Bull Yamaguchi Med Sch 47(3-4):55-68, 2001

Expression of mRNAs Encoding Adenylate Kinase Isozymes 1, 2, 3, and 4 in Mouse Tissues and during Neuronal Differentiation of P19 Embryonal Carcinoma Cells

Yasuhiro Yamashiro

Department of Biochemistry, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan.
(Received December 26, 2000. revised January 18, 2001)

Abstract We cloned cDNAs encoding four adenylate kinase (AK) isozymes from a mouse kidney cDNA library. The *AK1, AK2, AK3,* and *AK4* cDNAs encode the 194-, 232-, 227-, and 223-amino acid proteins, respectively. *AK4* is a recently isolated gene that is highly homologous to the reported human *AK3*. Northern blot analysis and reverse transcription-polymerase chain reaction analysis revealed that *AK1* mRNA was predominantly expressed in skeletal muscle, heart, and testis; *AK2* mRNA in liver, heart, kidney, and testis; *AK3* mRNA almost uniformly in all tissues examined; and *AK4* mRNA prominently in kidney. Subcellular and submitochondrial fractionation analysis suggested that AK4 was localized in the mitochondrial matrix. Further, we found a 76-fold induction of *AK1* mRNA expression concomitant with a 53-fold induction of *NeuroD* expression during retinoic acid-induced neuronal differentiation of P19 embryonic carcinoma cell. *AK2* and *AK3* mRNA expression was increased by 4- to 6-fold during differentiation, whereas *AK4* transcription was first down-regulated and subsequently returned to the original level. Our data on *AK* isozyme gene expression may provide basic information for production and evaluation of transgenic mice as well as knockout mice to further understand the physiological role of AK isozymes.

Key words: a denylate kinase, isozymes, Northern blot, RT-PCR, tissue distribution

Introduction

Adenylate kinase (AK) is a ubiquitous enzyme that catalyzes the interconversion of adenine nucleotides. Three isozymes were well characterized in vertebrates: AK1 and AK2, ATP:AMP phosphotransferase (EC 2. 7.4.3); and AK3, GTP:AMP phosphotransferase (EC 2.7.4.10).[1-5] AK1 is present in the cytosol of skeletal muscle, brain, and erythrocytes, while AK2 is found mainly in the mitochondrial intermembrane space of liver, kidney, spleen, and heart. AK3 is present in the mitochondrial matrix of many tissues. Regarding the physiological role of AK, two possibilities have thus far been proposed. First, AK contributes to the adenine nucleotide homeostasis by maintaining the "energy charge" in the subcellular milieu.[6] Second, AK participates in energy transfer from the sites of ATP synthesis to the sites of ATP consumption via a high-energy-phosphoryl transfer chain consisting of multiples of AK molecules.[7-8]

AK is indispensable for the growth of *Escherichia coli* [9] and *Schizosaccharomyces pombe*[10], indicating that it is an essential enzyme for life in single cell organisms. However, it was reported that no phenotypic change excepting hematological abnormality, due to homozygous base substitution in the exon 6 of the *AK1* gene, was recognized in a

patient with AK1 deficiency.[11] Thus the physiological function of AK isozymes in multicellular organisms should be further analyzed with organisms having mutation in each isozyme gene or some combination of the mutations. Recently, genetically engineered mice such as transgenic and knockout mice have widely been used for investigating the function of a specific gene [12]. In order to create a model mouse, it is necessary to obtain structural information about the genes encoding mouse AK isozymes as well as functional information about their gene expression with respect to tissue-specificity, region-specificity, cell-type-specificity, and changes during differentiation.

A fourth gene encoding putative AK isozyme (AK4) that is homologous to AK3 has recently been identified in the rat and the mouse.[13] During the course of this study, we independently found the same gene and obtained evidence indicating that AK4 is encoded by a gene separate from that of *AK3* in the mouse genome. Here, we describe the isolation of mouse cDNAs encoding AK isozymes including AK4, and the steady state levels of mRNA for these four AK isozymes in various tissues. We also document the change in levels of *AK* mRNAs during in vitro differentiation of P19 mouse embryonal carcinoma cells as a model system for analyzing the *AK* expression during development.

Materials and methods

Preparation of screening probes

The probes were prepared by reverse transcription-polymerase chain reaction (RT-PCR) using an LA RNA PCR kit (Takara) according to the manufacturer's instructions. One μg of the total RNA extracted from brains and hearts of 6-week old BALB/c mice was reverse-transcribed for 30 min at 42°C using an oligo dT primer as described previously[14]. PCR amplification was performed using the cDNA and following oligos : a sense primer, 5'-GCA GGA TGG AAG AGA AGC TGA AGA A-3', and an antisense primer, 5'-GCC TGC GTC CAC ATA CAG CAG CAG T-3', for *AK1* ; a sense primer, 5'-GCT TCT GGC TCA GAG CTA GGA AAA A-3', and an antisense primer, 5'-AGC TTC TCT TTC CTC TTC TCC ATG A-3', for *AK2*; a sense primer, 5'-AGC TGA AGC ACC TCT CCA GCG G-3', and an antisense primer, 5'-CTC TAT CCA GGG CTT CTG CCT G-3', for *AK3*. The primers were designed on the basis of homology of reported cDNA sequences and this study.[15–18] PCR amplification, subsequent isolation, and sequencing analysis of the products were performed as described previously.[14,19]

Isolation of mouse AK cDNAs and sequence analysis

A λgt11 mouse kidney cDNA library (Clontech) was screened by plaque hybridization using the fragment of each *AK* cDNA as a screening probe as described previously.[18] Positive phage clones were purified, and each cDNA fragment of the clones was cloned into a pBluescript SK(−) plasmid vector (Stratagene) at the *EcoR* I site. A technique of 5'-rapid amplification of cDNA ends (RACE) was applied to isolate the 5'-portion of the m*AK4* transcript using mouse liver Marathon-Ready cDNA (Clontech) as described previously.[20] PCR was performed under the same conditions as above using 5'-TTC CGT CCA GGG CTT CTG CCT G-3' as an *AK4* specific primer and 5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3' as an adapter primer at the 5' end of the cDNA. The PCR products were purified and recloned into a pGEM-Teasy vector, then sequenced by the dideoxy chain-termination method[21].

