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# Precursor of cdk5 activator, the 23 kDa subunit of tau protein kinase II: its sequence and developmental change in brain

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**Abstract** Tau protein kinase II (TPKII) is shown by immunoprecipitation to be a complex composed of two subunits, a catalytic subunit, cdk5, and regulatory subunit, p23. By sequence analysis of p23 cDNA, p23 was found to occupy a region from the 99th amino acid residue to the C-terminus of a novel protein with a molecular weight of 34,000 Da, suggesting that this 34 kDa protein is a precursor of p23 (pre-p23). These findings suggest that p23 results from the processing of the precursor protein, pre-p23. The precursor mRNA was expressed most abundantly in rat brain just before and after birth. Expression of pre-p23, but not of cdk5, mRNA changed, coinciding with the developmental change of TPKII activity, suggesting that its expression controls the phosphorylation of tau by the TPKII/TPKI system in the neonatal brain. p23 appears to be a cdk5 activator in neuronal cells.

*Key words:* Cyclin-dependent kinase 5; Tau protein; Paired helical filament; Alzheimer's disease

## 1. Introduction

Paired helical filaments (PHF) are one of the hallmarks of the brain of Alzheimer's disease patients. PHF contain abnormally phosphorylated tau protein, reflecting the physiological state in the brain of an Alzheimer's disease patients. It is important to determine the protein kinase responsible for the phosphorylation in order to understand the etiology of the disease. Tau protein kinases (TPK) I and II (EC 2.7.1.135) are considered as candidates for the kinases involved [1,2].

TPKI, identical to glycogen synthase kinase (GSK) 3$\beta$ [3], phosphorylates native tau in a PHF-like manner [2,3], and is involved in $\beta$-amyloid-mediated neuronal death [4], suggesting that TPKI/GSK3$\beta$ might be a key enzyme in the disease.

Native tau is already phosphorylated to some extent, and this normal phosphorylation enhanced the abnormal phosphorylation by TPKI/GSK3$\beta$. TPKII is considered as a candidate for the kinases involved in this normal phosphorylation, indicating that TPKII regulates the abnormal phosphorylation via normal phosphorylation indirectly [5]. Sites phosphorylated by TPKI [6] and TPKII [7] were occupied significantly by phosphate groups in human PHF-tau [8,9] and rat juvenile tau [10], suggesting that these TPKs are involved in sprouting [11] and/or neuronal death, which are considered to be two common features in Alzheimer's disease brain and neonatal brain.

TPKII is composed of a 30 kDa catalytic subunit and a 23 kDa regulatory subunit (p23) [2]. The 30 kDa subunit is cyclin-dependent kinase (cdk) 5 [12], which was originally known as PSSALRE kinase [13] and later named cdk5 because of its ability of association with cyclin D [14]. cDNA cloning [15] and enzyme purification [16,17] gave the means to search for cdc2-related kinases in mammalian brain, which indicated that cdk5 is the most prominent.

p23, a partner of cdk5, is regarded as an activator of cdk5, because active cdk5 co-eluted with the 23 kDa protein and cdk5 monomer was inactive [18]. The cdk5 of bovine brain proline-directed protein kinase associates with a 25 kDa protein [16], which seems to be identical to p23 although the estimated molecular weight is different. Cyclin-dependent kinases are known to be active only in complexes with their regulatory subunits known as cyclins [19]. cdk5 was reported to associate with D-type cyclin in human fibroblasts [14]. In brain, however, its regulatory subunit is not a well-known cyclin such as cyclin D. p23 is a C-terminal fragment of a novel protein (precursor of p23, pre-p23) [18] containing a slight similarity to amino acid sequences in the cyclin box of cyclin D1 [20]. The cyclin box is important for the interaction with cdc2-related kinase [21]. Considering that the function of cyclin is emphasized as a cell cycle promoting activity, p23 should not be called a cyclin because the neurons where TPKII exists are terminal differentiated cells that are out of the cell cycle. It is interesting that cdk5 binds to cyclin D1 in fibroblasts [14] and p23 in neurons. These accessory proteins may determine the different functions of cdk5 in different cells.

