J. Biochem. 123, 47-54 (1998)

Cloning and Expression of Human Adenylate Kinase 2 Isozymes: Differential Expression of Adenylate Kinase 1 and 2 in Human Muscle Tissues[1]

Younghee Lee,* Jae Wha Kim,* Sang Min Lee,† Hyo Joon Kim,† Kwang Soo Lee,‡ Chankyu Park,§ and In Seong Choe*,[2]

*Molecular and Cellular Biology Research Division, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea; †Department of Biochemistry, Hanyang University, Ansan 425-791, Korea; ‡Department of Surgery, Hanyang University Medical School, Seoul 133-792, Korea; and §Department of Biological Sciences, KAIST, Taejon 305-701, Korea

Received for publication, June 23, 1997

A cDNA clone coding for adenylate kinase 2B was isolated from fetal liver, and the expression of AK2 was investigated in human tissues. The ORF in the cDNA clone for human AK2B predicted a protein comprising 232 amino acids (25.6 kDa). The features of AK2A and AK2B sequences in human were the same as those in the bovine system. Each of the recombinant proteins, AK2A and AK2B, was expressed in *Escherichia coli* cells, and the purified recombinant proteins were enzymatically active. The distribution of AK2 transcripts in various human tissues was examined by Northern analysis. Unlike in the bovine system, it was found that the AK2A transcript was the major form of AK2 mRNA species in all human tissues. The transcripts of AK2 isozymes were relatively abundant in heart, liver, and also in skeletal muscle, where the expression level of AK2 was known to be low. Western blot analysis of AK isozymes in human heart and skeletal muscle revealed that AK2 protein was found only in heart, whereas AK1 was detected in both tissues. These tissue-specific expressions of the AK isozymes in human might suggest the presence of organ-specific regulation of the AK2 gene including a post-transcriptional control in skeletal muscle.

Key words: AK activity, human AK2 isozymes, post-transcriptional regulation, recombinant protein, tissue specificity.

Adenylate kinase (AK, ATP:AMP phosphotransferase, EC 2.7.4.3.) is a key enzyme involved in energy metabolism of procaryotic and eucaryotic cells (*1*). The enzyme has been extensively studied in enzymology, human genetics, protein chemistry, crystallography, and molecular spectroscopy.

In vertebrates, three isozymes (AK1, AK2, and AK3) have been identified. AK1 is present in the cytosol of skeletal muscle, brain and erythrocytes, while AK2 is localized in the mitochondrial intermembrane space of liver, kidney, spleen, and heart (*2*). AK3, also called GTP:AMP phosphotransferase, exists in the mitochondrial matrix of liver and heart (*3*). All three isozymes are encoded in the nuclear genome and synthesized in cytoplasm, and AK2 and AK3 are transported into mitochondria. The expression of these isozymes is tissue-specific and developmentally regulated (*4*).

The presence of two isozymes of AK2 (AK2A and AK2B) was reported in the bovine system. AK2A and AK2B differ in amino acid sequence at the C-terminal portion as well as in tissue distribution (*5, 6*). The AK2A enzyme protein is composed of 241 amino acids, and the AK2B protein of 234 amino acids. Both proteins share a common sequence from the 1st to the 233rd amino acid residue. Eight amino acids at the carboxy terminus of AK2A (Cys Lys Asp Leu Val Met Phe Ile) are replaced by one amino acid, Ser, in AK2B (*5*). The bovine AK2 gene consists of seven exons and six introns, and it has been suggested that an alternative splicing mechanism of the gene generates two types of mRNAs encoding AK2A and AK2B isozymes (*6*).

In human, cDNAs for AK1 (*7*) and AK3 (*8*) have been cloned and their nucleotide sequences have been determined. Except for their enzymatic activities, little has been found about the physiological functions of AK isozymes. It has been reported that congenital chronic non-spherocytic haemolytic anemia was associated with AK1 deficiency in

[1] This work was supported by a grant from the Ministry of Science and Technology (HAN Project HS050M). The nucleotide sequence data reported in this paper will appear in the EMBL, DDBJ, and GenBank nucleotide sequence databases with the accession number U39945 (human adenylate kinase 2A) and U54645 (human adenylate kinase 2B).

[2] To whom correspondence should be addressed at: P.O. Box 115, Yusung, Taejon, 305-600, Korea. Tel: +82-42-860-4180, Fax: +82-42-860-4593, E-mail: choemcbg@kribb4680.kribb.re.kr

Abbreviations: AK, adenylate kinase; AK1, cytosolic ATP:AMP phosphotransferase or adenylate kinase isozyme 1; AK2, mitochondrial ATP:AMP phosphotransferase or adenylate kinase isozyme 2; AK3, mitochondrial GTP:AMP phosphotransferase or adenylate kinase isozyme 3; ORF, open reading frame; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate-buffered saline; GST, glutathione-S-transferase.

erythrocytes, and that AK1 deficiency was transmitted by an autosomal recessive gene (*7, 9*).

Recently, we have cloned and reported the cDNA sequence for AK2A isozyme isolated from human fetal liver (*10*). Here, we have cloned the cDNA coding for AK2B, another AK2 isozyme, and investigated the expression of AK isozymes in human heart and skeletal muscle. Interestingly, the AK2 mRNA level did not parallel the protein level in skeletal muscle, which suggests the presence of post-transcriptional control in the expression of AK2 gene.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, Klenow enzyme, T4 DNA ligase, and calf intestine phosphatase were purchased from Kosco. Recombinant *Taq* DNA polymerase was from Takara. Oligonucleotides were purchased from Bioneer or NEB. [α-^{35}S]dATP and [α-^{32}P]dCTP were obtained from Amersham. Nucleoside mono- and triphosphate, lactate dehydrogenase (rabbit muscle), pyruvate kinase, phosphoenolpyruvate, isopropyl-β-D-thiogalactopyranoside (IPTG), and reduced glutathione were from Sigma. Glutathione Sepharose 4B was from Pharmacia LKB, and factor Xa was from NEB. All other reagents were of the highest purity commercially available.

