Regulation of rat AMP deaminase 3 (isoform C) by development and skeletal muscle fibre type

Donna K. MAHNKE-ZIZELMAN*, Jonathan D'CUNHA†, Jean M. WOJNAR*, Michele A. BROGLEY† and Richard L. SABINA*[1]

*Department of Biochemistry, Graduate School of Biomedical Sciences, The Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A., †Graduate School of Biomedical Sciences, The Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.

AMP deaminase (AMPD) is characterized by a multigene family in rodents and man. Highly conserved rat and human *AMPD1* and *AMPD2* genes produce protein products that exhibit cross-species immunoreactivities (*AMPD1*, rat isoform A and human isoform M; *AMPD2*, rat isoform B and human isoform L). A third gene, *AMPD3*, has been described in humans, but antisera raised against its purified protein product (isoform E) reportedly does not cross-react with a third activity purified from rat tissues (isoform C). This study was designed to address this latter issue by cloning, sequencing and expressing rat *AMPD3* cDNA species. Similarly to the human *AMPD3* gene, the rat *AMPD3* gene produces multiple transcripts that differ at or near their 5′ ends. The boundary at which these alternative sequences diverge is precisely conserved in both species. Across the region that is common to all rat and human *AMPD3* cDNA species, nucleotide and predicted amino acid sequences are 89 % and 93 % identical respectively, although the rat open reading frame is lacking two separate in-frame codons in the 5′ end. Extreme 5′ regions between the two species are entirely divergent, and one alternative rat sequence is predicted to confer at least 36 additional N-terminal residues to its encoded *AMPD3* polypeptide. A comparison of 3′ untranslated regions indicates that the rat sequence is 250 bp longer and contains multiple consensus polyadenylation signals. Examination of relative rat *AMPD3* gene expression shows (1) variable patterns of alternative mRNA abundance across adult tissues, (2) developmental regulation in skeletal muscle and liver, and (3) greater mRNA abundance in adult red (soleus) than in mixed (plantaris) and white (outer gastrocnemius) skeletal muscle. Finally, baculoviral expression of rat and human *AMPD3* proteins produces enzymes that are chromatographically and kinetically similar. Moreover, both recombinant activities immunoreact with anti-C and anti-E serum. These combined results demonstrate that rat isoform C and human isoform E are homologous cross-species *AMPD3* proteins.

INTRODUCTION

AMP deaminase (AMPD; EC 3.5.4.6) catalyses the irreversible deamination of AMP to IMP and ammonia. Multiple rat and human AMPD isoforms have been characterized and each intraspecies variant exhibits a distinct kinetic, chromatographic and immunological profile. Three AMPD isoforms have been reported in the rat and are termed A (muscle), B (liver and kidney) and C (heart) [1,2]. In humans, four AMPD isoforms have been described and are named after the source from which they were initially purified: M (muscle), L (liver), E1 and E2 (erythrocyte) [3,4]. With the exception of the E isoforms, only very weak or no cross-immunoreactivities are observed between intraspecies AMPD isoenzymes [1,3]. A likely explanation for the immunological similarity of isoforms E1 and E2 assumes a common genetic origin based on their combined absence in an inherited deficiency in erythrocytes [5]. Conversely, similar kinetic, chromatographic and immunological profiles have been demonstrated between the rat A and human M and the rat B and human L isoenzymes respectively [1–4]. Although the rat C and human E isoenzymes exhibit similar chromatographic and electrophoretic behaviours, they are reportedly distinct at the immunological level [3].

Corresponding rat and human AMPD multigene families have also been identified [6]. The *AMPD1* genes produce transcripts encoding isoforms A and M [7–12], whereas the *AMPD2* genes produce transcripts encoding isoforms B and L [12–15]. An alignment across the entire rat and human *AMPD1* open reading frames shows 87 % nucleotide identity [9], and a rat partial *AMPD2* cDNA is 91 % identical with the corresponding region of the human nucleotide sequence [13]. These cross-species sequences would encode polypeptides with 93 % and 100 % predicted amino acid identities respectively. Conversely, alignments between *AMPD1* and *AMPD2* sequences generate much lower nucleotide and predicted amino acid identities [13]. Therefore homologous AMPD genes across mammalian species are more alike than are those within the same species. As detailed above, this observation is consistent with available information about their encoded polypeptide products.

A third human gene, *AMPD3*, produces transcripts encoding the E isoforms [16,17]. To achieve a complete understanding of all known rat variants, the current study was designed to delineate the molecular basis for AMPD isoform C expression. To accomplish this goal, near full-length rat *AMPD3* cDNA species have been isolated and expressed to partly characterize the recombinant protein. The combined data are used to show that isoform C is the encoded protein product of the rat *AMPD3* gene.

Abbreviations used: AMPD, AMP deaminase; ORF, open reading frame; pBS, plasmid Bluescribe; RT–PCR, reverse transcriptase–PCR.

[1] To whom correspondence should be addressed.

The nucleotide sequence data reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U90888.

EXPERIMENTAL

Materials

Biochemicals were purchased from Sigma Chemical Co. unless otherwise specified. Bacterial (Luria broth) and insect cell culture (Grace's) growth media were obtained from Gibco-BRL Life Sciences Technologies. Fetal calf serum was purchased from HyClone Laboratories. Immobilon nitrocellulose filters (0.45 μm pore size) used for library screening were supplied by Millipore Corp. Restriction endonucleases, nucleotides, modifying enzymes and ultrapure reagents were purchased from Boehringer-Mannheim Biochemicals. Seaplaque agarose was obtained from FMC Corp. and *Staphylococcus aureus* cells (Pansorbin) were supplied by Calbiochem Corp. Phosphocellulose P-11 was purchased from Whatman. Disposable glass columns (1.5 cm × 30 cm) and nitrocellulose membranes were obtained from Bio-Rad. Amersham supplied all radioisotopes used for labelling probes and sequencing ([^{32}P]dCTP, [^{32}P]UTP and [^{35}S]dATP). Kits for random-prime labelling of probes and Western blot chemiluminescence detection were also purchased from Amersham, and a kit for DNA sequencing was obtained from United States Biochemical Corp. A PolyATtract mRNA isolation kit for purifying poly(A)$^+$ mRNA was supplied by Promega Corp. A 5′-AmpliFINDER kit for producing and cloning reverse transcriptase–PCR (RT–PCR) products was obtained from CLONTECH Laboratories (Palo Alto, CA, U.S.A.).

