Characterization of human AMP deaminase 2 (AMPD2) gene expression reveals alternative transcripts encoding variable N-terminal extensions of isoform L

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AMP deaminase (AMPD) is a highly regulated enzymic activity and multiple isoforms of this enzyme are coded for by a multigene family in mammalian species, including man. Isoform L (liver) is the main activity present in adult human liver and is the protein product of the AMPD2 gene, which is widely expressed in non-muscle tissues and cells. A previous report described almost the full-length cDNA sequence and part of the human AMPD2 gene and also presented Northern blot evidence for multiple transcripts in brain. This study was performed to further characterize the AMPD2 gene and its expression in human tissues. AMPD2 genomic and human cerebellum cDNA clones were isolated, sequenced and used as probes in RNase protection analyses which together demonstrated the following: (1) an intervening sequence near the 5′-end of the published AMPD2 cDNA, which affects the predicted N-terminal amino acid sequence of isoform L; (2) alternative transcripts resulting from exon shuffling at, or near, the 5′-end of the AMPD2 gene that exhibit tissue-specific patterns of relative abundance; (3) predicted usage of three different initiation codons to confer variable N-terminal extensions on isoform L polypeptides; and (4) an extension of a 3′ untranslated sequence in some AMPD2 transcripts. In addition, reverse transcriptase PCR and additional RNase protection analyses were used to map the 5′-ends of two mutually-exclusive exon 1 sequences, both of which contain multiple transcription-initiation sites. These results are discussed in relation to predicted isoform L diversity across human tissues and cells.

INTRODUCTION

AMP deaminase (AMPD; EC 3.5.4.6) is a highly regulated enzyme involved in purine nucleotide catabolism and interconversion (i.e. $AMP + H_2O \rightarrow IMP + NH_3$). Multiple isoforms, each exhibiting unique patterns of tissue distribution and kinetic, immunologic and chromatographic properties, can be isolated from rat [1,2] and human [3,4] tissues. In humans, four homotetrameric variants have been described, including isoforms M (muscle), L (liver) and the E1 and E2 (erythrocyte) isoforms. Most tissues and cells express more than one AMPD isoform [1–4] and evidence has been presented suggesting that in addition to homotetramers, heterotetramers also exist that form among individual isoform-specific subunits [5].

Molecular studies indicate that mammalian AMPDs are encoded by transcripts produced from a multigene family. In humans, the AMPD1 gene is specific for isoform M [6], the AMPD2 gene for isoform L [7] and the AMPD3 gene for the E isoforms [8]. Alignments among predicted amino acid sequences across the human AMPD multigene family show that the C-terminal region is conserved whereas the N-terminal region is divergent [7,8]. Alternative transcripts are produced from the human AMPD1 [9] and AMPD3 [8] genes, each containing different sequences at, or near, their 5′-ends. In most cases, alternative AMPD transcripts are predicted to confer N-terminal variation on their respective isoform, contributing to the complexity of AMPD expression.

A single report, describing the cloning, sequencing and bacterial expression of AMPD2 cDNA, represents currently available knowledge of this human gene [7]. Prokaryotic expression of AMPD2 cDNA generates isoform L activity. Northern blot analysis of multiple human tissues indicates that the AMPD2 transcript is approximately 4 kb in size, significantly larger than the reported 3.3 kb cDNA sequence. The presence of a poly(A)$^+$ tail on the 3′-most extending AMPD2 cDNA (HuPl 10B/16) infers that the 5′-end of this sequence is most likely missing. In addition, a slightly smaller and more abundant AMPD2 transcript is observed predominantly, if not exclusively, in brain.

This study was designed to identify missing AMPD2 cDNA sequences and to address the molecular basis for production of multiple transcripts. To accomplish these goals, a human cerebellum (HuCe) cDNA library was screened using a previously described human insert comprising most of the available 5′ AMPD2 cDNA sequence (HuT 6A). The results of this study correct and extend previous information regarding the AMPD2 cDNA sequence and show regulated expression of multiple AMPD2 transcripts, similar to other members of this multigene family.