Bacterial Strains, Plasmids, and Media—Escherichia coli XL1-Blue MRF′ [*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* {*F′ proAB lacIqZΔM15 Tn10(Tet^r)*}] cells were used as host. Plasmids pBluescript SK(−) obtained from Stratagene and pCRII from Invitrogen were used for routine cloning and sequencing. Plasmid pGEX-3X was used for gene expression. Bacteria were grown in Luria-Bertani broth (LB broth; 1% tryptone, 0.5% NaCl, 0.5% yeast extract) containing 100 μg/ml ampicillin.

Screening of Full Length AK2B cDNA Clones—λDR2 human fetal liver cDNA library (Clontech) was screened with human AK2A cDNA sequence obtained by rapid amplification of cDNA ends (RACE) as reported previously (*10*). The probe DNA was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by random priming (*11*). Unincorporated isotope was eliminated by spun column chromatography. Specific activity was 2.2×10^8 cpm/μg. Plaque hybridization was performed with the labeled cDNA probes at 42°C overnight in the hybridization solution (50% formamide, 5× Denhart solution, 5× SSC, 0.5% SDS, 0.1% pyrophosphate, 10 mM EDTA, 100 μg/ml denatured salmon sperm DNA). The membranes were washed twice with 2× SSC containing 0.1% SDS at room temperature for 10 min and twice with 0.2× SSC containing 0.1% SDS at 55 to 60°C for 20 min. After autoradiography, the plaques positive for hybridization were purified and rescreened. Recombinant pDR2 plasmids were excised *in vivo* by the method recommended by the manufacturer.

DNA Sequencing—Plasmid DNAs were purified by the alkaline method (*12*) and affinity chromatography using Wizard minipreps DNA purification system (Promega). DNA sequences were determined by the chain termination method (*13*) using Sequenase Kit Version 2.0 (USB) and [α-^{35}S]dATP.

Strategy for the Construction of Expression Vectors—Plasmid pGEX-3X (*14*) was used to express the AK2 isozymes as GST fusion proteins. The DNA fragment covering the entire ORF for AK2A was obtained by PCR method with the cDNA clone pDR5-1 as a template. ADK5 primer (5′-GAAGATCTCCATGGCTCCCAGCGTGC-3′) and ADK3 primer (5′-GAAGATCTTAGATAAACATAACCA-AGTCTT-3′), each respectively encoding the 5′ and the 3′-end region of AK2A gene (*10*), were used as amplimers. The resulting 730-bp DNA fragment was digested with *Bgl*II and ligated with the *Bam*HI-digested pGEX-3X. The resulting recombinant plasmid was named pGEX-AK2A. Using the cDNA clone pDR7-1 as a template, PCR was performed to obtain DNA fragments containing the ORF sequence for AK2B with or without the 3′-untranslated region. To amplify a 710-bp DNA fragment covering only the ORF sequence for AK2B, ADK5 primer was used as an amplimer in combination with AK2B-3 primer (5′-GAAG-ATCTGCAGCTAGGATGTGGCTTTGG-3′) containing the 3′-end region of AK2B ORF sequence and newly inserted *Bgl*II enzyme site. On the other hand, DR21 primer (5′-CA-GTGCCAAGCTTGCATGCCT-3′) complementary to the downstream sequence of the vector cloning site was used as an amplimer along with ADK5 primer to get a 2,100-bp DNA fragment covering not only the AK2B ORF sequence but also the following 3′-end untranslated region. The 710-bp fragment was digested by *Bgl*II and ligated with *Bam*HI-digested pGEX-3X to construct the plasmid pGEX-AK2B. The 2,100-bp DNA fragment was digested by *Bgl*II and *Hinc*II, and ligated with *Bam*HI, *Sma*I-cut pGEX-3X. The resulting plasmid was named pGEX-AK2B-1.

Production and Purification of Recombinant GST-AK2 Fusion Proteins from E. coli—Overnight cultures of *E. coli* transformed with parental or recombinant pGEX-3X plasmids were diluted 50 times with fresh medium, grown to the absorbance of 0.6 at 600 nm, and IPTG was added to a final concentration of 0.5 mM. Five hours after the induction, bacterial cells were harvested by centrifugation and resuspended in PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) containing 0.1 mM PMSF and 100 μg/ml of lysozyme. Cell lysates were prepared by ultrasonication and soluble fractions were separated by centrifugation at 12,000 rpm using Sorvall SS34 rotor. To each 5 mg of GST fusion protein in PBS containing 1% Triton X-100, 2 ml of Glutathione Sepharose 4B-bead suspension (50% v/v) equilibrated with PBS was added and incubated at 4°C on an Orbitron Rotator for 30 min. The beads were washed three times with ice-cold PBS by repeated centrifugation. The GST fusion protein was released from the beads by 1 ml of the elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) per ml bed volume of Glutathione Sepharose 4B.

Enzyme Activity Assay—Adenylate kinase activity was measured spectrophotometrically by the coupled enzyme assay method (*15*). Reaction mixture was composed of 75 mM triethanolamine (pH 7.6), 1.2 mM AMP, 1.0 mM ATP, 1 mM $MgSO_4$, 120 mM KCl, 0.2 mM NADH, 0.3 mM phosphoenolpyruvate, 16.5 units of lactate dehydrogenase, and 6 units of pyruvate kinase. After preincubation at 25°C for about 10 min, enzyme sample was added to the reaction mixture and the reaction was carried out at 25°C measuring the decrease in absorbance at 340 nm. The extinction coefficient of 6,220 was used to convert the absorbance change to the molar concentration of the product formed. One unit of enzyme activity was defined as 1 μmol of ADP produced per min at 25°C. Protein concentration was

determined by the method of Bradford (*16*).

Chromosomal Localization—Somatic cell hybrid blot (Oncor) was used to localize the AK2A cDNA clone on the human chromosome by Southern hybridization (*12*). The membrane was hybridized with the ^{32}P-labeled cDNA probe and analyzed as described above.