In our previous paper [18], the molecular size of pre-p23 was unknown because the sequencing was incomplete. Here, we show its complete sequence and characterize it further, especially the developmental change of its expression.

## 2. Materials and methods

### 2.1. Materials

Bovine TPKII was purified by the method described previously [2]. The anti-cdk5 antibody was described previously as an anti-30 kDa subunit antibody against peptide 2 [12].

### 2.2. Sequencing

Two cDNA clones, no. 2-1 and no.5, were ligated to *Eco*RI linker and inserted into the vector, pUC19. DNA was sequenced by dideoxy-mediated sequencing [22].

### 2.3. Northern blot analysis and probes

Total RNA was prepared from rat brains of various ages and from various rat tissues at day 8 postnatally with TRIzol reagents [23]. Using magnetic oligo dT beads (Dynabeads; Dynal), poly(A)$^+$ RNA was separated from total RNA. Each aliquot of poly(A)$^+$ RNA (1 $\mu$g/lane) was separated on a formaldehyde-agarose gel, and blotted to Hybond-N$^+$ membranes (Amersham). These blots were hybridized with the following DNA probes prepared by random-primed labelling (Takara): the 23 kDa subunit and its precursor (probe 1 and 2, respectively, in

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Fig. 1A); cdk5, the *Eco*RI 1.1 kb fragment of bovine cdk5 cDNA [15]; G3PDH, the *Eco*RI 1.1 kb fragment of human glycerol 3-phosphate dehydrogenase cDNA (Clontech). The blots were analyzed by a bioimaging analyzer BAS2000. Expressed amounts of mRNA were normalized with respect to the amounts of G3PDH. RNA size was measured using a 0.24–9.5 kb RNA ladder (Gibco BRL). With this marker, 28 S and 18 S RNAs were estimated to be 4.7 and 1.8 kb, respectively.

*2.4. Synthesis of peptides and preparation of antisera against the peptides*

Peptides were synthesized by the solid-phase method, using a Biosearch model 9500 peptide synthesizer. According to the amino acid residue number of pre-p23 (Fig. 1A,B), the sequence of: (i) pre-p23-N is $Lys^{13}$–$Ser^{33}$-Cys; (ii) p23-N is Lys-$Ala^{99}$–$Val^{121}$; (iii) p23-C peptide is $Lys^{271}$–$Lys^{290}$. The peptides were conjugated with keyhole limpet hemocyanin (KLH). Preparation of antibodies against the peptide-KLH conjugants were described previously [12].

*2.5. Preparation of rat brain extract*

Wistar rat brain was homogenized at 4°C in 2 vols. of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 10 nM okadaic acid, 50 mM β-glycerophosphate, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 0.5 mg/ml benzamidine. The homogenate was centrifuged at 15,000 × *g* for 20 min and the supernatant retained.

*2.6. Immunoprecipitation*

Immunoglobulin was purified from antisera against p23-N or p23-C by the method reported previously [5]. The purified immunoglobulin (40 μg) was added to 20 μl of protein G-Sepharose 4 fast flow (Pharmacia). Immunoprecipitation was as described previously [24]. The immunoprecipitates were used for measurement of TPKII activity and immunoblotting.

*2.7. TPKII-specific assay*

TPKII activity in the immunoprecipitate or TPKII fraction was measured by the method reported previously [2] using 0.2 mg/ml pro-Src peptide [15] as a substrate.

*2.8. Immunoblotting*

Polypeptides were separated on SDS-polyacrylamide gels and transfered to nitrocellulose membranes. Immunoblotting was performed with the ABC kit (Vector Laboratories) using 4-chloro-1-naphthol (Wako) as a chromogen. For crude brain extract, blocking of the membrane and reaction with primary antibody were done using Tris-buffered saline containing 5% skimmed milk.