Northern blot analysis

Tissues (liver, kidney, lung, heart, testis, brain, pancreas, spleen, thymus, and skeletal muscle) were separated from 6-week old BALB/c male mice. Total RNA was extracted from the mouse tissues and P19 cells by the acid guanidinium thiocyanate phenolchloroform method,[22] and poly(A)+RNA was isolated by using an oligo (dT) column. Total RNA (20 μg) was fractionated by electrophoresis in a 1.0% agarose gel containing 20 mM 3-(N-morpholino) propanesulfonic acid, 1 mM EDTA, 8 mM sodium acetate, pH 4.0, and 2.2 M formamide, then transferred onto a nylon membrane. The membrane and MTN blot filter (Clontech), on which poly(A) + RNA

was loaded, were prehybridized at 42℃ for 6 h in prehybridization buffer (1 % bovine serum albumin, 1 mM EDTA, 7% SDS, and 5% formamide in 0.5 M sodium phosphate, pH 7.2). The filter was first hybridized to a 774-bp fragment of *AK1* cDNA under the same hybridizing conditions as those used for screening. After detaching the *AK1* probe, the filter was used for hybridization of a 724-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA [23] and a 1.8-kb fragment of 18S *rRNA* cDNA [24] to confirm the integrity and quantity of mRNA in each sample. The previous probe was stripped by washing the filter in boiled 10 mM Tris-HCl and 1 mM EDTA (pH 7.4). For *AK2, AK3,* and *AK4* mRNA, hybridization was performed with a 1.0-kb fragment of the *AK2* cDNA, a 1.0-kb fragment of the *AK3* cDNA, and a 0.5-kb fragment of the *AK4* cDNA, respectively. Each DNA fragment was labeled with [α-^{32}P] dCTP by the random priming method. Washing was performed two times in 2 X SCC at room temperature followed by three washes in 0.5 X SCC, 0.1% SDS 55 ℃ for 20 min. Radioactivity of hybridizing signals was visualized with a BAS 2000 image analyzer (Fuji), and relative levels of mRNA for AK isozymes in mouse tissues were determined.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was incubated with RNase-free DNase I for 10 min at room temperature to remove residual genomic DNA. After cDNA synthesis using 1 μg of the RNA, PCR reaction was performed using specific primers for each isozyme as described previously.[14] The PCR products of each cycle were subjected to 5% polyacrylamide gel electrophoresis (PAGE), and exposed to an imaging plate for 30 min after drying the gel. Optimal cycle numbers for *AK1, AK2, AK3, AK4, NeuroD, GAPDH,* and 18S *rRNA* were determined as 24, 24, 24, 24, 24, 24, and 10, respectively. Primers used for PCR were the same *AK1* and *AK2* oligos as those used for preparation of the screening probes. Primers used for *AK3, AK4, NeuroD, GAPDH,* and 18S *rRNA* were as follows. *AK3* sense primer, 5'-AGC TGA AGC ACC TCT CCA GCG G-3', and antisense primer, 5'-CTC TAT CCA GGG CTT CTG CCT G-3'; *AK4* sense primer, 5'-AAG CAG TTG CTG CCA GGC TAA *GAC-3'*, and anti-sense primer, 5'-ATG GCA GGC TCA GTA GGC TTC TTT-3';*NeuroD* sense primer, 5'-AAC CAT CCG CCG AGT TTG AA-3', and antisense primer, 5'-AGT GAA ACT GAC GTG CCT CT-3'[20]; *GAPDH* sense primer, 5'-CAT TGA CCT CAA CTA CAT GG-3', and antisense primer, 5'-CTC AGT GTA GCC CAG GAT GC-3'[23]; 18S *rRNA,* sense primer, 5'-TAC CTG GTT GAT CCT GCC AGT AGC AT-3', and antisense primer, 5'-CCC GTC GGC ATG TAT TAG CTC TAG AA-3'[24]. The plasmids containing each PCR product were recloned in the pGEM-Teasy vector and used for calculation of copy number of the templates and as a positive control. Control plasmids (0.1 ng, 1 ng, and 10 ng) were also amplified by PCR using the same primer sets, and the signal intensity of PCR products was also analyzed as described above. The copy numbers of mRNA were calculated by comparing the signal intensity of the analyzed products with those of each control plasmid. We assumed that each cell contained the same content of 18S *rRNA,* and that 2% of total RNA corresponds to poly(A)$^+$ RNA.[25] The copy numbers of mRNA in a single cell were calculated from the above results assuming that the average weight of a cell is 40 pg, and that the RNA content in the cell is 1%.[25]

Western blot analysis

Tissue extracts (100 μg protein) were subjected to SDS-PAGE according to Laemmli[26] and electroblotted onto nitrocellulose membrane (Schleicher & Schuell). After soaking in blocking buffer [10% nonfat dry milk/0.1% Tween 20 in phosphate-buffered saline (PBS)] for 1 h at room temperature, the membranes were incubated with a 1 : 1,000 dilution of anti-AK1, AK2, and AK3 antibodies[27,28] for 1 h at room temperature. After washing with PBS-T (0.2% Tween 20 in PBS), membranes were incubated with a 1 : 5,000 dilution of horseradish-peroxidase-labeled goat anti-rabbit IgG antibodies for 1 h at room temperature, then visualized using the ECL Western blotting detection system (Amersham).

Subcellular localization of AK4 isozymes

Cytosol and mitochondrial fractions were obtained from mouse liver and kidney as described previously.[29,30] Protein concentrations were determined according to the Lowry method or Bradford method using bovine serum albumin as a standard. Proteins were subjected to 0.1% SDS-12% PAGE, then electrophoretically transferred to nitrocellulose membrane. The membrane was analyzed with rabbit antibody against bovine AK2 and AK3 and ELC Western blotting detection reagents.

The submitochondrial location of AK4 protein was also determined. Mitochondria were suspended in the isolation buffer at a concentration of 50 mg protein/ml. Intactness of isolated mitochondria was confirmed by t he respiratory control ratio of each mitochondrial preparation, which was determined to be higher than 3.0 with the oxygen electrode method using succinate as a substrate.[31] For the preparation of mitoplasts, mitochondria (300 μg protein) were suspended in 60 μl of 20 mM HEPES-KOH, pH 7.4, then placed in a test tube on ice for 30 min. [32]The mitoplasts were recovered by centrifugation at 4,000 X*g* at 4°C for 10 min and resuspended in 10 mM HEPES-KOH (pH7.4) containing 220 mM mannitol and 70 mM sucrose. The mitochondria and mitoplasts were treated with 250 μg/ml proteinase K with or without 1% Triton X-100 at 4°C for 30 min, followed by the addition of 400 μg/ml PMSF to terminate the protease reaction. The reaction mixture was centrifuged in a microcentrifuge tube at 12,000 X*g* for 10min, then the precipitates were subjected to 0.1%SDS-12% PAGE. Separated proteins in the gel were electrophoretically transferred to a nitrocellulose membrane. The membrane was analyzed with the same antibodies as those described above.

P19 cell culture and differentiation

Embryonal carcinoma P19 cells were kindly provided by Dr. H. Hamada (Osaka University). The cells were maintained in α-modified minimal essential medium (αMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C and in 5% CO_2. Neuronal differentiation was initiated by plating cells into bacterial dishes at 10^5 cel l/ml in αMEM supplemented with 10% FBS and 1μM retinoic acid (RA). After 4 days, the cellular aggregates were dispersed into single cells by treatment with 0.01% trypsin. The cell suspension (2×10^6 cells) was then re-plated onto a 100mm tissue-culture-grade dish. At various time points, cell samples were removed from the culture and total RNA was isolated. The steady-state levels of the different transcripts were quantitated by RT-PCR. The morphological differentiation was confirmed by microscopic examination : neurons were identified as round cells with elongated axonal or dentritic processes, while glial and other non-neuronal cells were recognized as flat fibroblastic cells as previously described.[33] Days of differentiation were numbered consecutively after the first day of RA treatment.