Northern Blot Analysis—As the probe for the analysis of mRNAs for both AK2 isozymes, a 730-bp cDNA fragment covering the entire ORF of AK2A, which contained the common nucleotide sequence shared by AK2A and AK2B transcripts, was obtained as described above. For the detection of AK2A-specific mRNA, a 180-bp fragment coding for the C-terminal 14 amino acids and the following 3′ end untranslated region of AK2A was amplified by PCR using the cDNA clone pDR5-1 as a template. ADK103 primer (5′-GCCTTCTCCAAAGCCACAT-3′) including the nucleotide sequence immediately before the splicing junction and ADK104 primer (5′-TTTACATCAAGCAAGTGC-3′) complementary to the 3′-end region of AK2A cDNA sequence were used as amplimers. For the measurement of AK2B-specific mRNA, a 270-bp fragment including the codon for the C-terminal end serine residue and a part of the following 3′-end untranslated region specific only to AK2B was amplified by PCR using the cDNA clone pDR7-1 as a template. ADK103 primer described above, and ADK105 primer (5′-CCCTCCAAGCATGGTGT-3′) complementary to the sequence located 230-bp downstream of ADK103 primer along the nucleotide sequence of AK2B cDNA were used as amplimers. The probe used for the mRNA of human AK1 isozyme was a 500-bp *Eco*RI–*Pvu*II restriction fragment from partial cDNA clone encoding the C-terminal region (81–194) of AK1 obtained during the analysis of fetal liver-related genes by random sequencing (*17*). All the DNA probes were ^{32}P-labeled. The specific activities of the labeled probes were about $1\text{–}2\times10^9$ cpm/μg. A Northern blot membrane containing a total RNA preparation (*18*) isolated from human fetal liver and Multiple Tissue Northern (MTN) blot membrane containing 2 μg each of poly(A)$^+$-RNAs from different human tissues (Clontech) were used. The membranes were hybrid-

```
   1  GCGAACTGGTGGCAGTGAGAGACTTCGGCGGACATGGCTCCCAGCGTGCCAGCGGCAGAACCCGAGTATCCTAAAGGCATCCGGGCCGTG    90
   1                                    M  A  P  S  V  P  A  A  E  P  E  Y  P  K  G  I  R  A  V     19

  91  CTGCTGGGGCCTCCCGGGGCCGGTAAAGGGACCCAGGCACCCAGATTGGCTGAAAACTTCTGTGTCTGCCATTTAGCTACTGGGGACATG   180
  20   L  L  G  P  P  G  A  G  K  G  T  Q  A  P  R  L  A  E  N  F  C  V  C  H  L  A  T  G  D  M     49

 181  CTGAGGGCCATGGTGGCTTCTGGCTCAGAGCTAGGAAAAAAGCTGAAGGCAACTATGGATGCTGGGAAACTGGTGAGTGATGAAATGGTA   270
  50   L  R  A  M  V  A  S  G  S  E  L  G  K  K  L  K  A  T  M  D  A  G  K  L  V  S  D  E  M  V     79

 271  GTGGAGCTCATTGAGAAGAATTTGGAGACCCCCTTGTGCAAAAATGGTTTTCTTCTGGATGGCTTCCCTCGGACTGTGAGGCAGGCAGAA   360
  80   V  E  L  I  E  K  N  L  E  T  P  L  C  K  N  G  F  L  L  D  G  F  P  R  T  V  R  Q  A  E    109

 361  ATGCTCGATGACCTCATGGAGAAGAGGAAAGAGAAGCTTGATTCTGTGATTGAATTCAGCATCCCAGACTCTCTGCTGATCCGAAGAATC   450
 110   M  L  D  D  L  M  E  K  R  K  E  K  L  D  S  V  I  E  F  S  I  P  D  S  L  L  I  R  R  I    139

 451  ACAGGAAGGCTGATTCACCCCAAGAGTGGCCGTTCCTACCACGAGGAGTTCAACCCTCCAAAAGAGCCCATGAAAGATGACATCACCGGG   540
 140   T  G  R  L  I  H  P  K  S  G  R  S  Y  H  E  E  F  N  P  P  K  E  P  M  K  D  D  I  T  G    169

 541  GAACCCTTGATCCGTCGATCAGATGATAATGAAAAGGCCTTGAAAATCCGCCTGCAAGCCTACCACACTCAAACCACCCCACTCATAGAG   630
 170   E  P  L  I  R  R  S  D  D  N  E  K  A  L  K  I  R  L  Q  A  Y  H  T  Q  T  T  P  L  I  E    199

 631  TACTACAGGAAACGGGGGATCCACTCCGCCATCGATGCATCCCAGACCCCCGATGTCGTGTTCGCAAGCATCCTAGCAGCCTTCTCCAAA   720
 200   Y  Y  R  K  R  G  I  H  S  A  I  D  A  S  Q  T  P  D  V  V  F  A  S  I  L  A  A  F  S  K    229


 721  GCCACATGTAAAGACTTGGTTATGTTTATCTAATGTTGGGTCCAAGAAGGAATTTCTTTCCATCCCTGTGAGGCAATGGGTGGGAATGAT   810   AK2A
 230   A  T  C  K  D  L  V  M  F  I  *                                                            239

       |
 728  CCTAGTATCAGAAGGCCAGGCGAGACTGCAACACTGCTCATCACCCCGCGGCGTGATCCCTGCTCTTAGGTGCTGGGCAGAGG   810   AK2B
      S  *                                                                                    232


 811  AGGACAGGCAAAGAGAAGCTTCCTCAGGCTAGCAAAAATATCATTTGATGTATTGATTAAAAAAGCACTTGCTTGATGT              889   AK2A

 811  GGAAGGGTGGTCAGGGTGAGGATGGTGAGGGAGGGCTGGTGAGGGGCTCAGAGGAATACTTGGAACAACAGCAGTGTTATTGTAGTGTGG   900   AK2B
 901  CAGTTTCTTTTATACATAGGTGAGAGTTTTTAAAGTGTAAGGGAAAAATTAATTTTTTAAAAAACACCATGCTTGGAGGGTGGGGGTAGA   990
 991  AATAGACACAATATTATTTCTAAGGAATCGGGTTTTCATTTACTCTGGACTGGTGAAAATATTTTTTAAAGCCAGTGCTCTAAGACCTCA  1080
1081  GCTTTTATCTCAGAACCCCATGGGTTCCAGACCAAGAGTACAGGAAATCAAATTGTTGTCCTGTCTGTCTATAGCTTGGAACAGGGAGCT  1170
1171  TTGATTACTGACTCCGGTTCCACACACTGTAAGATCAAAAAACCATCTCCACATTTGAAAGAGATGTAAGGTGTATTCATAGGGATGGTG  1260
1261  GCTCAACAAATCAAGCAAACTGGAATCAAGGGGAGGGGGAAGGGAATGAAATGGAAAGGGAGGCTGATTCCCTTCCCCTGACTTACCACT  1350
1351  AATTTACTAGGCTACCTACTTTCATGAGTAACCTCTCACAGCTACCCAGCACATGCCACAATCCTATCGTCTTGCCTTCTTTTATCTGCA  1440
1441  CTGTGTGAAGGGACTCTTTTAAATAAATGAGCAAGTGTCCTAAGCTATGTCATCCAAAGATTGTCCTTTCCATTCTCAAATCCTGTGACT  1530
1531  GGGATCACTCAACAGCACTGTGATGTATTATTTTCAATGAGGTGCCTTTCTTAACTGACCAAATGCTGCCTTGTTTGGCCCCTAAATCAA  1620
1621  TAAAATATGTTAAAATTTGTATCCCCTGTTGTGGCATTTTTTTTAGATAATCTAAGCTAGAAAAATGACATTGAATTCTGGACCTGGCTG  1710
1711  GAAGGAAAAGAAGCCCTTTCTTGTCGCTGGCAGCTGTGTGGTAGGAGGTCCAAGTATGTGCATATGAGATAAGCCTGCAACCTCTTGACC  1800
1801  TTCAGCTCCTATGCAGGCTTCTCTTGAGCCCAGAGACAAGGCAGCTTGGTCTAGTGGAGATAGCACTGTGCTTGGAGTTCAGGGGACCTA  1890
1891  GGACAAATCCCAGCCAGTTAGTTATTCACTGTGCTCCTGTTTCCTCAGCTGAAAAAGGAAGTTGGTTATGCCACCTTCTTGGCCTTAATG  1980
1981  GCATTAAATGAAATTTATAGGAAGAAGGTTTTTGCTCAGTACCTGGCATGCAACAGACATTGGATAAATGTTAGTTGGATCCAGATATAC  2070
2071  ACAGAAAGATATCTGCTTCCTGCCAGGCTGGATAACTGTTGAATGACACTTGTCCATAGTCTAGA                            2135
```