Library screening

A cDNA library prepared from adult rat soleus muscle and housed in the lambda gt10 vector [7] was screened with a previously described human *AMPD3* cDNA insert, HuT17 [16]. Approximately 10^6 plaque-forming units were infected into *Escherichia coli* strain C600 Hfl and plated in ten 150 mm^2 dishes on Luria broth agar. Duplicate filters were prepared and screened at relatively high stringency (0.6 M NaCl at 65 °C) with the ^{32}P[dCTP]-labelled cDNA insert as the probe. One positive recombinant was identified, plaque-purified and phage DNA was prepared. Two cDNA inserts were evident after digestion of the purified recombinant phage DNA with *Eco*R1 (results not shown), which were recovered by agarose gel electrophoresis and cloned into a unique *Eco*R1 restriction endonuclease site in plasmid Bluescribe (pBS; Stratagene Cloning Systems, San Diego, CA, U.S.A.). The ends of both recombinant cDNA inserts were sequenced by the dideoxy chain-termination method of Sanger [18] with universal and reverse primers. An alignment with available human *AMPD3* cDNA sequence demonstrated that the two recombinant inserts were most probably a continuous sequence connected by a natural *Eco*R1 restriction endonuclease site (see the Results and discussion section). The 5′-most cDNA fragment was used to rescreen the same rat soleus muscle library and a second positive recombinant was identified, plaque-purified and phage DNA was prepared. Again, two *Eco*R1 cDNA fragments were evident, which were subcloned into pBS and sequenced as described above. Finally, a commercially available rat kidney cDNA library (CLONTECH Laboratories) was screened by using the 5′-most cDNA fragment of the second isolate as the probe; a third recombinant phage was identified and characterized as described above.

Isolation of RNA species from rat tissues and cells

Total cellular RNA species were isolated from selected rat tissues and cells by the guanidinium isothiocyanate/caesium chloride method ([19], p. 196) and used in RT–PCR and RNase protection analyses. RNA species from rat hindlimb and L6 cells at different developmental stages, and from individual skeletal muscle groups of adult hindlimb, were taken from frozen aliquots of previously described preparations used in RNase protection [20] and Northern blot [7] analyses of relative *AMPD1* gene expression. Separate aliquots of total cellular RNA species were fractionated on denaturing (formaldehyde) agarose gels and revealed by Acridine Orange stain to verify the quality and quantity of each preparation.

RT–PCR cloning of additional *AMPD3* cDNA species

RT–PCR was performed with poly(A)$^+$ mRNA prepared from total cellular RNA isolated from adult rat heart and nested oligonucleotide primers designed from near the 5′ end of the rat soleus muscle *AMPD3* cDNA clone, RSol-2: RT primer, 5′-TGTGTACTTGATGAAACGCAGCAG-3′; PCR primer, 5′-TCTAGAAGCTTCTGATTCATGCAGGCCGCTGC-3′. The PCR primer had seven anomalous bases constructed onto its 5′ end to create restriction endonuclease sites (*Xba*1 and *Hin*dIII) that facilitated cloning of the resultant products. RT–PCR was performed in accordance with the procedure contained in the commercially available kit (5′-AmpliFINDER; CLONTECH Laboratories). Briefly, 6 μg of poly(A)$^+$ mRNA was incubated with 10 pmol of the RT oligonucleotide at 65 °C for 5 min. First-strand cDNA synthesis was performed by adding 20 μl of a master mix containing dNTPs, RNase inhibitor and AMV reverse transcriptase, and incubating at 52 °C for 30 min. The reaction was stopped by the addition of 16 mM EDTA, and RNA was hydrolysed in 0.4 M NaOH at 65 °C for 30 min. The hydrolysed solution was neutralized by the addition of 0.4 M acetic acid. Single-strand cDNA species were purified, precipitated and ligated to a 5′ anchor oligonucleotide (supplied in the kit) with T4 RNA ligase at 21 °C for 18 h. PCR was then performed on the anchor-ligated cDNA mixture with the PCR oligonucleotide primer and a primer complementary to the 5′ anchor sequence (supplied in the kit). PCR parameters were as follows: hot start at 100 °C for 2.5 min, followed by 40 cycles of PCR (denaturation, 94 °C for 45 s; annealing, 60 °C for 45 s; extension, 72 °C for 2 min), then a final extension at 72 °C for 7 min. The PCR product was digested with *Eco*R1 (restriction site located in the 5′ anchor region) and *Hin*dIII, then fractionated on a 0.7 % low-melting-temperature agarose gel. cDNA fragments in the 0.6–3 kb region were isolated and subcloned into pBS for subsequent sequencing analysis.

RNase protection analysis

Relative abundances of alternative *AMPD3* mRNA species were estimated in rat tissues and cells by RNase protection analysis [21]. A 397 bp *Eco*R1 (5′)/*Pst*1 (3′) fragment, representing the 5′ end of the longest RT–PCR cDNA from rat *AMPD3*, was subcloned into polycloning sites of pBS. The resultant recombinant plasmid was linearized at the *Eco*R1 site, and T3 RNA polymerase was used to produce a 420-base ^{32}P[UTP]-labelled cRNA probe. Of the 392 bases of *AMPD3* sequence in this probe, 272 are contained in the region common to all *AMPD3* cDNA species and 120 are specific to the 5′ end of the longest isolated variant. This probe also contains 20 bases complementary to pBS polycloning sequence at its 5′ end and eight bases complementary to the *Eco*R1 anchor-linker sequence from RT–PCR cloning at its 3′ end. Total cellular RNA species isolated from prenatal hindlimb muscle, postnatal liver and hindlimb muscle, several adult tissues, and L6 myocytes at different stages of development were used in protection assays

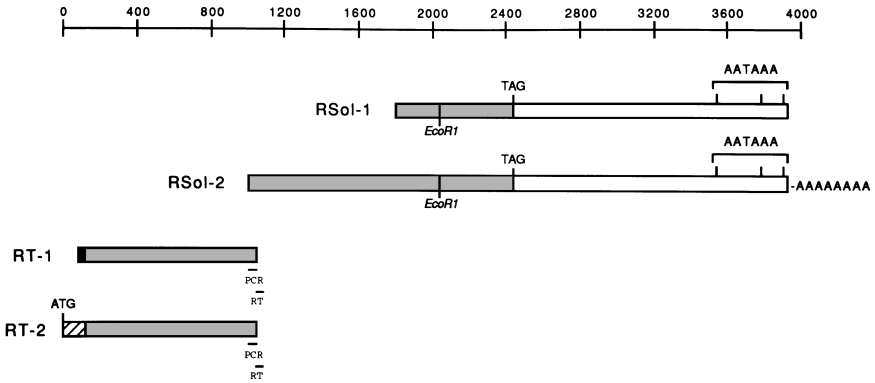


Figure 1 Schematic of cloned rat *AMPD3* cDNA species

Clones are illustrated as horizontal bars and are aligned in the 5′ → 3′ orientation and drawn to scale (in bp) as shown by the vertical line above the diagrams. Horizontal bars are filled to reflect the nature of predicted transcribed sequence: white, 3′ untranslated region; stippled, coding sequence common to all rat *AMPD3* mRNA species; black and hatched, alternative 5′ sequences. Two clones (RSol-1 and RSol-2) were isolated by conventional screening of a rat soleus muscle cDNA library constructed from oligo-dT primed mRNA, although only one of the clones (RSol-2) has a poly(A)$^+$ tail (-AAAAAAAA). Multiple potential consensus polyadenylation signals (AATAAA) found in the 3′ untranslated region are bracketed and denoted by vertical ticks. Both RSol clones contain two recombinant *Eco*R1 inserts joined at an endogenous restriction site. RT clones were isolated by RT–PCR of poly(A)$^+$ mRNA prepared from adult rat heart. The relative locations of oligonucleotides used in the RT and PCR are denoted by the short horizontal lines beneath the RT clones (see the text and Figure 2 for more detailed information). Other abbreviations: TAG, predicted stop codon for all rat *AMPD3* mRNA species; ATG, predicted start codon in the RT-2 clone sequence.

with this probe (see the Results and discussion section for additional details).