## 3. Results

We have previously determined the sequence of p23 which started just from the N-terminal residue of p23. No initiation codon was present near this N-terminus. Therefore, further sequence analysis of cDNA was undertaken. The overlapping sequences of the two independent clones, 2-1 (nucleotide −188 to +840) and 5 (nucleotide +117 to +1130), were identical, and the nucleotide sequence and a deduced amino acid sequence are shown in Fig. 1A, B. The first ATG codon occurred at nucleotide +1, and an in-frame termination codon occurred at nucleotide +922. The nucleotide sequence surrounding this ATG codon (nucleotide −6 to +4) was in a good agreement with Kozak's context (GCC[A/G]CCAUGG) for efficient translation in higher eukaryotes, except for the G at position −5 [25]. Thus, we conclude that the first ATG is the actual initiation codon. This ATG is followed by a long reading frame (ORF) encoding a novel protein composed of 307 amino acids with a molecular weight of 33,992 Da. This ORF contains essentially the same sequence of p23 downstream of the Ala codon at position 99, as reported previously, indicating that the 34 kDa protein is a precursor of p23 (pre-p23) and generates p23 by cleavage of the Phe–Ala linkage. A single change at +340 from G to C was observed when compared to previous results [18]. The N-terminal region of pre-p23 lacking p23 is rich in basic amino acids residues, but has no bipartite nuclear targeting sequence [26], and no destruction box as found in the N-terminus of G2-M cyclin [27]. By Western blot analysis of the crude extracts obtained from postnatal day 4 rat brain using anti-p23-N, anti-p23-C or anti-pre-p23-N antibodies, a band of 34 kDa was detected, but nothing at the 23 kDa position in every case (Fig. 2). At least, a detectable amount of pre-p23 was actually present in rat brain.

Northern blot analysis of poly(A)$^+$ RNA from various rat tissues at day 8 postnatally showed that a single pre-p23 mRNA was expressed only in the brain, and was not detectable in other tissues (Fig. 3), while both mRNAs (1.2 kb and 2.1 kb) of cdk5 were also expressed at the highest level in brain as reported previously [16]. In other tissues tested, the short mRNA was expressed at levels lower than observed in brain, whereas the long mRNA was detected at levels only slightly above the threshold level for detection.

We have previously suggested that p23 is an activator of cdk5 in neuronal cells playing a role analogous to cyclin in proliferating cells [18] because of the co-existence of p23 and cdk5 in the active fraction of TPKII. To reinforce the evidence for this suggestion, immunoprecipitates of TPKII with anti-p23 antibodies were examined (Fig. 4). Purified bovine TPKII was immmunoprecipitated either with anti-p23-N or anti-p23-C antibody. TPKII activity was detected in both precipitates with antibodies, while little activity remained in supernatants. By Western blotting using the same antibodies and anti-cdk5 antibody, both immunoprecipitates were found to contain cdk5 in addition to p23, indicating that p23 associates with cdk5 to form an active complex, that is TPKII. This result proved our presumption clearly, and indicated that immunoprecipitation with anti-p23 antibody is useful as a specific assay for TPKII in tissue extracts.

It has been reported that phosphorylation of tau in rat brain is evident both pre- and postnatally, and is developmentally regulated [5]. Northern blot analysis of poly(A)$^+$ RNA from rat brains of various ages (Fig. 5A) showed that expression of pre-p23 mRNA was most abundant just before and after birth (from gestation day 18 to day 1 postnatally) and retained abundantly in neonatal brain (about 2 weeks). Later, it decreased gradually to reach a minimum level in young adult brain (5 weeks to 4 months) and a slightly increased amount in aged adult brain. Although expression of cdk5 mRNA also changed in a manner similar to that of pre-p23 mRNA, it decreased only about 50% from the highest expression to that in aged adult brain. Developmental change of TPKII activity in brain extracts was in good agreement with that of the expression of pre-p23, but not of cdk5 mRNA (Fig. 5B). Considering that phosphorylation at TPKII sites of tau is developmentally regulated, and reached its highest level within the first week postnatally, it is reasonable to conclude that the expression of pre-p23 controls the phosphorylation of tau by TPKII in neonatal rat brain.

