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Research report

Identification of a novel adenylate kinase system in the brain: Cloning of the fourth adenylate kinase

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Abstract

We identify a novel subtype of adenylate kinase, which is the 4th adenylate kinase (AK4), in the vertebrate. AK4 mRNA is expressed in the mammalian central nervous system in a region-specific manner from the middle stage of embryogenesis to the adulthood in the rodent. The presence of three isozymes of adenylate kinase (AK1, AK2 and AK3) that maintains the homeostasis of adenine and guanine nucleotide composition has been reported in the vertebrate. Obtained mouse AK4 cDNA is 3667 bp in size. The predicted open reading frame consists of 223 amino acid residues. Rat AK4 cDNA is also obtained, and the predicted open reading frame is the same length as that of the mouse. The predicted rat AK4 molecule shows 97.8% homology with mouse AK4. Rat AK4 protein is distinct from rat AK3, 53.8% homologous with rat AK3, although the adenylate kinase signature and the mitochondrial energy transfer protein signature are found in both sequences. Interestingly, rat AK4 is 89.2% homologous with the human AK3 over 223 amino acid residues and rat AK3 is 53.7% homologous with the human AK3 indicating that the reported human AK3 actually belongs to the AK4 group (therefore, it should be referred to as human AK4). Although the sequence of AK4 is most similar to that of AK3 among the AK isozymes, its in vivo expression is completely different from AK3; AK4 mRNA is expressed in the pyramidal cells in the hippocampus (mainly in the subfield CA3), the granular cells in the cerebellum, nasal neuroepithelium and the liver while AK3 mRNA is expressed ubiquitously in the body. It is probable that AK4 acts on the specific mechanism of energy metabolism rather than control of the homeostasis of the ADP pool ubiquitously.

Keywords: Adenylate kinase; ATP; GTP; Liver; Nasal cavity; Hippocampus

1. Introduction

The adenylate kinases (AKs) are ubiquitous enzymes which are involved in maintaining the homeostasis of adenine and guanine nucleotide composition in various organism [24]. Each AK has been demonstrated to catalyze a similar reaction, MgNTP + AMP ⇔ MgNDP + ADP (N = A or G) [26].

In the central nervous system, ATP and GTP are thought to be not only the energy source but also the principal neurotransmitter or neuromodulator at purinergic synapses, and the evidence for the presence of a synaptic plasma membrane associated AK has been reported [12,28]. In addition to these facts, recent studies have revealed that purines can act as trophic factors with potential to regulate neural development, proliferation and apoptosis of glial and brain capillary endothelial cells, neural plasticity and the response of the nervous system to disease processes [14]. Furthermore, it has been found that there is substantial AK activity in developing mouse brain during the period of rapid neural growth and it has been concluded that AK is involved in neural functioning [8].

The presence of three AK isozymes (AK1, AK2 and AK3) has been reported in vertebrates. These isozymes are characterized by significantly conserved sequences and their sequences are fairly comparable [23]. Each isozyme has a distinct intracellular distribution and a preferred substrate. AK1 is a cytosolic enzyme for which ATP is the substrate. AK2 catalyzes the same reaction as AK1, but it is localized in the mitochondrial intermembrane space.

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AK3 is present in the mitochondrial matrix and prefers GTP over ATP as the substrate [1,13,15,24]. Cytoplasmic AK1 participates in energy metabolism when local high energy phosphate levels are low, by sustaining ATP levels at the expense of ADP. Mitochondrial AKs may function in the salvage pathway of AMP because this adenine nucleotide which accumulates in periods of energy demand cannot enter the mitochondrion and will be lost by deamination if not phosphorylated [4,10].

In the course of our search for genes that are expressed predominantly in the rat forebrain during the middle stage of neurogenesis (embryonic day 11, E11), we have observed that the transcripts of apparent novel adenylate kinase gene are localized in specific tissues, especially in the central nervous system during the embryonic stage. Here we report the cloning and analysis of this newly identified rat and mouse AK4, the first 4th adenylate kinase in the vertebrates.

2. Materials and methods

2.1. Nucleic acid sequence data

DNA database accession number for the mouse adenylate kinase 4 (cloned mouse D94) is D85036 and the rat adenylate kinase 4 (cloned rat D94) is D87809.

2.2. Animals

Wistar rats at various embryonic and postnatal ages were used: Embryonic day 9 (E9, E0 is defined as the day of confirmation of the vaginal plug) ($n = 22$), E11 ($n = 86$), E15 ($n = 8$), E18 ($n = 46$), E21 ($n = 5$), postnatal day 0 (day of birth, P0) ($n = 3$), P2 ($n = 3$), P5 ($n = 3$), P7 ($n = 3$), P14 ($n = 3$), P21 ($n = 3$), and three two-months old male rats. Male adult rats and pregnant rats were purchased from a local vender (Keari, Japan), housed at a constant temperature and humidity and provided with food and water ad libitum. Diurnal lighting conditions were maintained with lights on from 08:00–20:00 h.

2.3. Isolation of a rat cDNA fragment by differential display

We employed the mRNA differential display method [9] to search for genes expressed transiently in the cortex on E11. First, polyadenylated (poly A(+)) RNA was extracted using a micro Fast Track kit (Invitrogen, USA) from the telencephalon of fifteen rat embryos on E11 and E18, respectively. Then we employed sets of two primers; one being anchored to polyadenylate tail of a subset of mRNAs and the other being short and arbitrary, to display mRNAs and isolate DNA fragments.

Complementary DNAs (cDNAs), synthesized with the former primer from poly A (+) RNAs extracted from the telencephalon of E11 and E18 rats, were subjected to polymerase chain reaction (PCR) using the same two sets of primers used for mRNA display in the presence of [$\alpha - ^{35}$S] dATP; then the products were separated by electrophoresis on a DNA sequencing gel. Amplified cDNA fragments were visualized as bands in order of their molecular weights by autoradiography. Some of these bands appeared only in the E11-derived lane. These E11-derived lane specific bands were considered to be cDNA fragments of genes which are expressed in the E11 telencephalon but not significantly in the E18. We extracted these apparent E11-derived lane specific DNAs from the gel, subcloned them into the pGEM plasmid (Promega, USA) and determined their nucleotide sequences by dideoxy DNA sequencing method using an automated sequencer (373A, ABI, USA). After an inquiry into DNA databank (DDBJ, EMBL, GenBank) using FASTA program, we found a novel DNA fragment which was temporarily designated #D94 among the clones we had obtained. The employed set of two primers for identification of #D94 was T_{12}AA and GATCTAACCG.