Expression and characterization of rat and human *AMPD3* recombinant enzymes

AMPD3 cDNA species were cloned into the *Nco*I (5′) and *Hin*dIII (3′) polycloning sites of the baculovirus transfer vector, pBlueBacIII (Invitrogen Carlsbad, CA, U.S.A.), and co-transfected into Sf9 (*Spodoptera frugiperda*) insect cells along with a modified linearized AcNPV baculoviral genome (BaculoGold DNA; PharMingen, San Diego, CA, U.S.A.). Positive viral plaques were identified, isolated, amplified and used to infect Sf9 cells (two to four T-185 flasks) for 48–96 h at a multiplicity of infection of 5. Infected cells were pooled and extracts prepared by sonication at 4 °C in 20 mM potassium phosphate, pH 7.0, containing 100 mM KCl and 0.1% (v/v) 2-mercaptoethanol. Extracts were cleared by centrifugation at 14000 ***g*** for 5 min at 4 °C and then batch-adsorbed at 4 °C for 30 min with gentle mixing to phosphocellulose resin that had been pre-equilibrated in extraction buffer. After adsorption, the resin was washed once in extraction buffer and poured into a glass column (1.5 cm × 30 cm). Protein was eluted with a 100 ml linear gradient of 0.1–2 M KCl. Each 2 ml fraction was monitored for relative protein (Bio-Rad microassay) and AMPD enzyme activity. Active fractions were pooled and concentrated in Centricon-30 concentrators (Amicon, Beverly, MA, U.S.A.). To decrease the phosphate concentration, each concentrated protein was dialysed overnight at 4 °C against 1000 vol. of 150 mM imidazole, pH 7.0, containing 500 mM KCl and 1 mM dithiothreitol. Kinetic and immunological properties of the dialysed recombinant enzymes were determined as previously described [12,16]. Polyclonal antiserum raised against purified rat isoform A has been described [22]. Polyclonal anti-C [1] and anti-E [3] sera were made available by Dr. N. Ogasawara (Aichi Prefectural Colony, Aichi, Japan). All three of these reagents were raised in rabbits and are specific to their AMPD activities in solution-hybridization immunoassays of rat and human tissues respectively [1,3,12,20]. An additional polyclonal antiserum was raised in a rabbit against human *AMPD1* recombinant protein. This antiserum was used in the Western blot analysis of recombinant AMPD proteins.

Computer analysis of nucleotide and predicted amino acid sequence

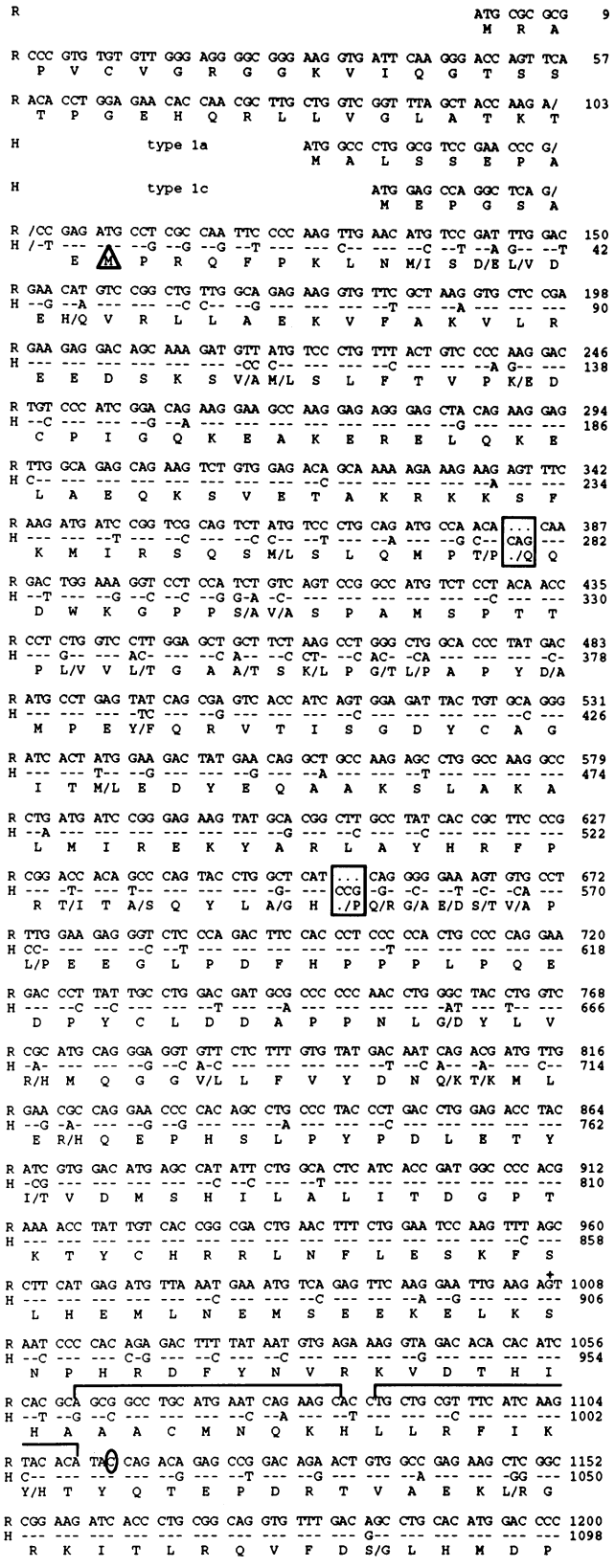
All computer-assisted analyses of nucleotide and predicted amino acid sequence were performed with the sequence analysis software package of the University of Wisconsin Genetics Computer Group (UWGCG; Madison, WI, U.S.A.).