EXPERIMENTAL

Materials

Chemicals were purchased from Sigma. Restriction endonucleases, nucleotides, modifying enzymes and molecular biology-grade reagents were obtained from Boehringer-Mannheim. Bacterial growth media were purchased from GIBCO-

The nucleotide sequence data reported in this paper appear in the GenBank Nucleotide Sequence Database under accession numbers U16267–U16272.

Abbreviations used: AMPD, AMP deaminase; HuCe, human cerebellum; RT, reverse transcriptase; RPE cells, retinal pigment epithelial cells; pBS, plasmid Bluescribe.

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BRL Life Technologies, Inc. Agar was obtained from Difco Laboratories and electrophoresis-grade agarose was supplied by Bio-Rad Laboratories. Seaplaque low-melting agarose was purchased from FMC Corp. Immobilon-NC nitrocellulose filters (0.45 μm) were obtained from Millipore Corp. The PolyATtract mRNA isolation system, used to purify poly(A)$^+$ mRNA, was purchased from Promega Corp. A 5′-AmpliFINDER kit, used to synthesize additional AMPD2 cDNAs, was supplied by CLONTECH Laboratories, Inc. All radioisotopes used for labelling probes ([α-^{32}P]dCTP and [α-^{32}P]UTP) and sequencing ([α-^{35}S]dATP) and a random-prime labelling kit were purchased from Amersham. A kit for sequencing was obtained from United States Biochemical Corp. Kodak X-OMAT AR Scientific Imaging Film was used for all autoradiography.

Library screening

Approximately 1.0×10^6 plaque-forming units of a commercially available HuCe 5′-STRETCH λgt10 cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA, U.S.A.) were used to infect *Escherichia coli* strain C600 Hfl, plated in 150 mm^2 dishes on Luria broth agar, and duplicate filters were prepared. Filters were hybridized at relatively high stringency (0.6 M NaCl and 67 °C) using an [α-^{32}P]dCTP-labelled insert of a previously described human AMPD2 cDNA, HuT 6A [7]. Overnight exposure of the washed filters yielded 16 duplicate positives, and 11 were plaque-purified in subsequent rounds of screening. Following *Eco*R1 restriction endonuclease digestion of isolated phage DNA, recombinant inserts were gel-purified and subcloned into the polylinker region of plasmid Bluescribe (pBS; Stratagene Cloning Systems, La Jolla, CA, U.S.A.). Both strands of all recombinant plasmid cDNAs were sequenced by the dideoxy chain-termination method [10] utilizing universal, reverse and designed oligonucleotide primers. The designed oligonucleotides were derived from cDNA sequences obtained from the use of the universal and reverse primers and synthesized commercially (Operon Technologies, Inc., Alameda, CA, U.S.A.) or from the Protein and DNA Synthesis facilities of the Medical College of Wisconsin (funded in part by United States Public Health Service Grant RR03326).

Reverse transcriptase (RT)-PCR amplification and cloning of AMPD2 cDNAs

RT-PCR analysis was used to generate additional human AMPD2 cDNAs representing the 5′-ends of alternative mRNAs identified by conventional library screening. Nested antisense oligonucleotides were designed from sequences contained in one or the other of two mutually-exclusive 5′ terminal exons (see underlined sense sequences in Figure 4b for relative locations).
Exon 1A:

RT-oligo, 5′-AACCCTGCAATCGAGAGGCTGGTA-3′

PCR-oligo, 5′-CCGAAACTCGTCCAGATTTCTCTC-3′

Exon 1B:

RT-oligo, 5′-AAGAGCCCTCACCCCATCATGCCA-3′

PCR-oligo, 5′-CCAGGCTAGCTGGTCCTTCCAGC-3′

Eight anomolous bases (CCCGGGAT) were added to the 5′-end of the PCR-oligonucleotides in order to create *Sma*1 and *Bam*H1 restriction endonuclease sites that facilitated subcloning of the resultant products. Employing the commercially available 5′-AmpliFINDER kit, these primers were used to synthesize AMPD2 cDNAs from poly(A)$^+$ mRNAs isolated from human retinal pigment epithelial (RPE) cells and placenta. RPE cell poly(A)$^+$ mRNA was prepared from isolated total cellular RNA using the commercially available polyATtract kit and placental poly(A)$^+$ mRNA was provided as a positive control in the 5′-AmpliFINDER kit. RT-PCR was performed according to the suppliers instructions.