## 4. Discussion

We report here that p23 is a regulatory subunit of TPKII and is derived from a precursor protein of 34 kDa by cleavage by

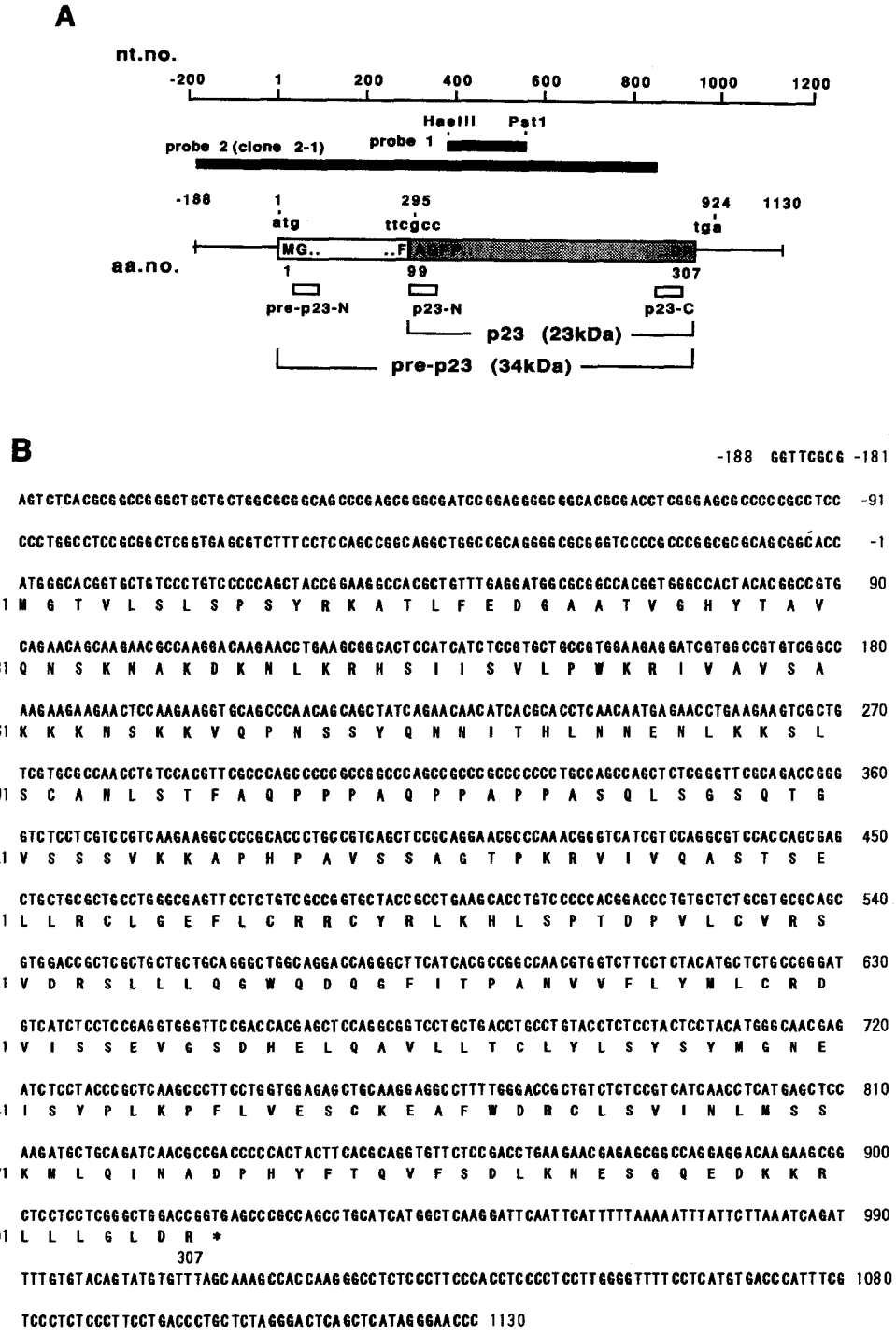


Fig. 1. Structure of cDNA of bovine pre-p23. (A) Map of the cDNA. The sequence of nucleotides (nt) −188 to +1130 was determined. An open reading frame is found at nt +1 to +924, and indicated by a rectangle. The amino acid sequence of pre-p23 and p23 are found at amino acid (aa) 1–307 and 99–307, respectively. Nucleotide probes 1 and 2 correspond to the regions indicated by black bars. Sequences of synthetic peptides, pre-p23-N, p23-N and p23-C, are indicated by open boxes. (B) Sequence of pre-p23. The nt sequence 295–970 and aa sequence 99 to 307 were already reported in our previous paper [24]. The accession number for the nucleotide and amino acid sequences is X78934 in the EMBL data library.