RESULTS AND DISCUSSION

Cloning, amplification and sequence of rat *AMPD3* cDNA species

Conventional library screening employing an available human *AMPD3* cDNA probe, together with RT–PCR analysis, was used to generate near full-length rat *AMPD3* cDNA species. A rat soleus muscle lambda gt10 library was initially surveyed by conventional screening. This yielded a single positive clone, RSol-1, that contained two *Eco*R1 inserts joined by an endogenous restriction site (Figure 1). The 3′ *Eco*R1 fragment was 1947 bp in length and had a consensus polyadenylation signal (AATAAA) near its 3′ end, but no poly(A)$^+$ tail. The 5′ *Eco*R1 fragment extended 262 bp upstream from the endogenous restriction endonuclease site. By using the 5′ *Eco*R1 fragment as a probe to rescreen the rat soleus muscle library, a second positive clone, RSol-2, was isolated that also contained two *Eco*R1 inserts joined at the same endogenous *Eco*R1 restriction endonuclease site (Figure 1). The 3′ fragment is identical with the one in clone RSol-1 except for the presence of a terminal poly(A)$^+$ tail composed of eight consecutive A residues. Moreover, the 5′ *Eco*R1 fragment of clone RSol-2 extends 1048 bp upstream from the endogenous restriction endonuclease site.

Further screening of the rat soleus muscle cDNA library using the 5′ *Eco*R1 insert of clone RSol-2 as the probe did not identify additional positive plaques, so a rat kidney cDNA library was surveyed. This resulted in the isolation of a third positive clone, RKid-1 (results not shown; see Figure 2 for further information on this clone). The 5′ end of the RKid-1 insert is identical with a region near the 5′ end of the RSol-2 clone. However, these two



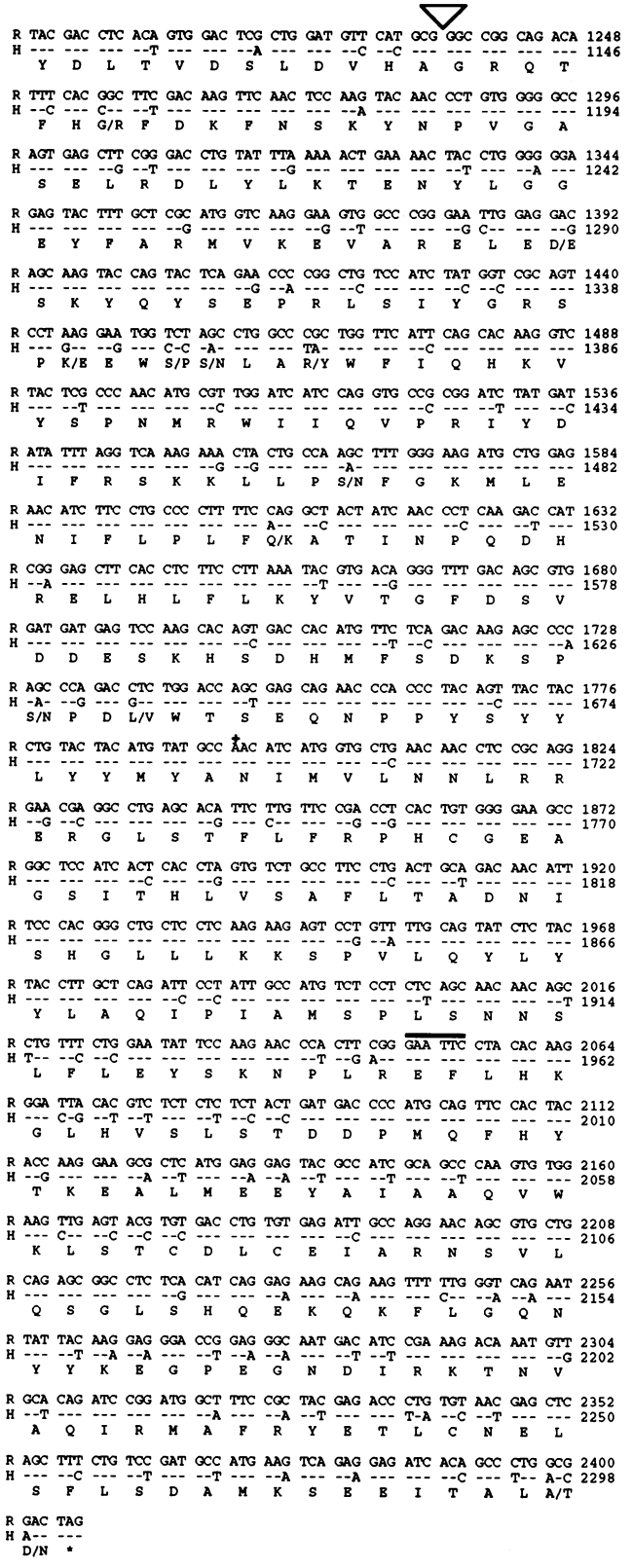


Figure 2 For legend see opposite page

recombinant inserts have divergent 3′ ends beginning at a gt dinucleotide in the kidney cDNA, indicative of an intervening sequence. In a related way there is also an intron at the same relative position in the human *AMPD3* gene [17].

RT–PCR was employed to generate additional cDNA species that extended more 5′ when conventional library screening became unsuccessful. Two oligonucleotides were designed from a sequence near the 5′ end of clone RSol-1 (one as a primer for the RT reaction and a second, nested sequence as a primer in the PCR reaction; see Figures 1 and 2) and used to synthesize and amplify cDNA species from adult rat heart poly(A)$^+$ mRNA. This yielded two additional cDNA species, RT-1 and RT-2 (Figure 1). Both began at a created *Hin*dIII restriction endonuclease site added to the 5′ end of the PCR primer (see the Experimental section) and extended upstream until a point of divergence was reached near their 5′ ends.

Comparison of rat and human *AMPD3* cDNA sequences

Predicted rat and human [16] *AMPD3* open reading frames are aligned in Figure 2. Nucleotide (89 % over 2309 bases) and predicted amino acid identities (93 % over 768 residues) are high across the sequence extending 5′ from the predicted termination codon in all *AMPD3* cDNA species, T(U)AG, to the point of divergence between variants. However, an inspection reveals more nucleotide substitutions in the 5′ end of this alignment. Even more striking is the predicted regional divergence in primary amino acid sequence. For example, only two C-terminal amino acids differ across 220 residues at the C-terminal end of the predicted polypeptides. Such strict primary amino acid sequence conservation might be expected in this portion of the *AMPD3* polypeptides, which contains proposed catalytic site residues found in all AMPDs [23]. Conversely, amino acid substitutions are more prevalent in the N-terminal two-thirds of the rat and human *AMPD3* polypeptides, including individual glutamine and proline residues that are missing from the rat sequence. Nevertheless, sizeable stretches of strictly conserved residues are also evident in the N-terminal portions of these polypeptides, including a predicted α-helical domain (QKEAKERELQKELAEQK) that is highly similar to homologous regions in spectrin and fodrin [16].

The human *AMPD3* gene produces alternative mRNA species that differ at or near their 5′ ends [16,17]. Figure 2 also shows that 5′ nucleotide sequences and corresponding predicted N-terminal amino acid residues diverge at the same relative position in all rat and human *AMPD3* cDNA species. This combined information suggests that, like its human counterpart, the rat *AMPD3* gene also produces multiple transcripts that differ at or near their 5′ ends.