Fig. 1. **Nucleotide and deduced amino acid sequences of human AK2A (7) and AK2B (this study).** The numbers at the right of each line denote the positions of the last nucleotide and amino acid. In-frame stop codons defining the 5′ and 3′ ends of the reading frame are indicated by an underline and an asterisk, respectively. The polyadenylation signal (*ATTAAA* or *AATAAA*) and the potential consensus motif (*ATTTA*) for mRNA instability are also indicated.

ized and analyzed as described above. To remove the hybridized probes, the filters were boiled in 0.5% SDS for 10 min. The stripped filters were reprobed for different mRNA species. The intactness and the amount of the loaded RNA were verified by ethidium bromide staining of 28S and 18S rRNAs or by probing β-actin mRNA as a control.

Antibody Preparation and Western Blot Analysis—Polyclonal mouse antibodies against human AK1 and AK2 were prepared using recombinant AK1 protein (*15*) and AK2 protein (this study), respectively. Human skeletal muscle and heart tissues were homogenized in 50 mM Tris-HCl (pH 7.4) containing 0.1 mM DTT and 0.2 mM PMSF, and centrifuged at $600 \times g$ for 10 min. The resulting supernatants were used directly for protein analysis. SDS-PAGE was performed using 100 μg of proteins per lane, and after electrophoresis, the protein bands were blotted onto a PVDF membrane (Bio-Rad). Partially purified anti-AK1 and anti-AK2 antibodies were bound to the corresponding proteins and these antigen-antibody complexes were visualized by ECL kit (Amersham). As a control, 3 μg of purified recombinant protein AK1 (*15*) or AK2 was used. Intact AK2 protein was prepared by partial digestion of GST-AK2A with factor Xa, SDS-PAGE, and electroelution.

RESULTS AND DISCUSSIONS

Cloning of Human AK2B cDNA—The λDR2 cDNA library constructed from human fetal liver was screened by plaque hybridization with human AK2A cDNA probe, and five positive clones were selected from approximately 9.6×10^4 plaques. Recombinant pDR2 plasmids were excised *in vivo* from λ DNAs, then subjected to nucleotide sequencing and database search. Three of them, pDR2-1, pDR5-1, and pDR4-1, contained cDNA inserts encoding human AK2A gene (*10*). The remaining clones, pDR1-1 and pDR7-1, contained a 2.1-kb cDNA insert coding for the putative human AK2B gene.

The Nucleotide Sequence of Human AK2B Gene and Its Deduced Amino Acid Sequence—The nucleotide and amino acid sequences of human AK2B are shown in Fig. 1 along with those of human AK2A. The DNA sequence had an ORF of 696 nucleotides which coded for a polypeptide of 232 amino acids starting from the first ATG codon found in the crucial context for efficient translation of eukaryotic mRNA [CC(A/G)CCAUGG: *19*]. The coding region showed 91 and 83% homology with the nucleotide sequences of bovine AK2B and rat AK2, respectively. Three stop codons (UGG or UGA) in-frame were detected upstream of the initiation codon, and three poly-A tail addition signals (AATAAA or ATTAAA) were recognized in the 3′-untranslated region. Three copies of an ATTTA motif, which has been shown to be associated with the susceptibility of mRNA to degradation (*20*), were also located in the 3′-untranslated region. The presence of these sequence motifs suggested that the AK2B mRNA has a short half-life *in vivo*. The predicted protein with the molecular mass of 25,600 Da exhibited 97% (95% identity and 2% favored substitution) and 95% (93% identity and 2% favored substitution) homology with the amino acid sequences of bovine AK2B and rat AK2, respectively. The amino acid sequence of human AK2B was different from that of human AK2A only in the C-terminal region. The same features have been reported in the bovine system (*5*).