2.4. Tissue preparation

All animals were deeply anaesthetized by hypothermia (for P0–P7) or sodium pentobarbital (for P8-adult including pregnant; 40 mg/kg, intraperitoneal injection). Whole bodies (E10–E21) or brains and livers (E21-adult) were immediately frozen with powdered dry-ice. Parasagittal sections (E10–E21) or frontal sections (P0-adult) were cut at a 15 μm thickness with a cryostat. Sections were then thaw-mounted onto glass-slides coated with TESTA (3-amino propyltriethoxysilane) (Sigma, USA) and stored at −80°C until use.

2.5. In situ hybridization histochemistry

The protocol for in situ hybridization histochemistry was based on the published method [11,27]. In brief, sections were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffer (PB), acetylated with 0.25% acetic anhydride, treated with 0.1 M triethanolamine and dehydrated with ethanol. [$\alpha - ^{35}$S] UTP-labeled single-strand RNA synthesized with the *Not*I-digested template plasmid (pGEM) containing the rat #D94 fragment and T7 RNA polymerase was used as an antisense probe. That synthesized with the *Nco*I-digested template plasmid containing the rat #D94 fragment and SP6 RNA polymerase was used as a sense probe. Hybridization and washing procedures were the same as those published [11,27]. Hybridization signals were visualized by macroautoradiography with New-RX X-ray film (Fuji, Japan) and microautoradiography using emulsion (Ilford, UK). The nomenclature of the brain is based on the published atlas [18].

On identification of positive hybridization signals, only cells with a grain density at least 3 times higher than the

background density were considered to be positive in this study [16]. No positive signal was observed in the sections hybridized with a sense probe. Based on this criteria, however, we found a limited number of cells and/or regions difficult to identify as either positive or negative. Such cells and/or regions were regarded as negative in our results.

2.6. Northern blot analysis

Poly A (+)-RNA was isolated from the forebrain of E11 embryos using a micro Fast track kit (Invitrogen, USA). For Northern blotting, 5 μg of poly A (+)-RNA was separated on formaldehyde-1% agarose gel (120 V, for 2 h) and transferred onto PVDF membrane (Immobilon-N, Millipore, USA). Membranes were hybridized with the fragment of rat #D94 labeled with [$\alpha - ^{32}$P] dCTP by Bca Best labeling kit (Takara, Japan). Hybridization was performed overnight at 55°C in 6× SSC (1× SSC: 15 mM sodium citrate, 150 mM NaCl), 0.5% SDS (sodium dodecyl sulfate), 50% formamide, 5× Denhardt's solution and 100 μg/ml denatured salmon testis DNA for 16 h. The membranes were washed at high stringency (0.1× SSC, 0.1% SDS at 65°C) and hybridization signals were visualized by macroautoradiography.

2.7. Screening of mouse and rat cDNA library

A mouse E11 whole body cDNA library (kindly provided by Dr. Akira Kakizuka, Osaka Bio-Science Inst.) and a rat E11 forebrain cDNA library constructed in lambda ZAP II were screened using #D94 DNA fragment labeled with [$\alpha - ^{32}$P] dCTP by the BcaBest labeling kit (Takara, Japan). Approximately 1×10^6 plaques were transferred to replicated filters (Hybond-N+; Amersham, UK). The filters were hybridized at 42°C in a buffer containing 5× SSPE (1× SSPE: 150 mM NaCl, 20 mM NaH_2PO_4, 20 mM ethylene diamine tetraacetic acid solution), 2× Denhardt's solution, 0.1% SDS, 40% formamide and 100 μg/ml denatured salmon testis DNA. We obtained mouse full length cDNA from the mouse cDNA library, and subsequently, we got the rat cDNA for open reading frame by RT-PCR based on the homology between mouse and rat. We applied the RT-PCR with degenerate primers. Degenerate oligonucleotide primers, TGGCCACCGCCAAAGC(A/C/G/T) and TGGTTCTTCCCATGGC(A/C/G/T), were designed based on the mouse #D94 cDNA sequence. Fifty ng of first-strand oligo d(T)-primed cDNA from E11 rat embryos, 1 μg of each primer, 200 μM dNTPs and 2 U of Taq polymerase (Perkin-Elmer, USA) were mixed in 50 μl of the supplied reaction buffer with Taq polymerase (Perkin-Elmer, USA); then 40 cycles of amplification procedures were performed at 94°C for 20 s; 50°C for 30 s; 72°C for 1 min. Amplified DNA fragments were then separated on a 1.8% agarose gel, subcloned into pGEM plasmid and sequenced. In order to avoid a replication error accompanied by PCR amplifications, four independent clones were isolated and sequenced. The same procedure was applied to detect #D94 (later, designated AK4) mRNA expression in tissues.

2.8. DNA and protein database inquiry and motif analysis

Sequence entry and analysis were performed using the DNASIS program (Hitachi, Japan). Values for percentage identity and similarity between sequences were obtained using the same program. DNA database searches were performed using the FASTA program of Pearson and Lipman [19] by directly accessing the database at DNA Data Bank of Japan (DDBJ). Consensus amino acid sequences were searched for with the motif analysis program written by Dr. Miki, N. (Osaka Univ.).

3. Results

3.1. #D94 clone was identified by differential display

We employed the mRNA differential display method [9] to search for genes expressed transiently during the middle stage of neurogenesis, around E11 in the case of rat. A cDNA fragment of 345 bp in size recognized in E11-derived lane but not in E18-derived lane was picked up as the candidate clone which is expressed in a stage-specific pattern in the brain during ontogenesis (Fig. 1). Its preference in expression for the middle stage of neurogenesis

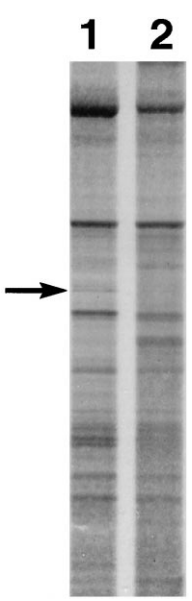

Fig. 1. An example of mRNA fingerprints. Lane 1 and 2 show examples of mRNA fingerprints amplified and displayed using the same set of primers (T_{12}AA and GATCTAACCG). mRNA used for the lane 1 display is extracted from E 11 (embryonic day 11) rat forebrains, whereas that for the lane 2 is from E18 rat forebrains. #D94 clone (later referred to as the 4th adenylate kinase, AK4) that is recognized only in lane 1 is indicated with an arrow.