Alternative 5′ sequences in human *AMPD3* mRNA species are largely non-coding, although N-terminal extensions of five and seven amino acids are predicted for two of the encoded polypeptides [16]. Conversely, an alternative sequence contained in the RT-2 cDNA is predicted to extend the rat *AMPD3* open reading frame by at least 108 bases and encode another 36 N-terminal residues. A computer-assisted analysis of this predicted N-terminal extension identifies two consensus casein kinase 2 phosphorylation sequences (T/SXXE/D), although these potential post-translational modification sites are not unique across the entire molecule. The RT-1 cDNA contains only 14 bp of divergent sequence at its 5′ end (5′-AGTGCTGGGAAGAG-3′) that would extend its open reading frame, although no start codon is present in this short stretch of bases.

Figure 3 shows an alignment between 3′ untranslated regions of rat and human *AMPD3* cDNA species. Although 40 gaps have to be inserted to compensate for the addition or deletion of stretches of nucleotides, these regions are 71 % (920/1292) identical up to the 3′ end of the human sequence, including a conserved consensus polyadenylation signal. However, the 3′ untranslated region of rat *AMPD3* cDNA extends another 250 bp and contains two downstream consensus polyadenylation signals. Figure 3 also includes an alignment between rat *AMPD3* cDNA and human genomic DNA sequence immediately downstream from the 3′ end of the available human cDNA sequence. After inserting 17 more gaps, 74 % (298/405) identity is revealed and human genomic DNA contains one of the two additional consensus polyadenylation signals found in the rat *AMPD3* cDNA sequence.

RNase protection analysis of alternative *AMPD3* mRNA abundance in adult rat tissues

RNase protection analysis was used to confirm the presence of alternative *AMPD3* mRNA species in rat tissues and cells and to estimate their relative abundances. A 420-base cRNA was produced from a linearized subclone of the rat *AMPD3* cDNA clone RT-2 (see the Methods section and Figure 2). As illustrated in the diagram in Figure 4, this probe spans the point of divergence at the 5′ ends of the RT clones and would be differentially protected by alternative mRNA species. RT-2 mRNA species would protect the entire 392 bases of *AMPD3* cRNA in this probe. Conversely, other alternative transcripts (e.g. RT-1 mRNA) would protect only the 272 bases of sequence in this probe common to both RT cDNA species. Total cellular RNA was isolated from a variety of adult rat tissues and 8 μg of each was used to protect this probe, with the exceptions of skeletal muscle (4 μg) and liver (200 μg).

Data presented in Figure 4 reveal both full (392-base band) and partial (272-base band) protection of this cRNA probe by alternative *AMPD3* mRNA species in most adult rat tissues, although different patterns are evident. Full protection of the probe is greatest in heart and kidney, whereas partial protection is most prominent in lung. Full protection would be expected in heart because this tissue is the source of the cDNA used as the template. Thyroid contains relatively little *AMPD3* mRNA, although most seems also to protect the cRNA probe fully. Liver is the only one among several adult tissues where no *AMPD3* mRNA species are detected, even though 200 μg of total cellular

Figure 2 Alignment of rat and human *AMPD3* open reading frames

Predicted amino acids are centred under each codon and designated by the single-letter code. The rat (R) nucleotide sequence is numbered from the A of the predicted start codon in the RT-2 clone, and the human (H) sequence is numbered from the A of the predicted start codon in the type 1b cDNA (denoted by the M in a triangle) located near the 5′ end of the common open reading frame [17]. Because no similarities are evident, alignments between alternative 5′ coding sequences are not possible (see the text for the 14 bp of divergent sequence at the 5′ end of the rat RT-1 cDNA clone). The boundary of divergence across all *AMPD3* open reading frames is denoted with a slash mark (/). Nucleotide sequence identities in the common region of the *AMPD3* open reading frames are indicated by dashes in the human sequence. Predicted amino acid differences in the common open reading frame are denoted with the rat residue listed first. Two separate in-frame codons deleted from the rat open reading frame are boxed. Brackets identify sense strand nucleotide sequences (+1062–1084, PCR; +1088–1110, RT) used to design anti-sense oligonucleotides for the RT–PCR generation of cDNA clones (see the text and Figure 1). 5′ ends of rat cDNA clones are denoted with a plus sign (+) over nucleotides +1007 (RSol-2), +1113 (RKid-1) and +1795 (RSol-1). The location of an intervening sequence in the RKid-1 cDNA clone (see the text) is illustrated by the inverted triangle at the nucleotide +1236/1237 boundary. A landmark *Eco*RI restriction endonuclease site (GAATTC) is overlined. Alignment was generated with the BESTFIT program of the GCG software.

Figure 3 Alignment between rat *AMPD3* 3′ untranslated sequence and the corresponding human cDNA and 3′ flanking genomic DNA sequence

The BESTFIT program of the GCG software package was used to generate an alignment requiring the insertion of 57 gaps. Rat (R) sequence is in lower case, except nucleotide +2406, which is the last base of the predicted stop codon (TAG). Human sequence is in capitals to the transcription termination site at nucleotide +3543. Thereafter the alignment continues with human genomic DNA immediately downstream of the transcription termination site (also lower case). Consensus polyadenylation signals (AATAAA) are boxed.

RNA was used in the assay. Together, these results demonstrate that adult rat tissues exhibit different relative abundances, and different ratios of alternative forms, of *AMPD3* mRNA species. This is similar to previous observations in human tissues and cells [16,17].

Developmental expression of alternative *AMPD3* mRNA species

The *AMPD1* [7,11,20] and *AMPD2* [6,20] genes are developmentally regulated during skeletal myogenesis, but so far *AMPD3* had not been examined. Therefore relative *AMPD3* mRNA abundance was estimated in developing skeletal muscle and cultured skeletal myocytes. Figure 4 shows that the relative abundances of all *AMPD3* mRNA species seemed to increase in developing hindlimb skeletal muscle. Amounts of the fully protected probe became progressively greater from 11–12 to 17–18 days of gestation, then remained relatively constant through 20 days of postnatal development. The profile of partial