Results

Isolation and nucleotide sequencing of cDNA clones for mouse AK isozymes

RT-PCR with the specific primer sets generated a 368-bp fragment for *AK1*, a 203-bp fragment for *AK2*, and a 219-bp fragment for *AK3*. Because sequence analysis revealed that the PCR products were highly homologous to the nucleotide sequences of published human, bovine, and rat *AK* sequences,[15–18,34,35] each fragment was used as a probe to screen a λgt11 mouse kidney cDNA library. About 500,000 phage-plaques were screened with each probe. The numbers of isolated clones were as follows: seven clones for *AK1*, 26 clones for *AK2*, and 64 clones for *AK3*. All of the *AK1* candidate clones and 20 each of *AK2* and *AK3* candidate clones were sequenced. Sequence analysis revealed that *AK1* cDNA has a 774-bp insert composed of a 73-bp 5'-untranslated region (UT), a 582-bp open reading frame (ORF) encoding a 194-amino acid protein, and a 119-bp 3'-UT. The *AK2* cDNA having 991 bp consisted of a 42-bp 5'-UT, a 696-bp ORF encoding a 232-amino acid protein, and 253 bp of the 3'-UT. Two types of partial *AK3* cDNA were obtained. One type, clone 9, has a 988-bp sequence homologous to rat *AK3* cDNA. We found an additional sequence of the remaining 5'-portion of cDNA in the database for mouse *AK3* (GenBank accession number AA638831).

We deduced a 1080-bp *AK3* cDNA consisting of 68 bp of the 5'-UT, 681 bp of an ORF encoding a protein of 227 amino acids, and 331 bp of the 3'-UT. The other type of cDNA, clone 2, contains a partial sequence homologous to human *AK3* cDNA. A missing 5'-portion of 400-bp was obtained by the 5'-RACE method using total RNA isolated from kidney as a template. The second type *AK3* cDNA was deduced to encode a protein of 223 amino acids. We named the second type sequence *AK4*. Both cDNA sequence were confirmed by sequencing mouse genomic DNAs that were located at separate locus (data not shown). Table 1 shows the comparison of homology among these four AK isozymes at both nucleotide and amino acid levels.

Isoelectric points (pI) of predicted mouse AK1, AK2, AK3, and AK4 were 5.55, 7.11, 9.25, and 7.39, respectively. pI of mouse AK1 is fairly deviated to the acidic side in c omparison with those of other eukaryotic AK1, including 8.61-9.21. We isolated only one type of *AK2*cDNA, the *AK2B*form, though we have

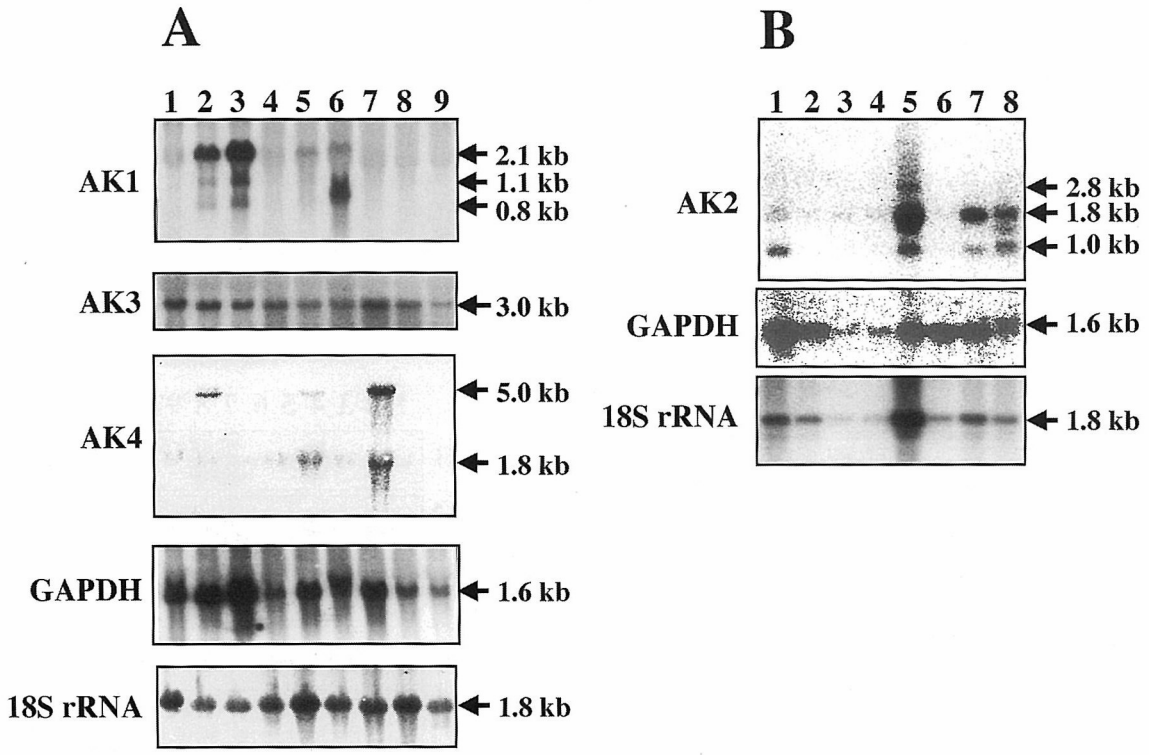

Fig. 1 Expression of *AK* mRNA in mouse tissues.

(A) Isolation of total RNA from mouse tissues; electrophoresis, blotting, hybridization with ^{32}P-labeled *AK1*, *AK3*, and *AK4* cDNA, and washing were described in Materials and methods. The tissue sources of the isolated RNA were: lane11, thymus; lane 2, heart; lane 3, skeletal muscle ; lane 4, lung; lane 5, brain; lane 6, testis ; lane 7, kidney; lane 8, liver; and lane 9, spleen. The *AK1* probe detected a strong 2.1-kb signal in skeletal muscle and heart, and a weak signal in testis and brain, and no signal in liver and spleen. The *AK3* probe hybridized to a single 3.0-kb transcript in all tissues at a similar level, while the *AK4* probe detected transcripts of 5.0 kb and 1.8 kb, predominantly in kidney.

(B) Mouse MTN blot filter (Clontech) was hybridized with ^{32}P-labeled *AK2* cDNA. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; and lane 8, testis. A 1.8-kb signal in addition to the signals of 2.8-kb and 1.0-kb were detected with the *AK2* probe in liver, kidney and testis, but no *AK2*transcript was detected in skeletal muscle, brain, spleen, lung, or skeletal muscle. Each blot was rehybridized with radiolabeled *GAPDH cDNA* (middle region) and 18S *rRNA* cDNA (lower region)

previously isolated two kinds of *AK2* cDNA, *AK2A* and *AK2B*, in human and bovine liver.[18,34] Mouse AK isozymes show markedly high homology in the NMP_{bind} domain and the LID domain, which are important for enzyme function.[36,37]

Tissue distribution of AK isozyme mRNA

To investigate the distribution of *AK* mRNA in mouse tissues, Northern blot analysis was performed using each cDNA as a probe. Fig. 1 shows an overview of hybridizing profiles. The *AK1* probe detected a strong 2.1-kb signal in skeletal muscle and heart, and a weak signal in testis and brain, and no signal in liver and spleen (Fig. 1A). In testis, a strong 0.8-kb signal was also observed. *AK2* transcripts were predominantly detected in liver as a 1.8-kb signal in addition to the signals of 2.8-kb and 1.0-kb (Fig.1B). In kidney and testis, *AK2* transcripts were detected at a moderate level. In heart, a 1.0-kb transcript was dominant. In spleen, lung, and skeletal muscle, *AK2* transcript was faintly observed. The results from other tissues were generally consistent with those from previous analysis.[16,18] The *AK3* probe hybridized to a single 3.0-kb t ranscript in all tissues examined at a similar level, while the *AK4* probe detected transcripts of 5.0 kb and 1.8 kb, predominantly in kidney, and weakly in heart and brain (Fig. 1A). The results indicated that the expression of *AK1, AK2,* and *AK4* are tissue-specific, whereas that of *AK3* is constitutive or occurs in a housekeeping manner.