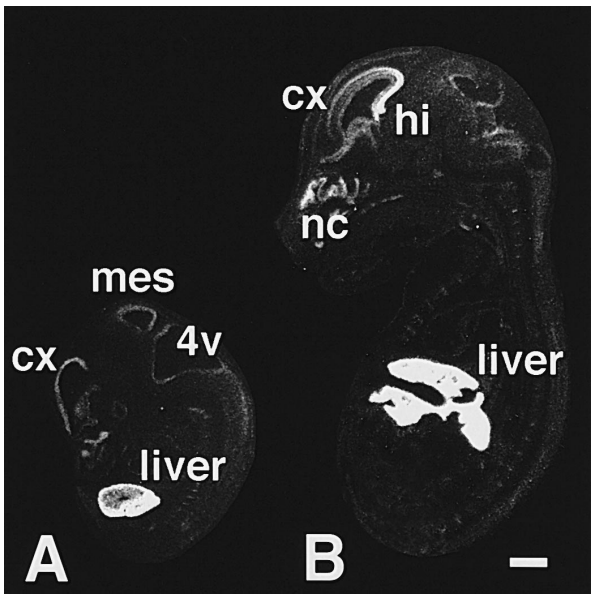

Fig. 2. In vivo expression of #D94 mRNAs in the E11 and E18 rat embryos. Parasagittal sections. Film-autoradiograms. Expression of #D94 mRNA is observed in the cerebral cortex and the inferior colliculus with rostro-caudal gradient. Significant signals are noticed in the hippocampal formation and in the liver. In the cortex, transcripts of AK (#D94) are gradually restricted to the subfield CA3 in the hippocampus during the embryonic period. 4V: 4th ventricle, cx: cortex, hi: hippocampal formation, mes: mesencephalon, nc: nasal cavity, scale bar = 1 mm.

(preplate stage of corticogenesis) was confirmed by in situ hybridization histochemistry (Fig. 2). This cDNA fragment was temporarily designated #D94 and used for further experiments.

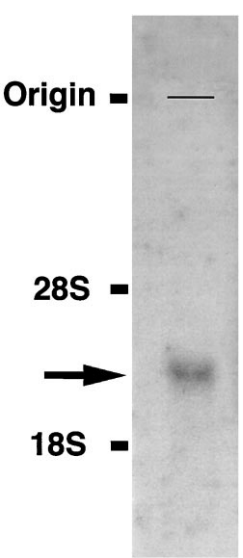

Fig. 3. #D94 mRNA transcript is approximately 3.6 kb in size. Northern blot analysis. mRNA (5 μg/lane) extracted from the E11 rat forebrains were used. Apparent #D94 mRNA transcript is indicated by an arrow.

3.2. Positive band of 3.6 kb in size was detected by Northern blot analysis

Northern blot analysis of the gene containing #D94 DNA fragment was performed with poly A(+) RNA extracted from the forebrain of E11 rat embryos (Fig. 3). A positive band of 3.6 kb in size was noticed.

3.3. Mouse #D94 homologous gene (AK4) was cloned

To obtain a full-length cDNA of #D94, a rat E11 forebrain cDNA library was screened using the #D94 DNA fragment as a probe. Since the mouse has an advantage for further gene manipulation, mouse E11 cDNA library was also screened at the same time. From the mouse cDNA library, three positive clones were identified but no obvious positive ones were obtained from the rat cDNA library. The cloned, apparent full-length mouse