RNase protection analyses

RNase protection analysis was used to estimate relative abundances of alternative AMPD2 transcripts in human tissues and cells and to map their 5′-ends. Total cellular RNAs were isolated from an available frozen human liver biopsy (provided by Dr. C. Myers, Department of Pharmacology, Medical College of Wisconsin, WI, U.S.A.) and from human RPE cells (provided by Dr. J. Burke, Department of Ophthamology, Medical College of Wisconsin) by the guanidinium/caesium chloride method [11]. Total cellular RNA isolated from HuCe was obtained commercially (CLONTECH Laboratories, Inc.). Total cellular RNAs were used to protect [α-^{32}P]UTP-labelled cRNA probes designed from human AMPD2 cDNA and genomic DNA inserts after the method of Krieg and Melton [12]. See text for descriptions of individual probes.

Computer analysis of predicted amino acid sequences

Computer-assisted analyses of predicted primary amino acid sequences were performed using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (UWGCG; Madison, WI, U.S.A.).

RESULTS

Isolation and sequencing of HuCe AMPD2 cDNA clones

Sixteen duplicate positives were identified in an initial screening of a HuCe cDNA library. Eleven were plaque-purified, their inserts isolated, subcloned into plasmid Bluescribe and sequenced. Alignments with the previously published HuPl 10B/16 cDNA sequence [7] demonstrate that all are AMPD2 cDNAs; their relative sizes and locations are illustrated in Figure 1. As expected, each overlaps the HuT 6A insert used as the probe for screening the library. Five are either completely contained within the probe sequence or extend only in the 3′ direction. Of these, four do not contribute any new information, i.e., two (HuCe 9B and 10B) are identical and extend only 638 bp from the 3′-end of the probe sequence, and two (HuCe 6B and 8) are contained entirely within the area covered by the probe, except for some anomalous sequences at their 5′-ends, as judged by alignments with other AMPD2 cDNA and genomic clones (results not shown). However, the fifth clone (HuCe 1) is nearly 3 kb in length and extends 85 bp beyond the 3′-end of the previously published AMPD2 cDNA sequence (see below).

The other six newly isolated AMPD2 cDNAs (HuCe 3B, 6A, 7A, 7B, 10A and 11) all extend 5′ to the HuT 6A sequence. Significantly, all diverge from the published AMPD2 cDNA sequence beginning at the nucleotide +27/+28 boundary (Figure 2). Moreover, five (HuCe 6A excluded) contain the published nucleotide sequence −175 to −181 immediately adjacent to nucleotide +28. Further inspection of the published AMPD2 nucleotide sequence from −174 to +27 shows GT and AG dinucleotides at the 5′- and 3′-boundaries respectively (see Figure 2 and [7]). Taken together, these data suggest the presence of an unprocessed intervening sequence in the original HuPl 10B/16 clone, extending from nucleotide −174 to +27.

Each of the five newly isolated AMPD2 cDNAs containing nucleotides −175 to −181 immediately adjacent to nucleotide