Hamada's group have purified and studied the physicochemical properties of AK enzyme proteins from mitochondrial preparations of normal human liver (*21*) and from whole liver tissue (*22*). After comparing their properties, they concluded that the liver-type AK was a mitochondrial type AK, AK2. As shown in Table I, the amino acid compositions of AK2A and AK2B predicted from the cDNA clones were closely related to those of AK2 proteins and clearly different from those of AK1 protein.

Purification of Recombinant Proteins and Enzyme Activity Assay—To express the human AK2 isozyme proteins in *E. coli* cells, the cDNA sequences were subcloned under the control of *tac* promoter on pGEX-3X plasmid vector, which could direct the expression of the cloned genes as fusion proteins with GST (Fig. 2). The recombinant strains harboring the parental or the recombinant pGEX plasmids were cultured in LB broth, and the expression of the cloned genes was induced by the addition of 0.5 mM IPTG. Analysis of the protein profiles by SDS-polyacrylamide gel electrophoresis (Fig. 3) showed the expression of novel proteins with molecular mass of approximately 53 kDa, as predicted. GST control protein and GST-AK2 fusion proteins were isolated by one-step affinity purification procedure using Glutathione-Sepharose beads. An attempt to separate the AK2 enzyme proteins from the GST fusion proteins failed due to the internal cleavage of the enzyme proteins (data not shown). This internal cleavage might be caused by the similarity of the amino acid sequence of the AK2 enzyme proteins, (139)Ile-Thr-Gly-Arg(142), to that of the specific cleavage site of factor Xa [Ile-Glu/Asp-Gly-Arg ↓ : *23*]. The enzymatic activities of the human AK2 isozymes were determined using the GST-enzyme fusion proteins. Specific activities of GST-AK2A and GST-AK2B were about 30 units/mg protein (Fig. 4), suggesting no prominent difference between the two isozymes in activity.

TABLE I. **Amino acid compositions of human AK2 and AK1.**

Amino acid	AK2			AK1
	Protein[a]	Clone AK2A	Clone AK2B	Protein[b]
Gly	16	15	15	19
Ala	19	21	21	8
Val	10	12	11	17
Leu	23	25	24	18
Ile	12	15	14	9
Ser	14	14	15	11
Thr	12	12	12	14
Cys	4	4	3	2
Met	6	9	8	5
Asx[c]	18	20	19	12
Glx[d]	23	23	23	27
Arg	13	15	15	13
Lys	18	19	18	19
His	4	5	5	2
Phe	7	8	7	5
Tyr	5	5	5	7
Trp	—	—	—	—
Pro	15	17	17	6
Expected total residues	220	239	232	194
Expected M_r	24,600	26,500	25,600	21,700

[a]Kuby *et al.* (*21*). [b]Matsuura *et al.* (*7*). [c]Asp and Asn. [d]Glu and Gln. Values are expressed as the number of the corresponding residues.

However, differences in physiological function *in vivo* could not be excluded. From the above results, we concluded that the cloned cDNA sequences encode functional human AK2 isozymes.

Chromosomal Localization of Human AK2—Southern blot analysis was done using a membrane on which the genomic DNA samples prepared from different human/rodent somatic cell hybrid cell lines and the control genomic DNAs from human, hamster and mouse had been blotted. Human AK2-specific signals were detected not only in the cell line carrying chromosome 1, on which the human AK2 gene has been localized by cytogenetic studies (*24, 25*), but also in the cell line carrying chromosome 2. Our result suggests the presence of multiple loci on human chromosomes for the AK2 gene, as for the human AK3 gene (*8*). AK3 gene was localized on chromosome 9 by previous cytogenetic studies (*26*), but another locus was found in the intron of NF1 on chromosome 17. The AK3 gene on chromosome 17 was a processed one containing the entire ORF sequence without an intron. As the DNA sequence detected by the AK2-specific probe on chromosome 2 was approximately 1.0-1.5 kb long, it also could be presumed that the sequence was a processed pseudogene.

mRNA Analysis—Northern blot hybridization was carried out with the total RNA preparations isolated from human fetal liver. Four RNA bands of 4.4, 3.4, 2.1, and 1.0 kb were detected when the PCR product encompassing the full ORF of the AK2A gene was used as a probe (Fig. 5B,

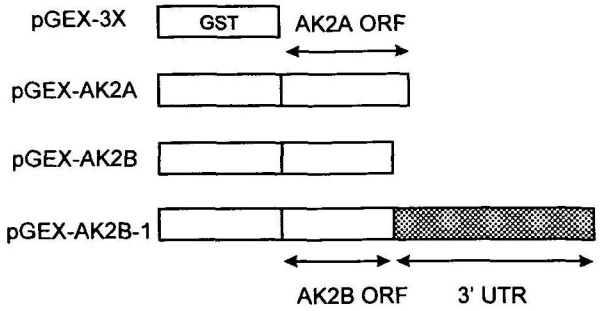

Fig. 2. **Schematic diagram of the expression plasmids.** Four plasmids, pGEX-3X, pGEX-AK2A, pGEX-AK2B, and pGEX-AK2B-1, were constructed to express GST protein and GST-AK2A, and GST-AK2B fusion proteins as described under "EXPERIMENTAL PROCEDURES."