Contents of AK isozyme mRNA in mouse tissues

In order to further assess the levels of *AK* isozyme mRNA expression, we performed quantitative RT-PCR analysis using RNA preparations isolated from kidney, heart, lung, brain, skeletal muscle, thymus, testis, spleen, and liver. First, we investigated the optimal

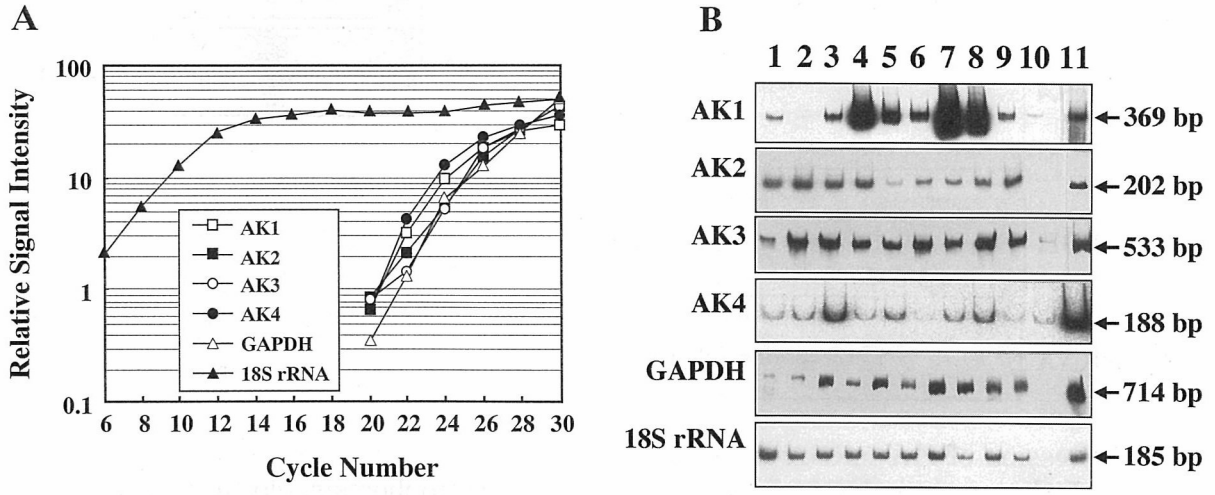

Fig. 2 RT-PCR analysis of *AK* mRNA levels in mouse tissues.

(A) Determination of the optimal number of amplification cycles for quantification of each *AK mRNA* and 18S *rRNA*. The PCR products were subjected to 5% PAGE and autoradiography, and associated radioactivity was analyzed with an image analyzer. PCR cycle and relative signal intensity was denoted by a logarithmic scale; AK1 (open square), AK2 (closed square), AK3 (open circle), AK4 (closed circle), GAPDH (open triangle), and 18S rRNA (closed triangle). The optimal cycle number was determined to be 24 except for 18S rRNA, for which it was 10.

(B) PCR products were separated in 5% PAGE and visualized by an image analyzer. Lane 1, spleen ; lane 2, liver ; lane 3, kidney ; lane 4, testis ; lane 5, brain; lane 6, lung ; lane 7, skeletal muscle ; lane 8, heart ; lane 9, thymus; lane 10, pancreas; lane 11, control plasmid containing each cDNA recloned in the pGEM-Teasy vector. Each mRNA expression was observed in parallel with the results of Northern blot analysis.

number of PCR cycles to determine RNA for *AK1, AK2, AK3, AK4, GAPDH,* and 18S *rRNA*. As shown in Fig. 2A, the optimal cycle number was determined to be 24 except for 18S *rRNA*, for which it was 10. The optimal number for *NeuroD* was also determined as 24 (data not shown). The RT-PCR products of RNAs from various tissues were separated in PAGE (Fig. 2B). Copy numbers of the *AK* templates in the RT-PCR samples were calculated using 18S *rRNA* as an internal control, on the assumption that each mouse cell contains the same copy number of 18S *rRNA* (Table 2). Comparatively higher levels of each *AK* mRNA were observed in the following tissues: skeletal muscle, heart, and testis for *AK1*; liver, kidney, heart, and testis for *AK2*; kidney for *AK4*. *AK* 3 mRNA was present in all the tissues examined.

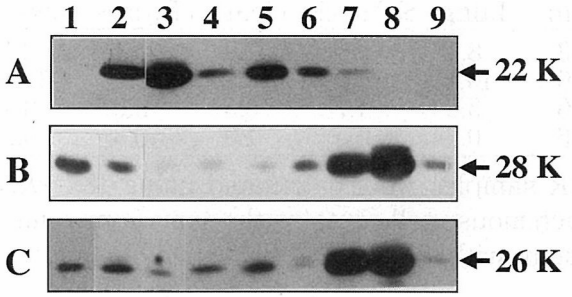

Fig. 3 Tissue distribution of mouse AK isozymes.
AK isozymes in mouse tissues were analyzed by Western blot analysis as described in Materials and methods. Extracts were prepared from 6-week old BALB/c mouse tissues, and proteins were separated in 10% SDS-PAGE. AK isozymes were visualized with anti-AK antibodies and an ECL system. AK1, AK2, and AK3 are represented by the upper (A), middle (B), and lower (C) lines, respectively. Lane 1, pancreas ; lane 2, heart ; lane 3, skeletal muscle; lane 4, lung ; lane 5, brain; lane 6, testis; lane 7, kidney ; lane 8, liver ; and lane 9, spleen. AK1 was detected abundantly in skeletal muscle, but faintly or not detectable in pancreas, spleen, and liver. AK2 was observed abundantly in liver and kidney, but faintly or not detected in skeletal muscle and brain. AK3 was present abundantly in liver and kidney. These findings were almost correlated with the expression pattern of each mRNA.

Tissue distribution of AK isozyme protein

We performed Western blot analysis to evaluate the protein contents of each AK isozyme in mouse tissues (Fig. 3). AK1 was detected abundantly in skeletal muscle, heart, and brain; moderately in testis, lung, and kidney; and faintly or not detectable in pancreas, spleen, and liver. AK2 was observed abundantly in liver and kidney; moderately in heart, pancreas, spleen, and testis; faintly or not detected in lung, skeletal muscle, and brain. AK3 was present abundantly in liver and kidney, and moderately in pancreas, heart, skeletal muscle, lung, brain, testis, and spleen.