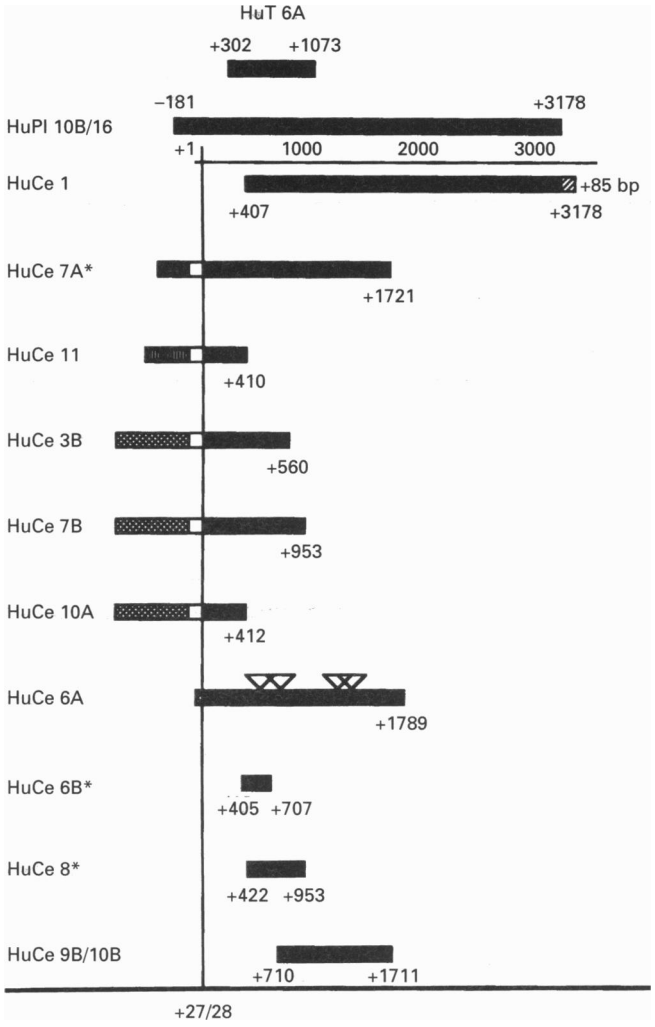


Figure 1 Schematic illustration of HuCe AMPD2 cDNAs

Insert sizes are relative to the upper horizontal axis, which is in bp. Where possible, the 5′- and 3′-end of each cDNA is numbered according to the previously published AMPD2 cDNA sequence [7], which is denoted by a black bar (■). Sequences extending 3′ to the previously published AMPD2 cDNA in the HuCe 1 clone are denoted by the hatched bars (▨). Stretches of identical sequence extending 5′ to nucleotide +28 of the previously published AMPD2 cDNA are indicated by white bars (□), vertically-striped (▥) and cross-hatched (▩) bars. The size and relative location of the original AMPD2 cDNA (HuPI 10B/16) and the one used as the probe for screening the HuCe library (HuT 6A) [7] are indicated above the upper horizontal axis. Asterisks (*) denote cDNAs found to contain anomalous sequences at their 5′-ends (see text). The relative position of intervening sequences in clone 6A are indicated by inverted triangles (▽).

+28 continue to align upstream for another 124 bp, at which point additional divergence is observed (see Figure 2). These observations indicate that the 131 bp sequence immediately upstream of nucleotide +28 comprises an exon (boxed sequences in Figure 2). Furthermore, absence of the 131 bp sequence from the sixth clone, Huce 6A, suggests that this putative exon is subject to an alternative splicing event which can remove it from some mature AMPD2 transcripts.

Additional upstream nucleotide sequence divergence is observed immediately adjacent to the putative 131 bp exon. Two of the HuCe cDNA clones, 7A and 11, contain 160 bp of identical sequence up to a point near the 5′-end of the former (see Figure 2). The HuCe 7A clone then has an additional sequence at its 5′-end that appears anomalous, based on alignments with AMPD2 genomic DNA (results not shown; see below). However, the HuCe 11 cDNA clone continues upstream for an additional 227 bp.

The HuCe 3B, 7B and 10A cDNAs differ from HuCe 7A and 11 immediately upstream of the putative 131 bp exon sequence (see Figure 2). Each has 538 bp of identical sequence to their 5′-ends, which all terminate in an *Eco*R1 restriction endonuclease site (GAATTC). This *Eco*R1 site appears to be included in the AMPD2 sequence, as judged by the absence of an adjacent linker sequence, i.e. GC, found in all other clones isolated from this library. The presence of an endogenous *Eco*R1 restriction endonuclease site in these three cDNA sequences suggested the possibility of an additional 5′-end sequence in the original phage insert. Therefore, more phage DNA was prepared from each of these three isolates and an additional 75 bp *Eco*R1 fragment was subsequently recovered from one, HuCe 10A, the 5′-end of which contains the appropriate linker sequence.