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   1 CGAGTCCCCGCGTGCGGAGGGTGTCGAGGCAGGGGCTGCTGGGCAAAGTGCGCGGGCTGC   60
  61 CGTCCTGGCCACCGCCAAAGCCATGGCTTCCAAACTCCTGCGCGGCGGTCATCCTCGGGCC  120
   1                   M  A  S  K  L  L  R  A  V  I  L  G  P       13
 121 GCCCGGCTCTGGCAAGGGCACCGTTTGCGAAAGGATCGCCCAGAACTTTGGCCTCCAGCA  180
  14  P  G  S  G  K  G  T  V  C  E  R  I  A  Q  N  F  G  L  Q  H   33
 181 TCTCTCCAGCGGCCACTTGTTGCGGGAGAACCTCAAGACCGGCACGGAAGTTGGTGACGT  240
  34  L  S  S  G  H  L  L  R  E  N  L  K  T  G  T  E  V  G  D  V   53
 241 GGCAAAACAGTACCTAGAAAAAGGTCTTTTGGTTCCAGATCACGTGATCACACGCCTAAT  300
  54  A  K  Q  Y  L  E  K  G  L  L  V  P  D  H  V  I  T  R  L  M   73
 301 GATGTCAGAACTGGAGACTCGGAGTGCCCAGCACTGGCTGTTAGATGGATTCCCGAGGAC  360
  74  M  S  E  L  E  T  R  S  A  Q  H  W  L  L  D  G  F  P  R  T   93
 361 ATTAGTACAGGCAGAAGCCCTGGACGGAATCTGTGATGTGGACCTAGTAATCAGTTTGAA  420
  94  L  V  Q  A  E  A  L  D  G  I  C  D  V  D  L  V  I  S  L  N  113
 421 TATTCCTTTTGAGACACTTAAAGATCGTCTGAGCCGACGGTGGATTCACCCTTCTAGCGG  480
 114  I  P  F  E  T  L  K  D  R  L  S  R  R  W  I  H  P  S  S  G  133
 481 GAGGGTCTATAACCTGGACTTCAACCCACCTCAAGTGCAGGGGATTGATGACATCACTGG  540
 134  R  V  Y  N  L  D  F  N  P  P  Q  V  Q  G  I  D  D  I  T  G  153
 541 TGAGCCACTTGTCCAACAGGAAGATGATAAACCTGAAGCAGTTGCTGCCAGGCTAAGACG  600
 154  E  P  L  V  Q  Q  E  D  D  K  P  E  A  V  A  A  R  L  R  R  173
 601 GTACAAGGATGCGGCAAAACCAGTCATCGAACTGTACAAGAGCCGTGGAGTGCTCCACCA  660
 174  Y  K  D  A  A  K  P  V  I  E  L  Y  K  S  R  G  V  L  H  Q  193
 661 GTTTTCTGGGACGGAGACTAACAGAATCTGGCCTTATGTTTACACACTTTCTCCAACAA  720
 194  F  S  G  T  E  T  N  R  I  W  P  Y  V  Y  T  L  F  S  N  K  213
 721 GATCACACCTATTCAATCCAAAGAAGCCTACTGAGCCTGCCATGGGAAGAACCAAGAAGA  780
 214  I  T  P  I  Q  S  K  E  A  Y  *
 781 CGTGGTCGCCCACTCGATCACACGTGTCGTCTTGGTGACCTGGCCCAGTTAGAAGCCAGC  840
 841 TGAGATAGCTTGCAGCATCTTTTCTAGTTTCAGTGATGAAGTAATGAGGAAACTCTAGTA  900
 901 GGTAGAAGGAATTCAGGAGGAAGGAAACCTCCTCTGCCTTTGAAAGGGGCTCTGCGGGT  960
 961 GCAACAGCCCTTCACAGGAACGAGTAGACTGGATCTCCACAGTGTGGAGCCCAGCGTG 1020
1021 CTAGCTATCAGAGCCTTTTTGGATGCAGTGGTGGGCTCTTTGGTTTATCCCACCTTCCAC 1080
1081 AGGCTATGAAACCACAGCGCTGTATGCCTGAGCACTTGAAGGGCTGTGGTCCTGGCTTGT 1140
1141 CCATTGCCATATGCTCTGACAAGTTGACGCTAATTGCACTCGGTGTGTTGCTTTTCTCCA 1200
1201 ATGACTGTCTCTCCCTGGCTCTCAGACATCCTCGTTTCTGTTGTCTGAATCTTCTCGTCT 1260
1261 GCTTGCTCCCTTCTCAGGCTATTCAGTCTCATGATGGCTTCTGCATTTACCCCTCGAATT 1320
1321 TCCTTTATACAAAACTGAGAAGTAGGTGACTGGGTTCAAGTCTTTTTCCTTCTTCCTAGG 1380
1381 CACTGATCCAGGGTAGAAGGCTTACTTGGGTTAAACAGCTGCTCTATCTTGAGCCCACTC 1440
1441 TGTACCTTTTATTGTTTAAAAAAAGATCCCCCCAAAGTCTTCAGGGTTCTCAAAAAACAA 1500
1501 GGGAAATGCTTACTTATGGAAAAGTGGGGTGATTGTATCTTCTATGTAGAAGTAGTAGTA 1560
1561 ATTGTATGGAAATCACTGGACTAATGAGATGGTTGGGTGCAAGAAAAGATGAATATTGAA 1620
1621 CATGCTGCTAAATATTAACGCTGTCAGTGTTTTTCTTCTGTGAGTGACTAACCAGACGGC 1680
1681 GAGAGGTCAGAAAAGGGAGCAAGCCTCTGAGGCTTGGAGCATCTTGCACTAGCTATACAC 1740
1741 AGCACCTGCACTGAGTAAGACAGCTTTACTGTTGTGGGGATGCAGAGTCCTTTTGTTTGT 1800
1801 TTTCTTCCTATTCCAAAGATGCAGTCTATAGGTGGTCATAAAGTCCAGAAATGAAGATAC 1860
1861 TAAGGTCTTATCAGTCATTGTGTGTCAAGAAAGCATTTGTGAAGACTTTCCTTCCTGGAT 1920
1921 CTCAGACTTGCTGGCCACCTTGTTACAATTTTTATTCTTTTCTGGGAAGAACAGGGTAAA 1980
1981 TTTCTTCCCACTGCCTTAAAAAGGTGTCCTGCACCTGCCTGTCCCCAGGAGGTGGTCTTT 2040
2041 TATTACTGGTGGTGACTTAGTGTGACATCTCATAGTCTGTGTGGGGTCAGGGTATGGGGT 2100
2101 GGGCTTAGCACTTAGTGGATTGAGACATTATTCATCTCTGAAGGTGTCGTGTGTTCTGT 2160
2161 GCTCCTGAGTCTGTCTGCCTTCGTTACTCCTGACTCCCATGGTTCCTCTCAGTCGTT 2220
2221 TTTTTCATGTGCATGTTCTGCGGCTGCCTTAGTGGAGATGCAGGCCAGCATTCCCACTCG 2280
2281 TGTTCTCAGTACAGAGAAGTAAGCTTCCAGAGTGCAGCTGCACGGGGCAGGTGACCATCT 2340
2341 GGGCAGTGTCGTGATGCAGAGCACCTTGGAAGCTTACATCTGGATGTCACTTGGTAGGGG 2400
2401 TTGGGTGATAAAAGCCCAGGCTTCCTGGAGGGGCAGGTCGGGAGTGGGAAACCCTGAGAT 2460
2461 TATTCCCAGGAACCAAGGGAAGTAGGGAGTTAAAGGGATTCAGGGTAATTCAAGAGAGGT 2520
2521 AGGAAAACTTCAAACTTTTCTTCCCCTCTTGTGGAGTCTGGGGAGAACAATAGGGTGTGA 2580
2581 GCAGCTCCTTGCCTGCACACTGCTGTTTCTCTATGCAGTTCAAGCTCAGTAGACTACGGT 2640
2641 AGCCCCAGTGGGACACCAGGCTCAGAAGAGCAGCAAGGGGCAAAGACATCTTTTCCAAAG 2700
2701 TCTTGGGTGATTTCATGTCTATGGGTGTAAAGGTGTTTTCTGCAGCAGGCTTGGCTGATT 2760
2761 TTGTGGAAATGGTCATAAATACTGACCGTGTCTTGACAAAGGTGCAATGGAAGCAGCATC 2820
2821 TTTGCCTTTCTCAGGCGACACATGGCCAAGAGTCTGGGTGCTCTCTGAGGTTCTGACTTT 2880
2881 ACATTCATGGACTCCCCGTTCAGCTTGCATCCCACAGGCCTCATGGGAAAGGCTGCATGT 2940