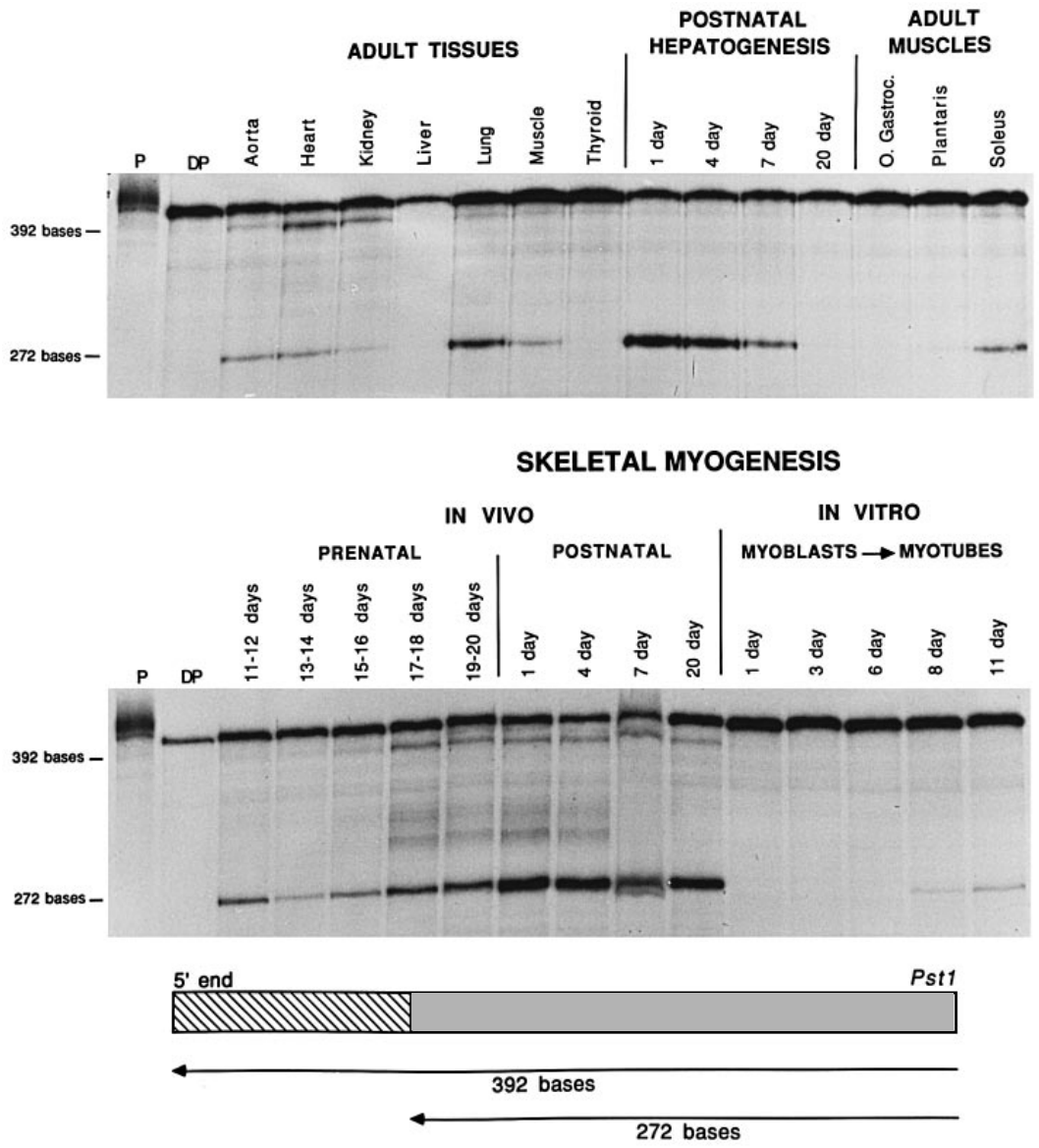


Figure 4 RNase protection analysis of relative *AMPD3* mRNA abundance

A 420-base ^{32}P[UTP]-labelled cRNA probe was produced from a linearized subclone of the RT-2 cDNA. This probe, which begins at an endogenous *Pst*1 restriction endonuclease site and extends upstream across the boundary of divergence between alternative mRNA species (see the diagram and Figure 2), contains 392 bases of rat *AMPD3* cRNA. As shown in the diagram, those mRNA species containing the alternative 5′ sequence found in the RT-2 cDNA clone (cross-hatched bar) would fully protect the 392 bases of *AMPD3* cRNA in this probe. Other alternative transcripts have different 5′ ends and would protect only the 272 bases of probe sequence that are complementary to that found in all rat *AMPD3* mRNA species (stippled bar). A series of RNase protection assays were performed by hybridizing this cRNA probe with total cellular RNA species isolated from a variety of adult rat tissues [8 μg each, except skeletal muscle (4 μg) and liver (200 μg)], prenatal skeletal muscle and postnatal skeletal muscle and liver (50 μg), adult hindlimb muscles (3 μg) and cultured L6 myocytes (8 μg). Abbreviations: P, undigested probe; DP, digested probe alone (tRNA control). Each sample contained detectable levels of the 420-base probe that were not digested by the RNase cocktail used in the assays.

protection is similar, although somewhat delayed as maximal levels are not reached until birth. Therefore steady-state levels of the transcript represented by the RT-2 cDNA reach a maximum at an earlier developmental time point than the combined accumulation of all other *AMPD3* mRNA species.

Cultured L6 cells also exhibited a developmental increase in relative *AMPD3* mRNA abundance, confirming that up-regulation occurs in skeletal myocytes. Partial protection of the cRNA probe was apparent during post-confluent differentiation, whereas no full protection was detected at any of the times examined (Figure 4). This latter observation might have been due to the relatively small amount of RNA used in this assay, which was 1/16 of that used in the analysis of hindlimb skeletal muscle. Alternatively, cultured myocytes might not reach a developmental stage where the *AMPD3* mRNA represented by the RT-2 cDNA is expressed, or perhaps its expression is confined to non-myocyte components of muscle.

The apparent up-regulation of *AMPD3* gene expression in differentiating skeletal myocytes has implications for a common inherited deficiency in the *AMPD1* gene (myoadenylate deaminase deficiency) that is variably associated with exercise-induced myalgia typically occurring in the early adult years [24].