The HuCe 6A clone, which is missing the putative 131 bp exon immediately upstream of nucleotide +28, does contain a 29 bp sequence at its 5′-end that is identical with the 3′-end of the 538 bp stretch in clones 3B, 7B and 10A (see Figure 2). HuCe 6A also contains four downstream intervening sequences (see Figure 1), information that will be presented in a separate manuscript detailing the structure of the entire human AMPD2 gene.

The combined sequence data obtained from these six HuCe isolates suggests that multiple transcripts are produced from the human AMPD2 gene. Figure 2 shows that the sequence 3′ to the nucleotide +27/+28 boundary is found in all AMPD2 cDNA clones. Conversely, the 131 bp sequence immediately upstream of nucleotide +28 in five of six HuCe cDNA clones appears to represent an exon subject to an alternative splicing event that can remove it from some mature AMPD2 transcripts (as evidenced by the HuCe 6A clone). Finally, the two divergent sequences at the extreme 5′-ends of all six HuCe cDNAs exhibit a mutually exclusive pattern of inclusion in these clones and appear to represent two additional upstream exons in the AMPD2 gene.

Isolation and partial characterization of AMPD2 genomic DNA

In order to validate the above hypotheses and clarify ambiguities derived from sequence analysis of the newly isolated cDNAs, it was necessary to examine AMPD2 genomic DNA. This was accomplished using subclones from two AMPD2 clones, RPMI-1s and RPMI-9, previously isolated from a human genomic library [7]. The significance of the extended 3′ sequence found in the HuCe 1 cDNA was addressed first by isolating a 6 kb *Sph*1 fragment from the RPMI-1s clone that begins in intron 10 (results not shown) and extends beyond the 3′-end of the gene. Exon 18 is co-linear with the previously published cDNA sequence and contains the 3′-end of the AMPD2 coding sequence, the predicted translation termination codon, T(U)GA, and the entire 895 bp 3′ untranslated region of the gene (Figure 3). Previously defined by the 3′-end of the HuPI 10B/16 cDNA clone, the 3′ untranslated region extends to nucleotide +3178, twelve base pairs downstream from a consensus poly(A)$^+$ signal (aataaa) and adjacent to the poly(A)$^+$ tail in the HuPI 10B/16 cDNA clone (Figure 3). However, an alignment between the newly isolated HuCe 1 cDNA and the AMPD2 genomic DNA sequence demonstrates nucleotide identity extending 85 bp beyond nucleotide +3178 to the 3′-end of the cDNA clone (Figure 3). This combined information extends the 3′-untranslated region and suggests multiple poly(A)$^+$ consensus signals may reside within the AMPD2 gene. However, no such sequence was found