2941 TTATTCCTCCAGTATTTATGACAAAAAAGGTGCTATGAAGGAGGCCCTCTGAGTAAAGTG 3000
3001 CGCACTCTCCGGACCTTTATAAGCATTGTGGTGAATCTTCCCAGGCACAACCAATATTTC 3060
3061 CTATAAACAAGAGTCCAGGATATGAAGTCTTGCCCAGGCTCTTCATATGAGTAAACACTA 3120
3121 AAGCTATGATTCAGTCCCAGGTTTTCCTGCTTCTGAAACCCTTGGCCTTTTACAGGATGT 3180
3181 TATTTGTCTGTCTTGACCTAAGCAACCTGGGGCCATGAGCCTGCTTAGGTCTGCTTGTGG 3240
3241 CCTCAGGGAGAGTGGCTGCGTGAGGCTATTTCTTTTTATGCTCTTTGCCTAAGCAAAAGG 3300
3301 AGTTCCCAGACACAAGACGTTAAGGCAGGCTGGGTTTAGACCAGGTCAATCTGCCTCAAT 3360
3361 TCTAGGCGTCAGGAAGCCATATGAAAGCTGACATCTACTGAAACATTAAAATTAACCATA 3420
3421 ACGGTTACCCTTATTTTTGGTGTGCAAACTGCACAATTCTTGCCAGAACAAATGCCATC 3480
3481 AACTGCCTCAGGGCACTCCTTGGTTTGACTGTCTTTATGTGGTTGGGTGTGAGGGAGAC 3540
3541 AGGAAGCCATTGCCCAGGACTCCATCCTGACTCCCTCTAGTGACAGACAGGAAGCACAGT 3600
3601 GTCTTTTAATAAAAGGCTCCTGTTTAAAACCTTTGCAGGGAGTACCGCACCTTCTCTCTG 3660
3661 GGATTTGAGCGGCCGCGAATT                                         3681
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Fig. 4. Nucleotide sequence of the mouse AK4 (#D94) cDNA. The complete nucleotide sequence and the predicted amino acid sequence of mouse #D94 cDNA. The location of the cDNA fragment (345 bp) obtained by the mRNA differential display (#D94 fragment) is underlined (nucleotide 1665–2009). Kozak's proposed translation initiation sequence (nucleotide 80–86) and the possible poly-adenylation signal (nucleotide 3608–3613) are indicated with bold underlines [7,21]. A translation stop codon at the end of the predicted open reading frame is indicated by an asterisk.

```
        10         20         30         40         50
MASKLLRAVI LGPPGSGKGT VCERIAQNFG LQHLSSGHLL RENLKTNTEV

        60         70         80         90        100
GDVAKQYLEK GLLVPDHVIT RLMMSELETR SAQHWLLDGF PRTLVQAEAL

       110        120        130        140        150
DRICDVDLVI SLNIPFETLK DRLSRRWIHP SSGRVYNLDF NPPQVLGVDD

       160        170        180        190        200
ITGEPLVQQE DDKPEALAAR LRRYKDAAKP VIELYKSRGV LHQFSGTETN

       210        220
RIWPYVYTLF SNKITPIQSK EAY
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Fig. 5. Predicted amino acid sequence of the rat AK4 (#D94). The adenylate kinase signature, [LIVMFYW]3-D-G-[FY]-P-R-x3-[NQ], is shown with a broken-underline and the mitochondrial energy transfer protein signature, P-x-[DE]-x-[LIVAT]-[RK]-x-[LRH]-[LIVMFY] is underlined [6,17,24]. These signatures are conserved between the AK3 and AK4.

cDNA identified with rat #D94 DNA fragment, was 3667 bp in size (Fig. 4). Predicted open reading frame encodes a protein of 223 amino acid residues (nucleotides 83–751). The predicted amino acid sequence of the mouse #D94 cDNA has an adenylate kinase signature, $[LIVMFYW]_3$-D-G-[FY]-P-R-x_3-[NQ] [24], shown with a broken-underline in Fig. 5. The underlined region matches a mitochondrial energy transfer protein signature (P-x-[DE]-x-[LIVAT]-[RK]-x-[LRH]-[LIVMFY]) [6,17]. The nucleotide sequence carries Kozak's proposed translation initiation sequence ((A/G)CC *AUG*G, 80–86 [7]) and the consensus polyadenylation signal (AAUAAA, 3608–3613 [21]).

3.4. Rat #D94 gene (AK4) was cloned

Since no obviously positive clones could be obtained by a rat cDNA library screening, PCR with degenerate oligonucleotide primers was carried out to acquire a rat gene containing #D94 fragment. The sequence of degenerate oligonucleotides was designed based on the identified mouse sequence in order to obtain an equivalent open reading frame sequence of the rat. Finally, one DNA fragment (rat #D94 cDNA) of 709 bp in size was obtained.

Sequence inquiry into the reported DNA database revealed that the obtained rat #D94 cDNA nucleotide sequence has a 95.1% homology with the mouse #D94 cDNA and 85.1% with the human adenylate kinase 3 (AK3) [29] over the corresponding open reading frame region (Table 1). Deduced amino acids sequence of the rat #D94 contains the adenylate kinase signature and the mitochondrial energy transfer protein signature, which are found in the mouse #D94 homologue.

3.5. #D94 encodes the fourth adenylate kinase (AK4)