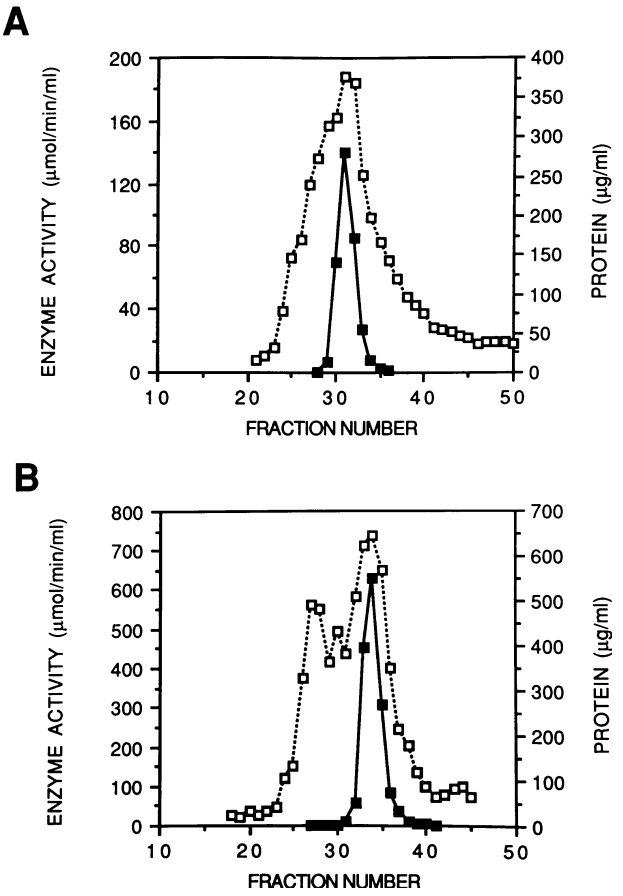


Figure 5 Phosphocellulose chromatography of *AMPD3* recombinant proteins

Rat (**A**) and human (**B**) *AMPD3* recombinant proteins were produced in insect cells (rat, two T-185 flasks infected for 2 days; human, four T-185 flasks infected for 4 days) infected with recombinant baculovirus. Cleared extracts were adsorbed on phosphocellulose resin equilibrated in 20 mM potassium phosphate, pH 7.0, containing 100 mM KCl and 0.1% (v/v) 2-mercaptoethanol. Adsorbed resins were poured into a 50 ml glass column (1.5 cm × 30 cm) and protein was eluted with a linear gradient of 0.1–2 M KCl. Fractions (2 ml) were collected and monitored for AMPD enzyme activity (■) and total protein (□).

Table 1 Kinetic and immunological properties of recombinant rat and human *AMPD3* enzymes

Enzyme assays were performed at 37 °C in the presence of 25 mM imidazole, pH 7.0, containing 100 mM KCl. The ATP concentration when added was 1.5 mM. Solution-hybridization immunoassays were performed as described in [13]. A solution-hybridization immunoassay of an adult rat heart extract yielded the following results: anti-A, 6%; anti-B, 30%; anti-C, 86%; anti-E, 83%. Values shown are means ± S.D. for *n* independent experiments.

	Kinetic				Immunological		
Organism	*n*	Condition	K_m (mM)	*h*	*n*	Antibody	Precipitation (%)
Rat	3	−ATP	2.4 ± 0.7	1	3	anti-A	< 5
		+ATP	0.6 ± 0.3	1		anti-B	< 5
						anti-C	85 ± 22
						anti-E	95 ± 3
Human	5	−ATP	5.0 ± 0.8	1	3	anti-A	< 5
		+ATP	0.7 ± 0.1	1		anti-B	< 5
						anti-C	84 ± 9
						anti-E	92 ± 7

AMPD is an integral component of skeletal muscle energy metabolism; partial compensation by the *AMPD3* enzyme (isoform E) during prenatal and early postnatal myogenesis might help prevent developmental abnormalities, which are not apparent in myoadenylate deaminase-deficient individuals [24]. Moreover, *AMPD3* expression in skeletal myocytes might contribute to clinical variability associated with myoadenylate deaminase deficiency in adults.

Relative *AMPD3* mRNA abundance was also assessed during postnatal hepatogenesis in the same rat pups used to examine postnatal hindlimb development. Figure 4 shows relatively high levels of partial protection of the cRNA probe at birth that subsequently became undetectable by 20 days of postnatal development. Full protection of the cRNA probe was not detected in any sample. Therefore alternative *AMPD3* mRNA species other than that represented by the RT-2 cDNA are expressed at birth, then are markedly down-regulated during postnatal hepatogenesis. These results are also consistent with the lack of any detectable *AMPD3* gene expression in adult liver.

Fibre type expression of alternative *AMPD3* mRNA species

Previous work has shown that the relative abundance of *AMPD1* mRNA species is greatest in adult hindlimb muscles composed predominantly of glycolytic fibre types [7,25]. Aliquots of the muscle RNA preparations used in this *AMPD1* analysis were employed to estimate relative *AMPD3* mRNA abundances in different muscles of the adult hindlimb. Figure 4 shows that soleus muscle, which is composed mostly of Type I, slow-twitch oxidative fibres [26], displayed the greatest abundance of *AMPD3* mRNA species. Plantaris, a mixed-fibre muscle [26], contained much smaller amounts of *AMPD3* mRNA species. Outer gastrocnemius muscle contained predominantly Type IIb, fast-twitch glycolytic fibres [26], and the abundance of *AMPD3* mRNA was below the limit of detection. Therefore, unlike *AMPD1*, relative *AMPD3* gene expression seems greatest in adult hindlimb oxidative muscle. These combined data are consistent with immunocytochemical analyses of adult human skeletal muscle showing that *AMPD1* (isoform M) and *AMPD3* (isoform E) proteins are distributed predominantly in Type II and Type I fibres respectively [27].

Characterization of rat and human *AMPD3* recombinant enzymes

Rat and human *AMPD3* cDNA species were cloned into a baculoviral vector and expressed in insect cells. Recombinant proteins were partly purified by phosphocellulose chromatography under conditions widely used to distinguish endogenous AMPD activities found in mammalian tissues and cells [1–4]. The rat and human *AMPD3* recombinant enzymes exhibited similar elution profiles from this adsorption resin during a 0.1–2 M salt gradient (Figure 5). Conversely, the human *AMPD1* recombinant enzyme was eluted distinctly later in this gradient (fractions 40–45; results not shown). Table 1 shows that rat and human *AMPD3* recombinant enzymes have greater, yet similar, affinities for substrate in the presence of 1 mM ATP. However, hyperbolic *V*–[S] profiles are exhibited regardless of whether this nucleotide effector is included in the assay.

Immunological properties of the *AMPD3* recombinant enzymes were evaluated by solution-hybridization immunoassay and Western blot analysis. Available polyclonal antisera raised against either purified rat isoform C (heart) or human isoform E (erythrocyte) were able to precipitate both *AMPD3* recombinant

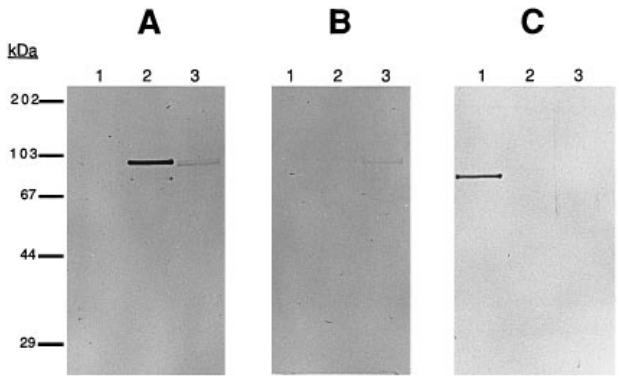


Figure 6 Western blot analysis of recombinant AMPD proteins

Human *AMPD1* (lane 1), human *AMPD3* (lane 2) and rat *AMPD3* (lane 3) recombinant proteins (0.45 unit of each) were fractionated by SDS/PAGE [9% (w/v) gel], electroblotted on nitrocellulose membranes and hybridized for 1 h at 30 °C with available polyclonal antisera raised against purified human isoform E (1:10000 dilution) (**A**), rat isoform C (1:5000 dilution) (**B**) and human *AMPD1* recombinant protein (1:5000 dilution) (**C**). Each membrane was then washed, incubated with a 1:5000 dilution of peroxidase-linked protein A for 1 h at 30 °C, re-washed and developed with commercially available luminescence reagents (Amersham). The rat *AMPD3* recombinant protein appears as a doublet, presumably reflecting a proteolytic event that produces heterogeneous N-terminal ends.

activities, whereas those raised against purified rat isoform A (skeletal muscle) or isoform B (kidney) did not interact with either of these proteins (Table 1). These results are confirmed in Western blot analyses shown in Figure 6, in which it is shown that anti-C and anti-E sera reacted with both *AMPD3* recombinant proteins, but not with the human *AMPD1* recombinant polypeptide.