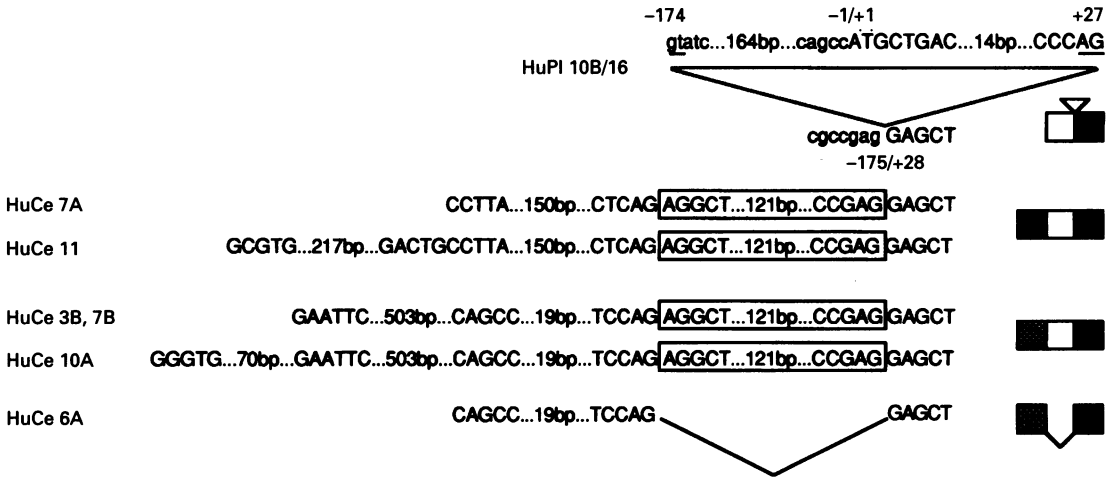


Figure 2 Schematic illustration of sequence heterogeneity found at the 5′-end of HuCe cDNA clones

All diverge from the original, near full-length AMPD2 cDNA, HuPI 10B/16 [7], at nucleotide +28. Five of the HuCe clones (3B, 7A, 7B, 10A, 11) contain a 131 bp sequence (boxed) immediately adjacent to nucleotide +28, the 3′- end of which is identical with nucleotides −175 to −181 in the HuPI 10B/16 cDNA. The suspected intervening sequence in the HuPI 10B/16 cDNA (nucleotides −174 to +27) is denoted above the inverted triangle and is bordered on its 5′- and 3′-ends by GT and AG dinucleotides (underlined) respectively. Two different patterns of 5′-end sequence are found adjacent to the 131 bp sequence in the HuCe cDNAs: clones 7A and 11 and clones 3B, 7B and 10A. The latter pattern is also found at the 5′-end of the HuCe 6A cDNA, although this clone is missing the internal 131 bp sequence found in the others. These two different sequences extend to the 5′-ends of each clone, except 7A, which has an anomalous sequence at its 5′-end (sequence not shown; see text). Taken together, these data suggest four different AMPD2 exons, three of which are variably expressed in different HuCe cDNA clones in the patterns presented to the right of their representative cDNA(s). The proposed exons, and their pattern of expression, are: one which is composed of a sequence 3′ to nucleotide +28, and common to all clones (■); a second containing the internal 131 bp sequence (□) that is either retained or excluded in these clones; a third (▥) and fourth (▩) that appear 5′ to the others, but never together in the same clone.

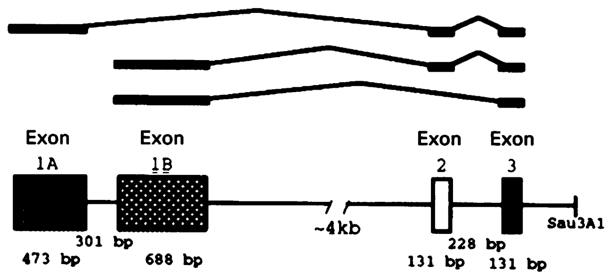
Figure 3 Evidence for heterogeneity at the 3′-end of human AMPD2 transcripts

Alignment across the 3′-ends of the previously published HuPI 10B/16 cDNA (top), the HuCe 1 cDNA (middle), and the corresponding area of the AMPD2 gene (bottom). Inspection of the latter demonstrates a 149 bp intron (intron 17) between the nucleotide +2073/+2074 boundary of the published AMPD2 cDNA sequence [7]. Intron 17 is bordered by GT and AG dinucleotides (underlined) at its 5′- and 3′-ends respectively. All three sequences are identical from nucleotides +2073/+2074 to the 3′-end of the HuPI 10B/16 cDNA sequence (nucleotide +3178), which ends in a poly(A)$^+$ tail. The predicted termination codon (TGA) and consensus poly(A)$^+$ signal (aataaa) are underlined. The HuCe 1 cDNA and AMPD2 genomic DNA sequences continue to align up to the 3′-end of the former, an additional 85 bp beyond nucleotide +3178.

in genomic DNA within 200 bp downstream of the 3′-end of the HuCe 1 clone (results not shown).