Demonstration of AK4 protein

Recently we isolated an authentic human *AK3* cDNA that is homologous to mouse *AK3* cDNA and distinct from the previously isolated human *AK3* cDNA, which was renamed AK4. The recombinant human AK3 and AK4 were both recognized by anti-AK3 antibody, and electrophoretic analysis demonstrated that the migration of AK4 (27 kDa) was retarded than that of AK3 (26 kDa) (T. Noma et al., manuscript in preparation). Homology of the AK isozymes among species is much higher than that between AK3 and AK4, as shown in Table 1. Human and mouse AK3 are 91% homologous to each other with the same amino acid length (227 residues), and human and mouse AK4 are 89% homologous with the same amino acid length (223 residues). The mobility difference could have been caused by the pI difference. In this study, we found that the expression of *AK4* mRNA was about 8-fold more abundant in kidney than in liver, while that of *AK3* mRNA in both tissues were nearly equivalent by RNA analysis (Table 2). Subcellular fractions were prepared from liver and kidney cells, and Western blot analysis was performed using anti-AK2 antibody and anti-AK3 antibody. When we examined the electrophoretic patterns of cellular extracts from liver and kidney by Western blot analysis using anti-AK2 antibody, AK2 was detected in both cytosol and mitochondrial fractions as previously reported[29,28] (Fig. 4A upper region).

Table 1. Sequence homology between AK isozymes at nucleotide and amino acid levels

	Similarity (%)			
	mAK1	mAK2	mAK3	mAK4
mAK1	100.0	(49.4)	(50.3)	(44.2)
mAK2	34.1	100.0	(48.3)	(50.0)
mAK3	34.2	40.9	100.0	(55.3)
mAK4	36.7	39.2	56.6	100.0

Sequence identities among for AK isozymes at nucleotide (in parentheses) and amino acid leveis were calculated with Mac DNASIS (Hitachi) using the Lipman-Pearson algorithm.

Table 2. RNA copy numbers of *AK* isozyme genes

	Copy numbers (fmol/μg RNA)									
	Spleen	Liver	Kidney	Testis	Brain	Lung	Sk.Muscle	Heart	Thymus	Pancreas
AK1	4.6	< 0.1	7.0	65.0	23.3	8.5	88.0	67.0	5.1	2.1
AK2	17.3	43.6	31.2	31.2	6.0	13.1	7.0	18.5	12.5	32.8
AK3	1.8	4.0	4.8	2.8	2.6	3.6	1.7	6.6	3.0	8.4
AK4	0.3	0.3	2.5	0.3	0.6	0.2	0.5	1.0	0.3	0.5

Copy numbers of *AK* templates in the RT-PCR samples were calculated using 18S *rRNA* as an internal control, on the assumption that each mouse cell contains the same copy number of 18S rRNA.The copy numbers were well consistent with the results of Northen blot analysis.

However, using anti-AK3 antibody displayed that whole cellular extracts from kidney gave doublet bands and those from liver gave a single band (Fig. 4A lower region). The positions of the upper and lower bands corresponded to those of recombinant human AK4 and AK3 in the parallel experiment (data not shown). The results indicated that electrophoretic analysis was able to distinguish AK4 from AK3. The ratio of the signal intensities between the doublet bands corresponded to the relative contents of *AK3* and *AK*4 mRNA in liver and kidney (Fig. 2, Table 2). Therefore, we concluded that the upper band of the doublet bands was due to AK4. AK3 was detected in the total cellular extracts and mitochondrial fraction of both tissues (Fig. 4A lower region, lanes 1, 3, 4, 6), but it was scarcely detected in the cytosol fractions (Fig. 4A lower region, lanes 2, 5).

Submitochondrial localization of AK4 protein

To further analyze the submitochondrial localization of AK4 protein, mitochondria and mitoplasts were prepared from liver and kidney, and the proteins were subjected to SDS-PAGE as described previously[29]. Western blot analysis using the antiserum against AK2 and AK3 indicated that AK2 was localized in mitochondria and not in mitoplast (Fig. 4B upper region, lanes 1, 2, 5, 6). On the other hand, AK3 and AK4 were detected in both mitochondria and mitoplasts, and the amounts of AK3 and AK4 in mitoplasts were the same as those in mitochondria (Fig. 4B lower region, lanes 5, 6). AK3 and AK4 in mitoplasts were resistant to proteinase K digestion (Fig. 4B lower region, lane 7), whereas they were completely destroyed by the protease treatment after rupturing of the mitoplasts with a detergent, Triton X-100 (Fig. 4B lower region, lane 8). Thus, we concluded that both AK3 and AK4 are located in the matrix of the mitochondria.

Expression of AK isozyme mRNAs during RA-induced neuronal differentiation of P19 cells

Our previous analysis indicated that AK isozymes were differentially induced during neuronal differentiation in P19 cells.[39] However, in the current literature, there is no

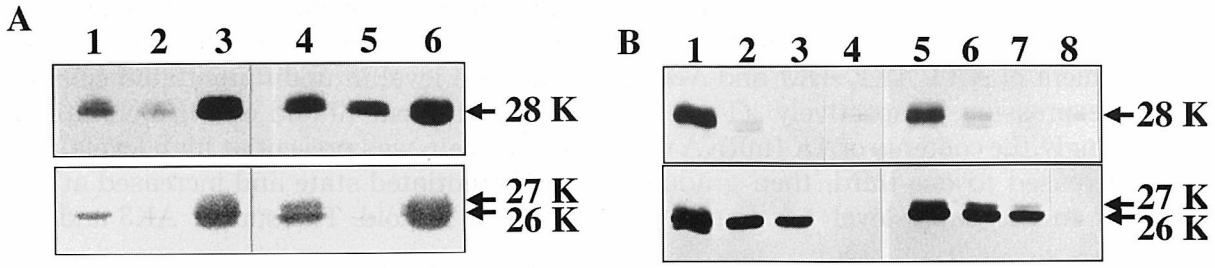

Fig. 4. Localization of AK4 in the cell.

(A) Subcellular localization of AK4. Subcellular fractionation of liver and kidney extracts was performed as described previously.[29] Western blot analysis was performed using anti-AK2 and -AK3 antibodies after electrophoresis with 0.1% SDS-12% PAGE and transfer of protein samples to the membrane. Lanes 1 to 3, liver samples ; lanes 4 to 6, kidney samples; lanes 1 and 4, total cellular extracts; lanes 2 and 5, cytosol fractions; lanes 3 and 6, mitochondrial fractions. Upper and lower regions are the results from anti-AK2 and -AK3 antibodies, respectively. AK2 was detected in both cytosol and mitochondrial fractions. Analysis with anti-AK3 antibody revealed that whole cellular extracts from kidney gave doublet bands (AK3 and AK4) and those from liver gave a single band (AK3), which is corresponding to the lower band from kidney sample (Lower region, lanes 1, 3, 4, 6).