an unknown protease at a Phe–Ala linkage to yield the mature p23 (C-terminal) protein. We previously reported that p23 has a sequence with a slight similarity to the cyclin box of cyclin D1, which might act as the binding site to cdk5 [18]. The complete amino acid sequence of the pre-p23 has no additional similarity to cyclins nor any relationship to any other motif. This result leads to the conclusion that pre-p23 is not a cyclin even though it interacts with cdk5, as does cyclin D of human fibroblasts.

Immunoprecipitation of p23 with anti-p23 antibodies co-precipitates cdk5 from preparations of purified TPKII. The

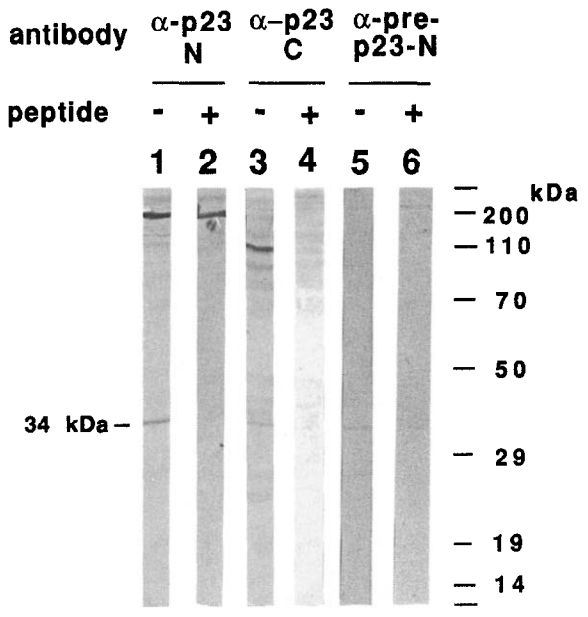


Fig. 2. Immunoblot of rat brain extract with anti-p23 and anti-pre-p23 antisera. 260 mg brain extract of 6-month-old rat was immunoblotted with anti-p23-N (1:30, lanes 1,2), anti-p23-C (1:15, lanes 3,4) or anti-pre-p23-N (1:50, lanes 5,6). Antiserum was preadsorbed by 0.1 mg/ml antigen peptide p23-N (lane 2), p23-C (lane 4) or pre-p23-N (lane 6) for 15 h at 4°C. Molecular weights of marker proteins are indicated at the right.