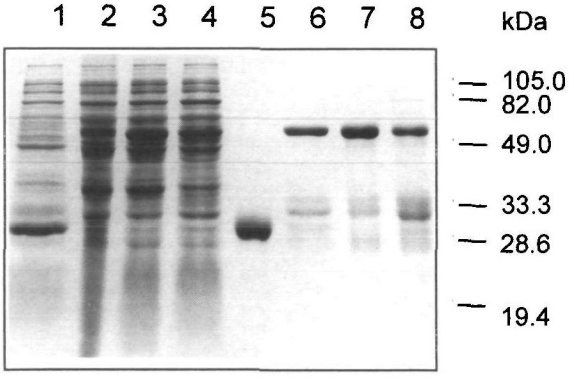

Fig. 3. **Production and isolation of recombinant GST-AK2 fusion proteins in *E. coli*.** Each protein preparation was purified from the same number of XL1-Blue MRF′ cells transformed with an expression plasmid and analyzed by SDS-PAGE (12% acrylamide gel) and Coomassie staining (*27*). Lanes 1-4: lysates of the cells containing pGEX-3X (lane 1), pGEX-AK2A (lane 2), pGEX-AK2B (lane 3), and pGEX-AK2B-1 (lane 4), respectively. Lanes 5-8: purified proteins of GST (lane 5), GST-AK2A (lane 6), and GST-AK2B from cells containing pGEX-AK2B (lane 7), and GST-AK2B from cells containing pGEX-AK2B-1 (lane 8), Standard molecular weight markers in kDa are indicated on the right.

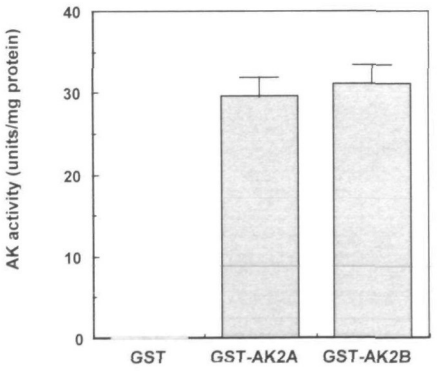

Fig. 4. **Enzyme activity of GST-AK2 fusion proteins expressed in *E. coli*.** The activity of purified GST or GST-AK2 fusion protein was examined by spectrophotometric assay. Enzymatic activity is indicated as specific activity (units/mg protein).

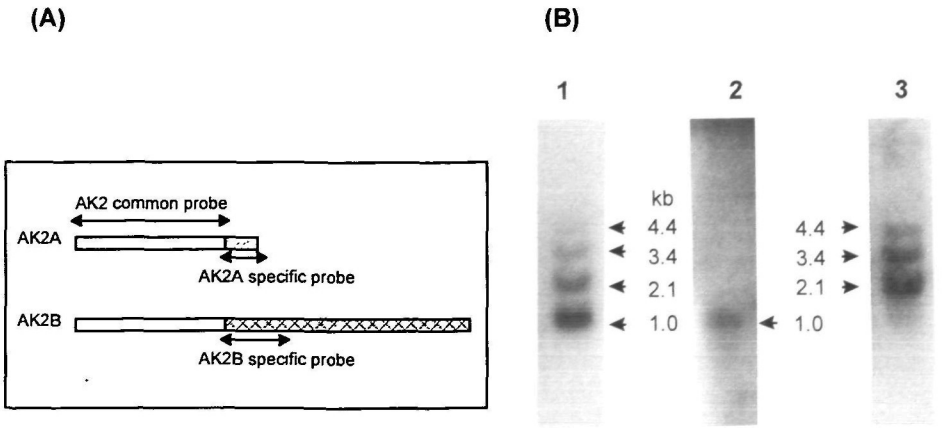

Fig. 5. **Identification of AK2 mRNA species by Northern blot analysis.** (A) Schematic map of the probes. The entire ORF of human AK2A gene was used as a common probe for AK2, while the 3′ end regions of AK2A (nt 709-889) and AK2B (nt 709-980) were used as AK2A-specific and AK2B-specific probes, respectively. (B) RNAs isolated from human fetal liver were resolved on formaldehyde gel and hybridized with the AK2 common probe (lane 1), AK2A-specific probe (lane 2), or AK2B-specific probe (lane 3). The estimated sizes of the mRNA species are marked.

lane 1). In the bovine system, Northern analysis revealed the presence of three AK2 mRNA species, of 1.7, 1.4, and 0.9 kb, of these, the 1.7- and 1.4-kb species were found to be messages of the AK2B isozyme (*5*). To identify the human AK2 mRNA species, AK2A-specific and AK2B-specific probes were prepared according to the sequence information (Fig. 5A). As shown in Fig. 5B, the AK2A-specific probe could recognize only the shortest mRNA band of the 1.0-kb transcript (lane 2), while the longer transcripts were detected by the AK2B-specific probe (lane 3). These results suggest that the alternative splicing mechanism involved in AK2 gene expression in the bovine system (*5, 6*) might also operate in the human system.

To study the distribution of AK2 isozyme mRNAs in

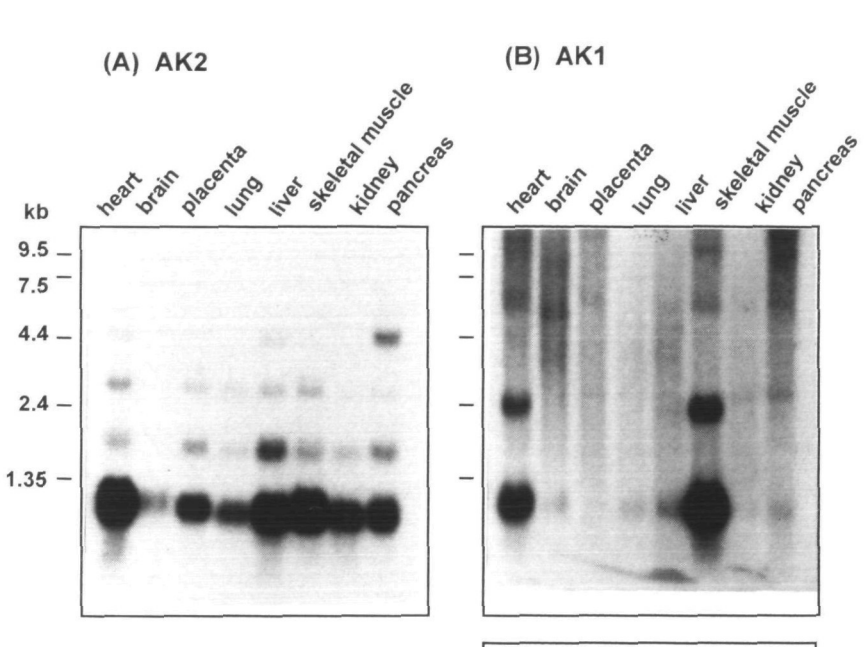

Fig. 6. **Distribution of AK2 (A) and AK1 (B) mRNAs in human tissues.** A multi-tissue blot membrane (Clontech) containing 2 μg of poly(A)$^+$ RNA per lane from several human tissues was hybridized with the ^{32}P-labeled AK2 common probe (A) or the AK1-specific probe encoding the C-terminus residues of AK1 (B), and analyzed as described under "EXPERIMENTAL PROCEDURES." Size markers in kb are denoted on the left. RNA contents were estimated by probing β-actin gene as a control (C).