(B) Submitochondrial localization of AK4. Submitochondrial fractions were prepared as described in Materials and methods. Each submitochondrial fraction was separated with SDS-PAGE. Separated proteins were electrophoretically transferred to a nitrocellulose membrane and analyzed with antiserum against AK3, then visualized with ECL Western blotting detection reagents. Subsequently, anti-AK3 antibody was detached from the same filter and analyzed with anti-AK2 antibody. Lanes 1 to 4, liver samples ; lanes 5 to 8, kidney samples; lanes 1 and 5, mitochondria ; lanes 2 and 6, mitoplasts ; lanes 3 and 7, proteinase K-treated mitoplast ; lanes 4 and 8, proteinase K- and Triton X-100-treated mitoplast. Upper and lower regions are the results from anti-AK2 antibody and anti-AK3 antibody, respectively. AK3 was detected in mitochondria, mitoplasts and proteinase K-treated mitoplast (Lower region, lanes 1 to 3, 5 to 7), but it disappeared by the detergent treatment (Lower region, lanes 4, 8), suggesting AK3 is located in the matrix of the mitochondria. Similarly, the 27-kD upper band (AK4) was resistant to hypotonic treatment (Lower region, lane 6) and proteinase K digestion (Lower region, lane 7), whereas they were completely disappeared by the protease treatment with an addition of Triton X-100 (Lower region, lane 8), indicating the AK4 was localized in the mitochondrial matrix.

information available on the levels of mRNA for AK isozymes. Therefore, we performed RT-PCR analysis to evaluate the copy number of each *AK* isozyme mRNA. We also examined *NeuroD* mRNA, because NeuroD, a neuronal transcription factor, was found to enhance *AK1* expression.[14,40] Typical morphological changes were observed during RA-induced differentiation of P19 cells (Fig. 5A). At an a ppropriate time, cells were harvested and total RNA was isolated for quantitative analysis by RT-PCR. The mRNA expression of AK isozymes as well as NeuroD was shown in Figure 5B. The copy numbers of *AK* isozyme mRNAs were quantitated by the densitometric analysis and summarized in Table 3. The results indicated that the copy numbers of each *AK* mRNA in undifferentiated P19 cells were 1.4 for *AK1*, 28.9 for *AK2*, 0.9 for *AK3*, 2.0 for *AK4*, and 0.2 for *NeuroD*, in which the unit is fmol/μg total RNA. On the other hand, the copy numbers in neuronally differentiated P19 cells at day 6 were 107 for AK1, 65.3 for AK2, 3.6 for AK3, 0.8 for AK4, and 10.5 for

NeuroD. Thus, treatment of P19 cells with 1μM RA gave 76-, 2.3-, 4.0-, and 53-fold enhancement of *AK1, AK2, AK3,* and *NeuroD* mRNA expression, respectively (Table 3). Interestingly, the contents of *AK4* mRNA were first decreased to one-third, then gradually returned to the same level as that of the undifferentiated cells. In the previous study, we found that AK1 protein was present only at a trace level in undifferentiated cells and increased about 10-fold on differentiation.[39] AK2 protein was present at high levels in the undifferentiated state and increased at most by about 1.2-fold. The sum of AK3 and AK4

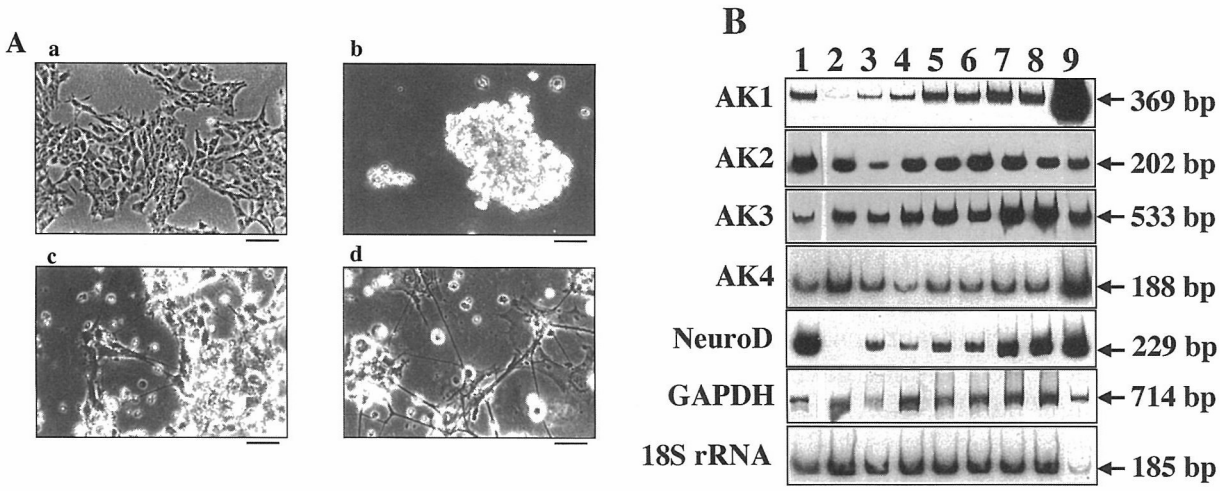

Fig. 5. RA-induced neuronal differentiation of P19 cells.
(A) Morphology of P19 cells during RA-induced neuronal differentiation. (a) Undifferentiated P19 cells grown on the tissue culture dish. (b) Cellular aggregates in a bacterial dish after 4 days of the RA treatment. (c) Differentiated P19 cells at 2 days after plating the cell suspension in the tissue culture dish. (d) Cells 5 days after the plating. Bars indicate 20 nm.
(B) RT-PCR analysis of *AK* and *NeuroD* expression during neuronal differentiation of P19 cells. Total RNAs were prepared from P19 cells at various stages of differentiation, and analyzed for the mRNA expression of *AK* isozymes, *NeuroD, GAPDH,* and 18S *rRNA* by RT-PCR. RNA isolated from AtT20 cells, a mouse pituitary-derived ACTH producing cell line,[43] which express all *AK* isozyme mRNA and *NeuroD* mRNA, was used as a positive control. The PCR products for *AK1* and *NeuroD* cDNA were tremendously increased in parallel. Those for AK2 and AK3 cDNA were gradually increased. On the other hand, the contents of *AK4* cDNA were first decreased to one-third, then gradually returned to the same level as that of the undifferentiated cells. The sources of the templates used for RT-PCR were as follows: lane 1, AtT20 cells ; lane 2, untreated P19 cells ; lane 3, treated P19 (day 2); lane 4, treated P19 (day 5); lane 5, treated P19 (day 6); lane 6, treated P19 (day 7); lane 7, treated P19 (day 8); lane 8, treated P19 (day 9); lane 9, control plasmid, which contained each cDNA recloned in the pGEM-Teasy vector.

had increased by over 10-fold at day 10. The increase in the level of each *AK* mRNA paralleled the evel of each protein. These results indicated that the expression of *AK* isozyme genes is differentially regulated primarily at the transcriptional level in neuronal differentiation of P19 cells.

Discussion

In order to understand the physiological function of AK isozymes, it is important to use a widely applied genetic system such as that using transgenic mice and knockout mice. As the first step toward development of a model system, we cloned cDNAs encoding mouse AK

isozymes and evaluated their mRNA expression in various tissues as well as in P19 mouse embryonal carcinoma cells.

During the course of isolation of cDNAs for *AK1, AK2,* and *AK3,* we found a new *AK* clone that is homologous to human *AK3.* Independently, Yoneda et al. reported the same cDNA clone from rat and mouse, named *AK4,* and demonstrated that it is expressed in the central nervous system.[13] Although Yoneda et al. documented that AK4 is highly expressed in brain and liver, we found that the level of *AK4* mRNA was much higher in kidney than in brain or liver (Fig. 1A, Table 2). We cloned mouse genomic DNAs encoding AK3 and AK4 as independent clones, indicating that AK3 and AK4 are encoded by distinct genes on the mouse genome. The sequence encoding human AK3 was originally identified as a processed pseudogene by Xu et al., who later isolated the *AK3* cDNA from a human brain cDNA library[17]. The clone had three base substitutions from the pseudogene in the coding region, but the deduced amino acid sequence was identical to that from the pseudogene. Taking these findings into account, the human *AK3* clone isolated by Xu et al.[17] should now be called the *AK4* clone. We have recently isolated an authentic human AK3 cDNA that is homologous to rat and bovine *AK3* cDNA. Northern blot analysis with human tissues revealed that *AK3* is almost uniformly expressed in many tissues, while *AK4* is expressed much more highly in kidney than in liver, heart, or brain (T. Noma, et al., unpublished results). Therefore, constitutive expression of AK3 and tissue-specific expression of AK4 may be a common feature of the mammalian *AK4* isozyme genes.