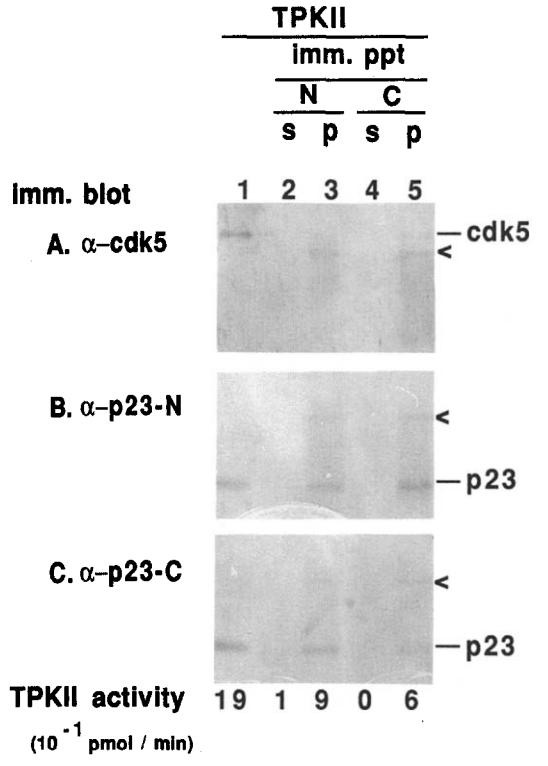


Fig. 4. Immunoprecipitation of TPKII with anti-p23 antibodies. TPKII (1.9 pmol/min) was immunoprecipitated by anti-p23-N (N, lanes 2 and 3) or anti-p23-C (C, lanes 4 and 5). TPKII (lane 1), supernatant (s, lanes 2 and 4) and precipitate (p, lanes 3 and 5) of immunoprecipitation were immunoblotted with anti-cdk5 (1:30, A), anti-p23-N (1:30, B) or anti-p23-C (1:30, C). The positions of cdk5 and p23 are indicated at the right. Arrowheads indicate the position of light chain of immunoglobulin co-precipitated and reacted with anti-rabbit immunoglobulin antibody used as the second antibody of the immunoblot. Recovery of TPKII is indicated in terms of activity at the bottom.

interaction between cdk5 and p23 is very strong, not dissociating even in the presence of 1 M NaCl (data not shown). We have also shown, as before [18], that a complex between cdk5 and p23 is required for kinase activity of TPKII. The 34 kDa protein, pre-p23, can also be found in crude extracts of rat brain, but it is unclear whether or not it is in a complex with cdk5.

The following observations lead us to the conclusion that p23 is a cdk5 activator: (i) cdk5 monomer alone has little activity [18]; (ii) active TPKII is a complex of cdk5 with p23; (iii) expression of pre-p23 changes in parallel with TPKII activity in rat brain during development. The cdk5 activator p23 is considered to be brain-specific because expression of pre-p23 specifically occurs in the brain.

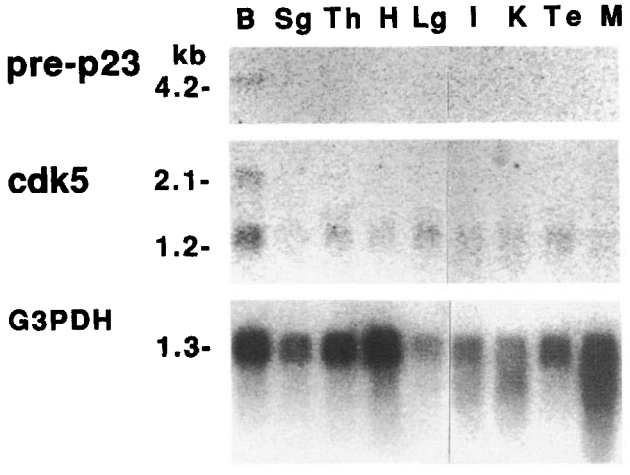


Fig. 3. Northern analysis of pre-p23 and cdk5 mRNA in various organs of rat at postnatal day 8. Poly(A)$^+$ RNA was extracted from brain (B), salivary gland (Sg), thymus (Th), heart (H), lung (Lg), intestine (I), kidney (K), testis (Te) and skeletal muscle (M). 1 μg of RNA was hybridized with probe 1 for detection of pre-p23 mRNA (upper), the *Eco*RI fragment of cdk5 cDNA for cdk5 mRNA (middle), and G3PDH cDNA as positive control (lower).

TPKII activity in rat brain extracts was retained at high levels within the first week postnatally, and then decreased gradually till postnatal week 5, indicating that TPKII is active in neonatal rat brain. This result is consistent with the previous observation that phosphorylation at TPKII sites of tau reached a maximum level within the first week postnatally [5]. Therefore, expression of pre-p23, but not of cdk5, controls the appearence of TPKII activity which controls the function of TPKII/TPKI phosphorylation system in neonatal brain, where sprouting of neurite and neuronal cell death occur extensively. Actually, TPKI was also shown to be present at a high level in neonatal brain [24]. In addition, TPKI was reported to play an important role in β-amyloid-mediated programmed cell death in the primary culture of hippocampal cells [4]. A slight increase in pre-p23 expression in the aged brain may reflect the involvement of the TPKII/TPKI phosphorylation system in neuronal cell death.