The predicted amino acid sequence of the rat #D94 cDNA reveals a 89.2% homology with the human adenylate kinase 3 (AK3) and a 53.8% identity with the rat AK3 [25,29] (Table 1). Alignment of the predicted amino acid sequence of rat and mouse #D94 cDNA with that of already reported adenylate kinases in other species is depicted in Fig. 6. This alignment suggests that the reported AK3s (and AK4s) can be divided into two groups, the authentic AK3 group (rat AK3, bovine AK3) and the unknown AK group (human AK3, rat #D94, mouse #D94). As shown in Fig. 6, there are highly conserved amino acid sequences (87.2%–97.8%) in each group, while 52.9%–56.1% sequences are maintained between the two groups (Table 1).

Thus, we concluded that the rat #D94 cDNA encodes the 4th adenylate kinase (adenylate kinase 4, AK4), although the existence of only 3 types of adenylate kinases has been reported in mammals. Rat AK4 is 53.8% homologous with that of rat AK3 over 223 amino acids (Fig. 6). Since the nucleotide sequence of mouse #D94 clone is

Table 1

	Rat AK4 (rat #D94)	Mouse AK4 (mouse #D94)	Human AK3	Rat AK3	Bovine AK3
Rat AK4 (rat #D94)	–	97.8 (95.1)	89.2 (85.1)	53.8 (56.8)	55.2 (59.3)
Mouse AK4 (mouse #D94)	97.8 (95.1)	–	90.1 (87.6)	54.7 (56.8)	56.1 (59.2)
Human AK3	89.2 (85.1)	90.1 (87.6)	–	54.7 (58.0)	56.1 (59.3)
Rat AK3	52.9 (55.8)	53.7 (55.8)	53.7 (57.0)	–	87.2 (84.6)
Bovine AK3	54.2 (58.3)	55.1 (58.1)	55.1 (58.3)	87.2 (84.6)	–

Simple homologous scores among amino acid and nucleotide sequences of AKs.

Values are matching percentages of amino acid sequences or nucleotide sequences of the open reading frame (given in parentheses). Difference in values between the same matching pairs are due to the difference in the length of compared sequences. Sequences of AKs listed in the first column are compared with those of AKs listed in the first row, e.g., when the amino acid sequence of rat AK3 is compared with that of rat AK4, 120 amino acids match against 227 amino acids of rat AK3 (52.9%), while the amino acid sequence of rat AK4 is compared with that of rat AK3, 120 amino acids match against 223 amino acids of rat AK4 (53.8%). Based on the sequence similarity, AK3s and AK4s can be divided into two groups; the authentic AK3 group (rat AK3, bovine AK3) and novel AK4 group (rat AK4, mouse AK4 and human AK3). There are highly conserved amino acid sequences within each group (87.2%–97.8%), while 52.9%–56.1% are maintained between the two groups. Sequences of human AK3, rat AK3 and bovine AK3 are based on the published reports [3,25,29].

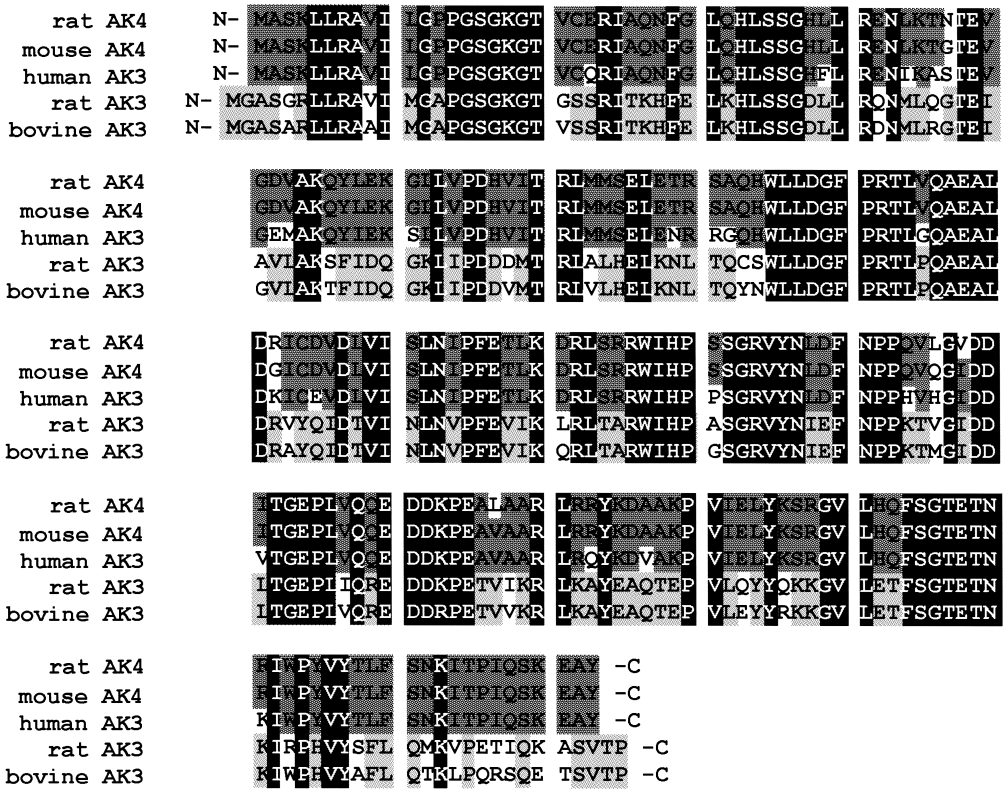

Fig. 6. Comparison of amino acid sequences of AK3 and AK4 among various species. Predicted amino acid sequences of the rat and mouse AK4s are compared with those of the already reported AK3s in other species. Based on the sequence similarity, AK3s and AK4s can be categorized into two groups: the authentic AK3 group (rat AK3 and bovine AK3) and the novel AK4 group (human AK3 group, the rat AK4 (#D94) and the mouse AK4 (#D94)). There are some group-specific conserved sequences (indicated by dark gray or light gray dotted boxes) and several sequences maintained through the two groups (indicated by black boxes). Homologous scores among AKs are listed in Table 1.

95.1% homologous with that of rat AK4 and 56.8% homologous with that of rat AK3, we concluded that cloned mouse #D94 is the mouse AK4. A phylogenetic tree of the AK3 and AK4 family in vertebrates is depicted in Fig. 7. Simple homologous scores among amino acid and nucleotide sequences of AKs are summarized in Table 1.

3.6. AK4 mRNA was expressed dominantly in the hippocampus (especially in the subfield of CA3) and the liver during ontogenesis

The spatial and temporal localization of the rat AK4 mRNA during embryogenesis was examined by in situ hybridization histochemistry (Fig. 8). No specific labeling for the AK4 mRNA was detected in rat E9 embryos nor obvious expression of rat AK4 mRNA could be confirmed by RT-PCR method (data not shown). Positive but weak signals were observed in E11 embryos. Signals were noticed in the telencephalon, especially in the cortex (including the primordium of hippocampal formation), the liver and the olfactory neuroepithelium (Figs. 2 and 8). In the cortex, signal intensity was continuously changed with a gradient. Intensity was stronger in the caudal region than in the rostral region. The strongest signals were observed in the primordium of the hippocampal formation.