Our analysis suggested that mouse AK4 is localized in the mitochondrial matrix. The structure of AK4 indicated that there is no cleavable signal sequence, but its N-terminal region can form an amphipathic-helical wheel consisting of several positive charged and hydrophobic residues, a characteristic of the potential mitochondrial targeting signal.[35,41,42] The finding that AK4 protein was purified with blue sepharose column and phosphocellulose column suggests that the AK4 protein may contain a nucleotide-binding domain as do other AK isozymes. However, with the recombinant human AK4, we were not able to detect GTP : AMP phosphotransferase or ATP : AMP phosphotransferase activity (data

Table 3. RNA copy numbers in neuronally differentiated P19 cells

	Copy numbers (fmol/μg RNA)				
	AK1	AK2	AK3	AK4	NeuroD
Day 0	1.4	28.9	0.9	2.0	0.2
Day 2	13.7	26.8	1.8	1.7	13.6
Day 5	37.9	57.1	5.2	0.3	13.2
Day 6	107.0	65.3	3.6	0.8	10.5
Day 7	80.5	98.6	2.2	0.8	8.4
Day 8	29.0	110.1	4.3	1.2	22.0
Day 9	37.9	72.8	5.2	1.8	30.6

Copy numbers of the templates in the RT-PCR samples were calculated using 18S *rRNA* as an internal control, on the assumption that each mouse cell contains the same copy number of 18S *rRNA.* The sources of the templates used for RT-PCR were as follows : Day 0, untreated P19 cells; Day 2, 2 days after RA treatment; Day 5, 5 days after RA treatment ; Day 6, 6 days after RA treatment; Day 7, 7 days after RA treatment; Day 8, 8 days after RA treatment; Day 9, 9 days after RA treatment.Three patterns were classified in the mRNA expression during the RA-induced neuronal differentiation of P19 cells. The first is a strong induction (~hundred fold ; *AK1* and *NeuroD*), the second is a weak enhancement (~several fold ; *AK2* and *AK3*), and the last is a down regulation (*AK4*).

not shown). The reason that we could not detect enzymatic activities in the recombinant AK4 protein remains to be determined.

When knockout mice are generated, proceeding to the heterozygous state in which one allele is knocked out while another is intact is inevitable. In applying the transgenic technique to assess the effect of antisense RNA, evaluation of the cellular mRNA levels should be critical. Therefore, we examined mRNA contents for each AK isozyme per one cell in each tissue by quantitative RT-PCR (Table 2). The estimation is based on the assumption that mRNA in each sample is transcribed at equal efficiency, and PCR reaction equally proceeds to the mRNA templates in each sample. The mRNA contents were calculated by comparing the amounts of the PCR products with control plasmids contaning the corresponding isozyme sequence. Given that it has been documented that a cell contains an average of 40 pg total RNA,[25] we can roughly estimate the maximum copy numbers of each isozyme mRNA in a cell as follows : the copy numbers of *AK1* are 2×10^6/cell in skeletal muscle and testis; those of *AK2* are 1×10^6/cell in liver; those of *AK3* are 2×10^5/cell in heart and pancreas; and those of *AK4* are 4.8×10^4/cell in kidney (Table 2).

P19 cells, from a mouse embryonal carcinoma cell line, are pluripotential cells that can be induced to differentiate into a wide variety of cell types. Using the P19 cells as a model system in vitro for differentiation, we examined the expression patterns of each isozyme. Neuronal differentiation of P19 cells with RA increased the mRNA of each isozyme except AK4 (Fig. 5B, Table 3). AK1 and its regulatory transcription factor, NeuroD, were significantly up-regulated during neuronal differentiation, which is in accord with findings in previous studies.[14,39,40] The mRNA levels of each *AK* as well as of *NeuroD* during RA-induced neuronal differentiation of P19 cells were correlated with the levels of each protein, as previously reported, except for those of AK4, which was first down-regulated then returned to the starting levels during RA-induced neuronal differentiation.

Acknowledgements

I sincerely thank Drs. Atsushi Nakazawa and Takafumi Noma for their advices in completing this study.

References

1) Noda, L.H. : Adenylate Kinase. *The Enzymes* Vol. Ⅷ, part A., Academic Press, Orlando, 1973, pp. 279-305.
2) Khoo, J.C. and Russell, P. J. : Isoenzymes of adenylate kinase in human tissue. *Biochem. Biophys. Acta.*, **268** : 98-101, 1972.
3) Heldt, H.W. and Schwalbach, K. : The participation of GTP-AMP-P transferase in substrate level phosphate transfer of rat liver mitochondria. *Eur. J. Biochem.*, **1** : : 199-206, 1967.
4) Tomasselli, A.G. and Noda, L.H. : Mitochondrial ATP : AMP phosphotransferase from beef heart : purification and properties. *Eur. J. Biochem.*, **103** : : 481-491, 1980.
5) Nakazawa, A., Yamada, M., Tanaka, H., Shahjahan, M. and Tanabe, T. : Gene structures of three vertebrate adenylate kinase isozymes. *Prog. Clin. Biol. Res.*, **344** : : 495-514, 1990.
6) Atkinson, D.E. : Adenylate Control and the Adenylate Energy Charge. *Cellular Energy Metabolism and Its Regulation,* Academic Press, Orlando, 1977, pp. 85-107.
7) Dzeja, P.P. and Terzic, A. : Phosphotransfer reactions in the regulation of ATP-sensitive K^+ channels. *FASEB J.*, **12** : : 523-529, 1998.
8) Zeleznikar, R.J., Dzeja, P.P. and Goldberg, N.D. : Adenylate kinase-catalyzed phosphoryl transfer couples ATP utilization with its generation by glycolysis in intact muscle. *J. Biol. Chem.*, **270** : : 7311-7319, 1995.
9) Cronan, J.E. and Godson, G.N. : Mutants of *Escherichia coli* with temperature-sensitive lesions in membrane phospholipid synthesis : genetic analysis of glycerol -3-phosphate acyltransferase mutants. *Mol. Gen. Genet.*, **116** : : 199-210, 1972.
10) Konrad, M. : Molecular analysis of the essential gene for adenylate kinase from

the fission yeast *Schizosaccharomyces pombe. J. Biol. Chem.,* **268** : 11326-11334, 1993.