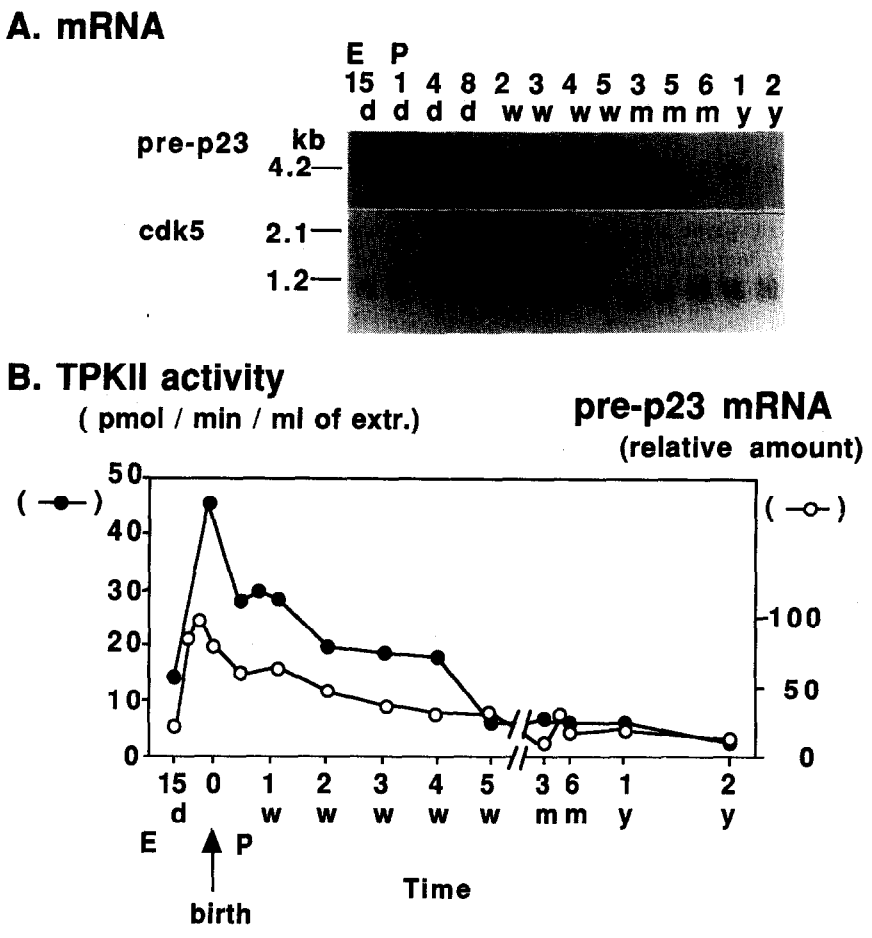


Fig. 5. Developmental change of expression of mRNA of cdk5 and pre-p23, and TPKII activity. (A) Total RNA was prepared from rat brain at embryonic day 15, 18, 20, and postnatal day (d) 1, 4, 8, week (w) 2, 3, 4, 5, month (m) 3, 5, 6, and year (y) 1, 2. 1 μg of poly(A)$^+$ RNA was subjected to Northern analysis. (B) TPKII activities in immunoprecipitates of brain extracts with anti-p23-C are indicated by filled circles. Relative amounts of pre-p23 mRNA in (A) were measured and are indicated by open circles.

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*Note added in proof*

After the submission of this manuscript, we found two reports on the same subject [28,29] as that described here. Several sequences, such as those from nt −188 to −136 in the 5′ non-coding region and from 1066 to 1130 in 3′ non-coding region, are not coincident with those reported by Lew et al. [29].