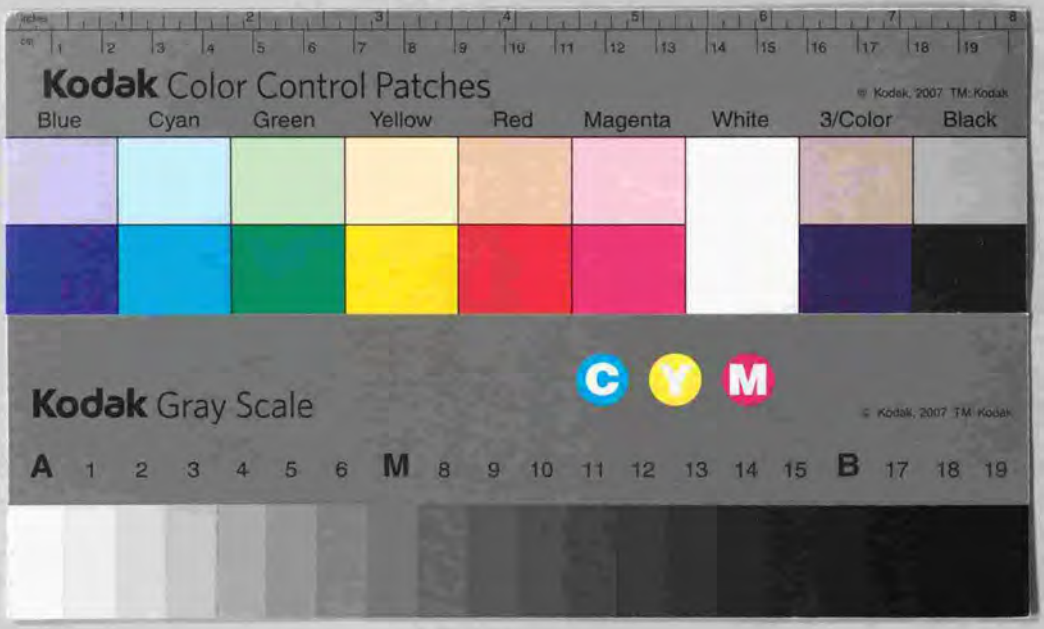
Neuroepithelial cells in embryo are actively dividing, and the number of mitotic cells are decreased during development. It is restricted in SVZ in adult brain. I addressed to analyze the distribution pattern of PAL31 and its intracellular localization. PAL31 protein was expressed in entire region of the E12 brain, and also in whole embryo. PAL31 was expressed also in the proliferating neuroprogenitor cells. PAL31 signal was detected in both nuclei and cytosols. PAL31 positive region corresponded well to poliferating cell region, and overwrapped the neural progenitor region. In postnatal day 5 (P5) brain, PAL31 positive cells were observed around ventricular zone. In adult SVZ, a few number of cells were found to be PAL31 positive. Mitotic cells were also expressed around ventricular zone in P5, and they were restricted to SVZ in adult brain.

PC12 cells stop mitosis and differentiate into neuron-like cells by nerve growth factor (NGF) treatment. In PC12, PAL31 transcripts and the protein signal in each cell was significantly decreased after NGF treatment for seven days. PAL31 was thus suggested to relate with cell proliferation. PAL31 was appeared to be a nuclear protein. PAL31 was also found in cytosol, implicating that this NLS was regulated. Signal intensity in the nucleus was variable among cells, while cytosolic signal of PAL31 was constantly observed. Interaction and its regulation between molecule and organelles were considered to be important for molecular function of PAL31.

Discussion

The one of the properties of embryonic CNS is its activity of cell proliferation. It has been longed to know the mechanism to stimulate and maintain this activity. This report shows that the factors from outside of the embryo, for example placenta can influence the embryonic brain, and

that PAL31 gene expression, subcellular localization and their regulation is suggested to relate with the activity of embyronic CNS growth.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