11) Qualtieri, A., Pedace, V., Bisconte, M.G., Bria, M., Gulino, B., Andreoli, V. and Brancati, C.: Severe erythrocyte adenylate kinase deficiency due to homozygous A → G substitution at codon 164 of human AK1 gene associated with chronic haemolytic anaemia. *Br. J. Haematol.,* **99** : 770-776 , 1997.
12) Gordon, J.W. : Production of transgenic mice. *Methods Enzymol.,* **225** : 747-771, 1993.
13) Yoneda, T., Sato, M., Maeda, M. and Takagi, H. : Identification of a novel adenylate kinase system in the brain: cloning of the fourth adenylate kinase. *Mol. Brain Res.,* **62** : 187-195, 1998.
14) Noma, T., Yoon, Y-S. and Nakazawa, A. : Overexpression of NeuroD in PC12 cells alters morphology and enhances expression of the adenylate kinase isozyme 1 gene. *Mol. Brain Res.,* **67** : 53-63, 1999.
15) Matsuura, S., Igarashi, M., Tanizawa, Y., Yamada, M., Kishi, F., Kajii, T., Fujii, H., Miwa, S., Sakurai, M. and Nakazawa, A. : Human adenylate kinase deficiency associated with hemolytic anemia. A single base substitution affecting solubility and catalytic activity of the cytosolic adenylate kinase. *J. Biol.Chem.,* **264** : 10148-10155, 1989.
16) Tanabe, T., Yamada, M., Noma, T., Kajii, T. and Nakazawa, A.: Tissue-specific and developmentally regulated expression of the genes encoding adenylate kinase isozymes. *J. Biochem.,* **113** : 200-207, 1993.
17) Xu, G., O'Connell, P., Stevens, J. and White, R. : Characterization of human adenylate kinase 3 (AK3) cDNA and mapping of the AK3 pseudogene to an intron of the NF1 gene. *Genomics,* **13** : 37-542, 1992.
18) Noma, T., Song, S., Yoon, Y-S., Tanaka, S. and Nakazawa, A. : cDNA cloning and tissue-specific expression of the gene encoding human adenylate kinase isozyme 2. *Biochem. Biophys. Acta.,* **1395** : 34-39, 1998.
19) Iizuka, N., Oka, M., Noma, T., Nakazawa, A., Hirose, K. and Suzuki, T. : NM23-H1 and NM23-H2 messenger RNA abundance in human hepatocellular *carcinoma. Cancer Res.,* **55** : 652-657, 1995.
20) Yoon, Y-S., Noma, T., Yamashiro, Y., Ito, H. and Nakazawa, A. : Molecular cloning and characterization of the gene encoding human NeuroD. *Neurosci. Lett.,* **251** : 17-20, 1998.
21) Sanger, F., Nicklen, S. and Couslon, A.R. : DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.,* **74** : 5463-5467, 1977.
22) Chomczynski, P. and Sacchi, N. : Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162** : 156-159, 1987.
23) Tso, J.Y., Sun, X.H., Kao, T.H., Reece, K.S. and Wu, R. : Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.,* **13** : 2485-2502, 1985.
24) Torczynski, R.M., Bollon, A.P. and Fuke, M. : The complete nucleotide sequence of the rat 18S ribosomal RNA gene and comparison with the respective yeast and frog genes. *Nucleic Acids Res.,* **11** : 4879-4890, 1983.
25) Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. : *Molecular Biology of the Cell,* 3rd ed., Garland Publishing, New York, 1994, pp. 90.
26) Laemmli, U.K. : Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227** : 680-685, 1970.
27) Kishi, F. and Nakazawa, A.: Development of myokinase mRNA during embryogenesis of the chick. *J. Biochem. (Tokyo),* **98** : 1091-1095, 1985.
28) Nobumoto, M., Yamada, M., Saeki, S., Song, S., Inouye, S. and Nakazawa, A. : Overproduction and simple purification of mitochondrial adenylte kinase isozyms. *J. Biochem. Mol. Biol. Biophys.,* **2** : 13-20, 1998.
29) Nobumoto, M., Yamada, M., Song, S., Inouye, S. and Nakazawa, A.: Mechanism of mitochondrial import of adenylate kinase isozymes. *J. Biochem.,* **123** : 128-135, 1998.

30) Murakami, K., Amaya, Y., Takiguchi, M., Ebina, Y. and Mori, M. : Reconstitution of mitochondrial protein transport with purified ornithine carbamoyltransferase precursor expressed in *Escherichia coli. J. Biol. Chem.,* **263** : 18437-18442, 1988.
31) Chance, B. and Williams, G. R.:Respiratory enzymes in oxidative phosphorylation. *J. Biol. Chem.,* **217** : 383-393, 1955.
32) Glick, B.S., Brandt, A., Cunningham, K., Muller, S., Hallberg, R.L. and Schatz, G. : Cytochromes c1 and b2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. *Cell,* **69** : 809-822, 1992.
33) Rudnicki, M.A. and McBurney, M.W. : Teratocarcinoma and Embryonic Stem Cells. In Robertson, E.J., (ed.), *A Practical Approach,* IRL press, Washington, D.C., 1987, pp.19-50.
34) Kishi, F., Tanizawa, Y. and Nakazawa, A. : Isolation and characterization of two types of cDNA for mitochondrial adenylate kinase and their expression in *Escherichia coli. J. Biol. Chem.,* **262** : 11785-11789, 1987.
35) Yamada. M., Shahjahan, M., Tanabe, T., Kishi, F. and Nakazawa, A. : Cloning and characterization of cDNA for mitochondrial GTP : AMP phosphotransferase of bovine liver. *J. Biol. Chem.,* **264** : 19192-19199, 1989.
36) Schlauderer, G.J. and Schulz, G.E. : The structure of bovine mitochondrial adenylate kinase: comparison with isoenzymes in other compartments. *Protein Sci.,* **5** : 434-441 , 1996.
37) Vonrhein, C., Schlauderer, G.J. and Schulz, G.E. : Movie of the structural changes during a catalytic cycle of nucleoside monophosphate kinases. *Structure,* **3** : 483-490, 1995.
38) Kohler, C., Gahm, A., Noma, T., Nakazawa, A., Orrenius, S. and Zhivotovsky,B. : Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis. *FEBS Lett.,* **447** : 10-12, 1999.
39) Inouye, S., Seo, M., Yamada, Y. and Nakazawa, A. : Increase of adenylate kinase isozyme 1 protein during neuronal differentiation in mouse embryonal carcinoma P19 cells and in rat brain primary cultured cells. *J. Neurochem.,* **71** : 125-133, 1998.
40) Noma, T., Yoon,Y-S., Yamashiro, Y., Fujisawa, K. and Nakazawa, A. : Regulation of NeuroD expression by activation of the protein kinase-C pathway in Y79 human retinoblastoma cells. *Neurosci. Lett.,* **272** : 45-48, 1999.
41) Bandlow, W., Strobel, G. and Schricker, R. : Influence of N-terminal sequence variation on the sorting of major adenylate kinase to the mitochondrial intermembrance space in yeast. *Biochem. J.,* **329** : 359-367, 1998.
42) Schricker, R., Magdolen, V., Strobel, G., Bogengruber, E., Breitebach, M. and Bandlow, W.:Strain-dependent occurrence of functional GTP:AMP phosphotransferase (AK3) in *Saccharomyces cerevisiae. J. Biol. Chem.,* **270** : 31103-31110, 1995.
43) Bounassisi, V., Sato, G. and Cohen, A.L. : Hormone-producing cultures of adrenal and pituitary tumor origin. *Proc. Natl. Acad. Sci. USA.,* **48** : 1184-1190, 1962.