During the later ontogenesis, expression of AK4 mRNA in the cortex was gradually confined to the hippocampal formation, particularly in the subfield CA3 of Ammon's

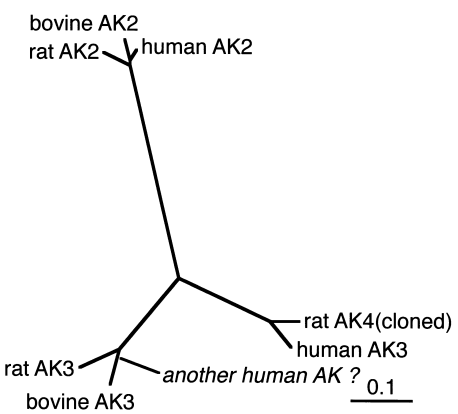

Fig. 7. A phylogenetic tree of the AK2, AK3 and AK4 family in vertebrates. Only the members of reported AKs 2, 3 and cloned AK4 in vertebrates are utilized. Sequence divergence is calculated by the Neighbor-joining Method [22] and the result is depicted as a tree using the CLASTAL algorithm (this program is available on the World Wide Web server at http://www.ddbj.nig.ac.jp/E-mail/homology-j.html). The existence of a human homologue of rat AK3 isozyme is suggested. The sequences used in the tree construction are bovine [30], rat [25] and human [29] AKs 2 and 3 and rat (cloned) AK4.

horn. This delineated distribution pattern in the cortex was substantially maintained into adulthood (Fig. 9). In the adult, distinct expression of the AK4 mRNA was detected in the pyramidal cell layer of the subfield CA3 of Ammon's horn, with faint expression in the CA1, CA2 and the dentate gyrus (Fig. 9). Another strong expression of AK4 mRNA was recognized in the granular cell layer of the cerebellum in the adult. Localization of AK4 mRNA in the cerebellum was detected only after postnatal day 3 during ontogenesis (Figs. 8 and 9). Positive cells bearing AK4 mRNA in the adult brain were identified as neurons based on their localization, although we could not exclude the possibility of some glial cells expressing AK4 mRNA because cells are arranged too compact in the pyramidal cell layer of the hippocampus or in the granular cell layer of the cerebellum to identify individual small glial cells.

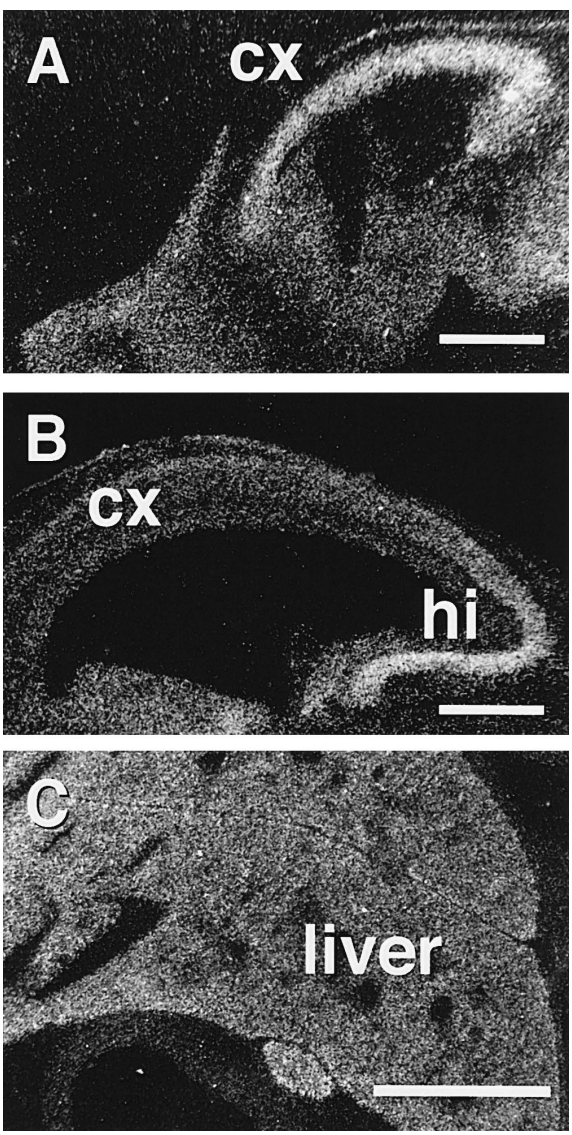

Fig. 8. In vivo localization of AK4 mRNA during the rat embryogenesis. Parasagittal sections of the rat embryos. (A) Forebrain of E 11 embryo, (B) Telencephalon of E18 embryo, (C) Liver of E18 embryo. Emulsion-autoradiograms. During the ontogenesis, expression of AK4 mRNA in the cortex is gradually confined to the hippocampus especially in the subfield CA3. cx: cortex, hi: hippocampus. Scale bar = 1 mm.

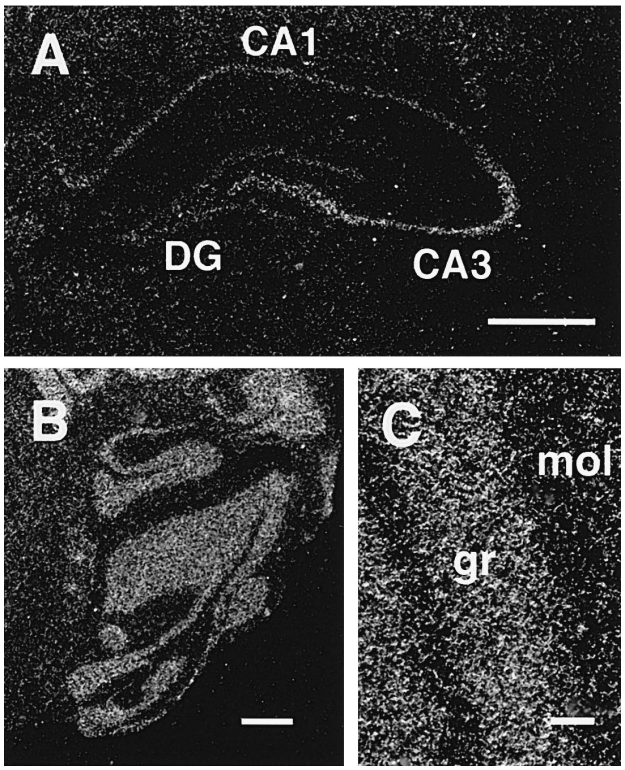

Fig. 9. In vivo localization of AK4 mRNA in the adult rat brain. Frontal sections of the adult rat. (A) Hippocampus. Distinct expression of AK4 mRNA is detected in the pyramidal cell layer of the subfield CA3 of Ammon's horn. (B) Cerebellum. Positive signals of AK4 mRNA are observed in the granular cell layer of the cerebellum. (C) High magnification of (B). CA1: the subfield CA1 of Ammon's horn, CA3: the subfield CA3 of Ammon's horn, DG: dentate gyrus, gr: granular cell layer, mol: molecular cell layer. Scale bar = 1 mm (A and B), 100 μm (C).

Positive signals were continuously observed in the liver during ontogenesis. Strong hybridization signals in the adult liver were also confirmed.

4. Discussion

To date, three types of adenylate kinase (AK) have been identified in mammals. In rat, all 3 isozymes have been studied [25]. The amino acid sequence of our rat clone is similar to rat AK3 (53.8%) but is different from the other two AKs. Therefore we designate this newly cloned molecule the rat adenylate kinase 4 (AK4) cDNA. Because our cloned mouse cDNA encodes a protein which is very similar (97.8% homology) to the rat AK4, we have concluded that our mouse cDNA clone encodes the mouse AK4.

Although the rat and mouse AK4s are newly identified, the cDNA encoding the probable human AK4 has already been cloned under the name of human AK3 [29]. Human AK3 is named based on its amino acid sequence similarity

to the bovine AK3 (56.1% homology, [29]). However the amino acid sequence of human AK3 is closer to that of rat AK4 (89.2% homology) than to rat AK3 (54.7% homology); rat AK3 and bovine AK3 amino acid sequences are very similar (87.2% homology). Thus, we propose here that the human AK3 should be called the human AK4. It is possible that an authentic human AK3, having a sequence closer to other AK3s than AK4s, could exist but has not been cloned to date.

4.1. On the distribution of AK4

The expression of AK4 mRNA is restricted in the brain (hippocampus and cerebellum), olfactory neuroepithelium and liver from the embryonic stage to the adult. This pattern of localization is in contrast to that of AK3 mRNA, which is expressed ubiquitously in the body [25].