Similar chromatographic, kinetic and immunological behaviours of the rat and human *AMPD3* recombinant enzymes can be expected owing to 93% identity between their predicted primary amino acid sequences. However, specificity for both anti-C and anti-E sera was not anticipated on the basis of a previous report stating 'no immunological cross-reactivity between the two enzymes' from which these reagents were raised [3]. Nevertheless, the combined data of the present study demonstrate that rat isoform C and human isoform E are homologous activities encoded by *AMPD3* mRNA species. Furthermore these data are consistent with the cross-species similarities previously established for the protein products of the rat and human *AMPD1* (isoforms A and M respectively) and *AMPD2* (isoforms B and L respectively) genes.

On the basis of the conclusion that rat *AMPD3* mRNA species encode isoform C, relative gene expression data can be related to previous observations pertaining to this AMPD activity. For example, steady-state levels of *AMPD3* mRNA species are relatively high in adult heart, the tissue from which isoform C was originally isolated and characterized [1]. Moreover, the apparent fibre type difference in skeletal muscle *AMPD3* gene expression is consistent with a report detailing an AMPD activity found in adult red (soleus), but not white (extensor digitorum longus), skeletal muscle that is chromatographically and kinetically identical with the adult heart enzyme [28]. Finally, the observed down-regulation in *AMPD3* mRNA abundance parallels diminishing levels of isoform C activity during postnatal hepatogenesis [2].

In conclusion, this study has extended the knowledge of the mammalian AMPD multigene family by detailing rat *AMPD3* cDNA sequences and showing developmental and skeletal muscle fibre type expression for alternative mRNA species produced from this gene. A nucleotide sequence common to alternative rat and human *AMPD3* mRNA species is highly conserved and predicted to encode the nearly identical isoforms C and E respectively. The delineation of the molecular basis for isoform C expression completes our understanding of all previously described rat and human AMPD activities.

This work was supported by Public Health Service Grant DK-50902 from the National Institutes of Health.

REFERENCES

1 Ogasawara, N., Goto, H. and Watanabe, T. (1975) Biochim. Biophys. Acta **403**, 530–537
2 Ogasawara, N., Goto, H., Yamada, Y. and Watanabe, T. (1978) Eur. J. Biochem. **87**, 297–304
3 Ogasawara, N., Goto, H., Yamada, Y., Watanabe, T. and Asano, T. (1982) Biochim. Biophys. Acta **714**, 298–306
4 Ogasawara, N., Goto, H., Yamada, Y. and Watanabe, T. (1984) Int. J. Biochem. **16**, 269–73
5 Ogasawara, N., Goto, H., Yamada, Y., Nishigaki, I., Itoh, T. and Hasegawa, I. (1984) Biochem. Biophys. Res. Commun. **122**, 1344–1349
6 Morisaki, T., Sabina, R. L. and Holmes, E. W. (1990) J. Biol. Chem. **265**, 11482–11486
7 Sabina, R. L., Marquetant, R., Desai, N. M., Kaletha, K. and Holmes, E. W. (1987) J. Biol. Chem. **262**, 12397–12400
8 Sabina, R. L., Morisaki, T., Clarke, P., Eddy, R., Shows, T. B., Morton, C. C. and Holmes, E. W. (1990) J. Biol. Chem. **265**, 9423–9433
9 Sabina, R. L., Fishbein, W. N., Pezeshpour, G., Clarke, P. R. H. and Holmes, E. W. (1992) Neurology **42**, 170–179
10 Morisaki, T., Gross, M., Morisaki, H., Pongratz, D., Zollner, N. and Holmes, E. W. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6457–6461
11 Morisaki, H., Morisaki, T., Newby, L. K. and Holmes, E. W. (1993) J. Clin. Invest. **91**, 2275–2280
12 Bausch-Jurken, M. T. and Sabina, R. L. (1995) Arch. Biochem. Biophys. **321**, 372–380
13 Bausch-Jurken, M. T., Mahnke-Zizelman, D. K., Morisaki, T. and Sabina, R. L. (1992) J. Biol. Chem. **267**, 22407–22413
14 Van den Bergh, F. and Sabina, R. L. (1995) Biochem. J. **312**, 401–410
15 Mahnke-Zizelman, D. K., Van den Bergh, F., Bausch-Jurken, M. T., Eddy, R., Sait, S., Shows, T. B. and Sabina, R. L. (1996) Biochim. Biophys. Acta **1308**, 122–132
16 Mahnke-Zizelman, D. K. and Sabina, R. L. (1992) J. Biol. Chem. **267**, 20866–20877
17 Mahnke-Zizelman, D. K., Eddy, R., Shows, T. B. and Sabina, R. L. (1996) Biochim. Biophys. Acta **1306**, 75–92
18 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5476
19 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20 Sabina, R. L., Ogasawara, N. and Holmes, E. W. (1989) Mol. Cell. Biol. **9**, 2244–2246
21 Krieg, P. A. and Melton, D. A. (1987) Methods Enzymol. **155**, 397–415
22 Marquetant, R., Desai, N. M., Sabina, R. L. and Holmes, E. W. (1986) Proc. Natl. Acad. Sci. U.S.A. **84**, 2345–2349
23 Chang, Z., Nygaard, P., Chinault, A. C. and Kellems, R. E. (1991) Biochemistry **30**, 2273–2280
24 Sabina, R. L. and Holmes, E. W. (1995) in The Metabolic and Molecular Bases of Inherited Disease, 7th edn., (Scriver, C., Beaudet, A., Sly, W. and Valle, D., eds.), pp. 1769–1780, McGraw-Hill, New York
25 Mineo, I., Clarke, P. R. H., Sabina, R. L. and Holmes, E. W. (1990) Mol. Cell. Biol. **10**, 5271–5278
26 Armstrong, R. B. and Phelps, R. O. (1984) Am. J. Anat. **171**, 259–272
27 van Kuppevelt, T. H., Veerkamp, J. H., Fishbein, W. N., Ogasawara, N. and Sabina, R. L. (1994) J. Histochem. Cytochem. **42**, 861–868
28 Raggi, A., Bergamini, C. and Ronca, G. (1975) FEBS Lett. **58**, 19–23

Received 28 January 1997/7 May 1997; accepted 15 May 1997