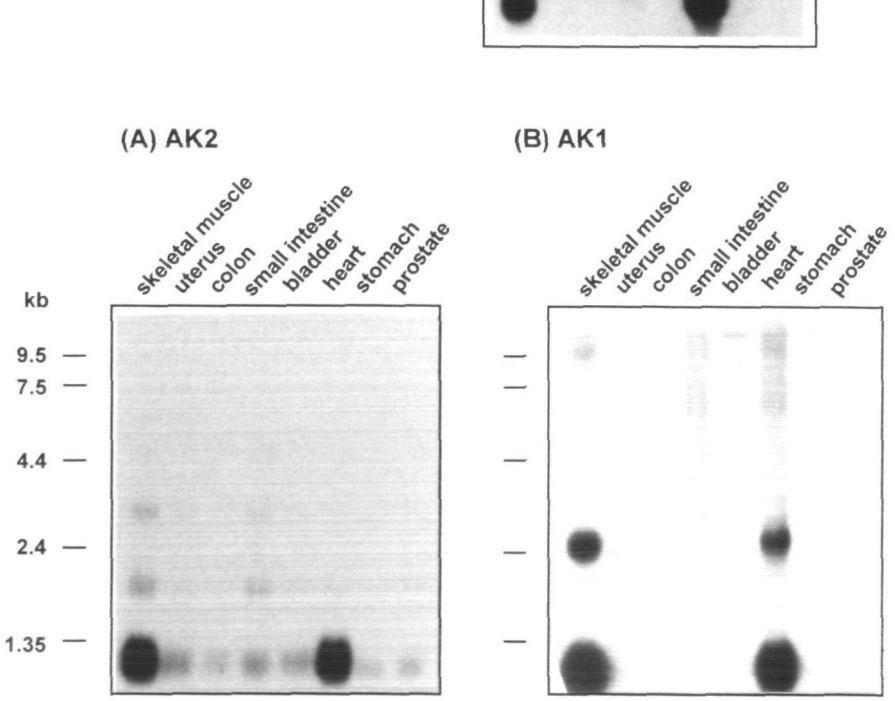

Fig. 7. **Distribution of AK2 (A) and AK1 (B) mRNAs in human muscle tissues.** A multi-tissue blot membrane (Clontech) containing 2 μg of poly(A)$^+$ RNA per lane from several human muscle tissues was hybridized and analyzed as described in Fig. 6.

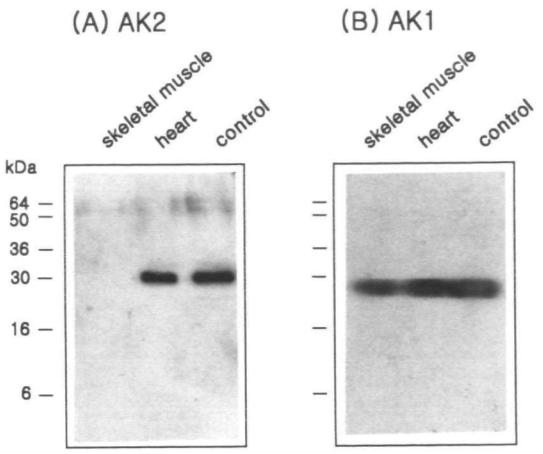

Fig. 8. **AK2 (A) and AK1 (B) proteins in skeletal muscle and heart.** 100 μg each of proteins prepared from human tissues or 3 μg of purified recombinant AK2 (A, control) or AK1 (B, control) protein were analyzed by SDS-PAGE and Western blot as described under "EXPERIMENTAL PROCEDURES." Size markers in kDa are denoted on the left.

human tissues, poly(A)$^+$ RNAs prepared from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Clontech, MTN blot) were subjected to Northern blot analysis using AK2A cDNA, which could recognize both isozyme mRNAs, as a probe. As shown in Fig. 6A, the AK2A mRNA of 1.0 kb was the major transcript in all human tissues. In the bovine system, the AK2B mRNA of 1.7 kb was the major transcript and the AK2A mRNA of 0.9 kb was the minor transcript in liver, and *vice versa* in heart (*5*). These differences in the expression patterns suggested the presence of species-specific gene expression. The expression pattern of each AK2 mRNA species, especially the mRNA of 4.4 kb, was not consistent in various human tissues and might reflect discrepancies in splicing events in various tissues.

An interesting feature of the AK2 distribution of mRNAs was their abundance not only in heart and liver but also in skeletal muscle. It has been reported that AK1 protein is the major type expressed in muscle and AK2 protein the major type in liver. In the rat system, the expression of AK1 and AK2 isozymes seemed to be inversely regulated (*4*). High expression level of AK1 was observed where the expression of AK2s was low or absent, and *vice versa*. In human, however, high expression level of AK2 was detected in liver, where the expression of AK1 was barely detected, and also in skeletal muscle, where the expression of AK1 was abundant (Fig. 6, A and B) at the transcriptional level. We investigated the mRNA levels of AK isozymes in several human muscle tissues and confirmed that both AK1 and AK2 transcripts were highly detected in heart and skeletal muscles (Fig. 7).