This result indicates that AK4 acts on the specific mechanism of unusual energy metabolism rather than the control of homeostasis of ADP pool ubiquitously, which AK3 acts on [25]. Thus, it is suggested that at least two types of AKs exist to deal with the different energy requirements in various tissues. Moreover, it is possible that the function of AK4 is distinct from energy metabolism, rather the control of ATP/GTP metabolism for the intracellular signal transduction, production of the neurotransmitters or the neurotrophic factors and neuroprotection in the case of ischemia (especially in the subfield CA3).

In addition, the restricted hippocampal distribution of AK4 gene expression from E11 suggests that AK4 could potentially be a good marker for the pyramidal cells of the developing and mature hippocampus, especially those in CA3 subfield, where very few other specific markers are known [2].

4.2. On the 4th adenylate kinase

Until now, the AKs (AK1, 2 and 3) have been categorized into two major subgroups; AK1 group and AK2, AK3 group based on their molecular weights [3]. The molecular mass of AK1 is approximately 21 kDa and that of AK2 and AK3 is approximately 25 kDa [24]. The size of AK4 is approximately the same as AK3, four amino acid residues shorter than the AK3. Therefore newly cloned AK4 is assumed to belong to the AK2 and AK3 group (bigger AK molecule group).

Phylogenetically this categorization is evident. The AK1 enzyme is considered to have diverged from the bigger AK molecule group prior to the separation of eukaryotes and prokaryotes [3]. Regardless, the analysis of the subcellular localization of AKs has revealed that AK1 resides in the cytoplasm, while both the AK2 and AK3 are located mainly in the mitochondria. It is reported that AK2 resides in the mitochondrial intermembrane space and that AK3 is in the mitochondrial matrix [24]. Marker [10] has reported that mitochondrial AK may function in the salvage pathway of AMP, since this adenine nucleotide which accumulates during periods of energy demand cannot enter the mitochondrion and will be lost by deamination if not phosphorylated. As AK4 is considered to belong in the bigger AK group and AK4 has a mitochondrial energy transfer signature in its sequence, it is likely that AK4 also resides in mitochondria. Although typical molecules located in mitochondria have a pair of mitochondrial energy transfer signatures while AK4 has one, the existence of a mitochondrial anchor sequence [6,17] reinforces the concept that AK4 is located in mitochondrial matrix.

AKs catalyze the same reaction, Mg-NTP + AMP ⇔ Mg-NDP + ADP (N = A or G), but an individual isozyme has been shown to be very specific for its substrate as a high energy donor. While ATP is the substrate for AK1 and AK2, GTP is the substrate for AK3 [26]. AK3 is assumed to play a role as a scavenger of mitochondrial AMP (or dAMP) at the interconversion of non-ATP nucleotide triphosphate, ITP and GTP hydrolysis, which are formed in the tricarboxylic acid cycle (the Krebs cycle) by substrate chain phosphorylation through succinic thiokinase [5]. Such a scavenger function has to be postulated in mitochondria, because on the one hand AMP is generated in this compartment in a number of hydrolytic reactions (e.g., turnover of DNA or RNA) or in ATP-dependent substrate activations yielding AMP (e.g., tRNA synthetase). On the other hand, mitochondrial inner membranes from mammals lack a transport system for guanosine nucleotides as well as for AMP [20]. It is likely that AK4 also preferentially utilizes GTP, although no such experiment has been carried out for human AK3 (human homologue of AK4 isozyme), and this issue is still open at present. The interrelationship between AK4 mRNA expression and its activity cannot be discussed, as no method has been established to distinguish the activity of AK4 from AK3. It is likely that the activity of AK3 is estimated as the combination of AK3 and AK4 activities. It would be desirable to measure the activity of AK3 and AK4 separately. On the other hand, the activities of AK1 and AK2 are found in proportion to the amount of their expressed mRNAs. However, this is not the case for AK3 [25].

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