AK Proteins in Skeletal Muscle and Heart—As the contents of AK1 and AK2 mRNAs were high in skeletal muscle and heart, the levels of these AK isozymes in the tissues were analyzed by Western blotting. The result revealed that anti-AK1 and anti-AK2 antibodies each detected a single protein band in human tissue showing the same molecular mass as the respective recombinant AK1 and AK2 protein (Fig. 8). AK2 protein was found only in heart (Fig. 8A), whereas AK1 protein was present in both tissues (Fig. 8B). The rare presence of AK2 proteins in skeletal muscle is in agreement with previous reports that AK1 is the major type in skeletal muscle and AK2 the major one in liver (*2*). In the rat system, co-linearity between the mRNA level and protein activity of AK2 was reported in all tissues including skeletal muscle (*4*). Our results suggest the existence of a tissue-specific post-transcriptional control in AK2 gene expression, especially in skeletal muscle. Evaluation of this possibility is in progress.

We thank Dr. Young Tak Lee of Buchon Sejong Hospital and Dr. Yong Sung Lee of Hanyang University Medical School for providing us with human tissue samples.

REFERENCES

1. Noda, L.H. (1973) Adenylate kinase in *The Enzymes* (Boyer, P.D., ed.) 3rd ed., Vol. 8, pp. 279-305, Academic Press, New York
2. Khoo, J.C. and Russell, P.J. (1972) Isoenzymes of adenylate kinase in human tissue. *Biochim. Biophys. Acta* **268**, 98-101
3. Tomasselli, A.G., Schirmer, R.H., and Noda, L.H. (1979) Mitochondrial GTP-AMP phosphotransferase: 1. Purification and properties. *Eur. J. Biochem.* **93**, 257-262
4. Tanabe, T., Yamada, M., Noma, T., Kajii, T., and Nakazawa, A. (1993) Tissue-specific and developmentally regulated expression of the genes encoding adenylate kinase isozymes. *J. Biochem.* **113**, 200-207
5. Kishi, F., Tanizawa, Y., and Nakazawa, A. (1987) Isolation and characterization of two types of cDNA for mitochondrial adenylate kinase and their expression in *Escherichia coli*. *J. Biol. Chem.* **261**, 11785-11789
6. Tanaka, H., Yamada, M., Kishi, F., and Nakazawa, A. (1990) Isolation and characterization of the gene encoding bovine adenylate kinase isozyme 2. *Gene* **93**, 221-227
7. Matsuura, S., Igarashi, M., Tanizawa, Y., Yamada, M., Kishi, F., Kajii, T., Fujii, H., Miwa, S., Sakurai, M., and Nakazawa, A. (1989) Human adenylate kinase deficiency associated with haemolytic anemia: A single base substitution affecting solubility and catalytic activity of the cytosolic adenylate kinase. *J. Biol. Chem.* **264**, 10148-10155
8. Xu, G., O'Connell, P., Stevens, J., and White, R. (1992) Characterization of human adenylate kinase 3 (AK3) cDNA and mapping of the AK3 pseudogene to an intron of the NF1 gene. *Genomics* **13**, 537-542
9. Toren, A., Brok-Simoni, F., Ben-Bassat, I., Holtzman, F., Mandel, M., Neumann, Y., Ramot, B., Rechavi, G., and Kende, G. (1994) Congenital haemolytic anemia associated with adenylate kinase deficiency. *Br. J. Haematol.* **87**, 376-380
10. Lee, Y., Kim, J.W., Lee, I.A., Kang, H.B., Choe, Y.-K., Lee, H.G., Lim, J.S., Kim, H.J., Park, C., and Choe, I.S. (1996) Cloning and characterization of cDNA for human adenylate kinase 2A. *Biochem. Mol. Biol. Int.* **39**, 833-842
11. Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13
12. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Lab., Cold Spring Harbor, NY
13. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
14. Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptide expressed in *Escherichia coli* as fusion with glutathione-S-transferase. *Gene* **67**, 31-40
15. Kim, H.J., Nishikawa, S., Tokutomi, Y., Takenaka, H., Hamada, M., Kubby, S.A., and Uesugi, S. (1990) *In vitro* mutagenesis studies at the arginine residues of adenylate kinase: A revised

binding site for AMP in the X-ray-deduced model. *Biochemistry* **29**, 1107-1111
16. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* **72**, 248-254
17. Kim, J.W., Lee, Y., Lee, I.A., Kang, H.B., Choe, Y.K., and Choe, I.S. (1997) Cloning and expression of human cDNA encoding Na^+,K^+-ATPase γ-subunit. *Biochim. Biophys. Acta* **1350**, 133-135
18. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
19. Kozak, M. (1989) The scanning model for translation: an update. *J. Cell. Biol.* **108**, 229-241
20. Shaw, G. and Kamen, R. (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667
21. Hamada, M., Sumida, M., Okuda, H., Watanabe, T., Nojima, M., and Kuby, S.A. (1982) Adenosine triphosphate-adenosine-5′-monophosphate phosphotransferase from normal human liver mitochondria. *J. Biol. Chem.* **257**, 13120-13128
22. Kuby, S.A., Fleming, G., Frischat, A., Cress, M.C., and Hamada, M. (1983) Studies on adenosine triphosphate transphosphorylases: Human isozymes of adenylate kinase: Isolation and physicochemical comparison of the crystalline human ATP-AMP transphosphorylases from muscle and liver. *J. Biol. Chem.* **258**, 1901-1907
23. Nagai, K., Perutz, M.F., and Poyart, C. (1985) Oxygen binding properties of human mutant hemoglobins synthesized in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **82**, 7252-7255
24. Shows, T.B. and Brown, J.A. (1975) Mapping chromosome 1 and 2 employing a 1/2 translocation in somatic cell hybrids. *Cytogenet. Cell Genet.* **14**, 421-425
25. Van Cong, N., Rebourcet, R., Weil, D., Couillin, P., Hors, M.C., Jami, J., and Frezal, J. (1974) Assignment of the second locus of adenylate kinase to chromosome 1p: Preliminary data. *Cytogenet. Cell Genet.* **13**, 173-178
26. Wilson, D.E., Povey, S., and Harris, H. (1976) Adenylate kinases in man: evidence for a third locus. *Ann. Hum. Genet.* **39**, 305-313
27. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685