A third overlapping genomic clone, labelled RPMI-2A, was not reported in the initial human library screening [7]. However, the 3′-end of this clone was subsequently found to contain the various divergent 5′-ends of the AMPD2 cDNA sequence. A physical map of the 3′-end of RPMI-2A is presented in Figure 4(a) and shows the relative location of four exons. The 3′-most exon, labelled exon 3, contains the published sequence from nucleotides +28 to +158 [7] and is bordered by AG and GT dinucleotides at its 5′- and 3′-ends respectively (Figure 4b). The next exon, labelled exon 2, is located 228 bp upstream and contains the 131 bp sequence found in five of the newly isolated AMPD2 cDNA clones (see above). Exon 2 is also bordered on its 5′-and 3′-ends by AG and GT dinucleotides respectively (Figure 4b). Moreover, the 228 bp intervening sequence between exons 2 and 3 is identical with nucleotides −175 to +27 in the published AMPD2 cDNA sequence, except for a 27 bp insertion at the nucleotide −39/−40 boundary (see Figure 4b and [7]). However, a re-examination of the original HuPI 10B/16 cDNA sequencing gel has revealed that these 27 nucleotides were inadvertently omitted from the original report.

(a)



(b)

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        1A                                                                1B
        -220 ggccctacct ctggccaggc actgtgtaaa ggacttacgt ttaatcttca -945
        -170 cagtaacccc tttgagtgag gtactgtcat taccccatct tactgatgag -895
        -120 gaactggacg cacggaactg gcccaagacc cagggtcggc tcaggcctgc -845
        - 70 cctaccgcgg tgtggtggct gcccacgtgg cgcccaggaa gagaaaaaaa -795
        - 20 tggcgaggcg gggtcggcag GCGGAGGCGG GGCCAGGCGG GGGCGGGGCC -745
        + 31 AAGGGCCGCA GAGCCTGGCG CGGAGCCGGC GAGATTTTGG TGGGGTCTCA -695
        + 81 CCTGTTGCGT GACTCCCCA CAGTCCGGCC GCGGGAGTCC GACCCTGAAT -645
        +131 GCCCAGGGAG TGTTGAGAGA AATCTGGACG AGTTTCGGGT CCCGCTCCCT -595
Exon 1A +181 TGGGAGCACG TGGCCTACCA GCCTCTCGAT TGCAGGGTTG GGTGGTCGCG -545
        +231 ACACCGGGGT CGCCTTGAGG CCAGTCCGGC TGCCGAGGTC TGCGGGAGTC -495
        +281 CACCTCCGGC CAGCTGGCAA TTTTGAAAGA CTCCTTACT TTCCCCATCT -445
        +331 CAGTGCCAGG GCAGGGGCCC TTGGAGTGAC TTGGCTGGGG TCTGTGGCCC -395
        +381 GATCCCCCTG CCGTCCCTCA GGACCCGGGC TTTCTGCTGT ACAGACTTCT -345
        +431 CGTGGGCAGC CTCCCCCTCG GAACTCGGGC ATCATGGCCT CAGgtgagtg -295
             tgtgtacgtg tgttggaggg agggtctctg ggacagagaa gtgctgtggc -245
             cagagtgaca agagggtggg agtcacagca cagcagcagg acctagggaa -195
             ggcggtcagc tcctcccagg agggcctggg gagcagagcc agagtgtgca -145
             gagacagagg aaaaatccga agaggctagg gtctctgtcc cagaccccca - 95
             gacaaggggg tctcctggcc ttcctgcctt cctgggaggt agctatttgg - 45
             cgacggtgga atttcccgg ggacaggccc ctccctcctg ggctGGGATT +  6
             CTCGGTTCTT CCCCCTCCTC CCAGAGCTGG GCTACCCCGG GAGCTGAGGG + 56
             TCAGATGTGG AAGGAAAGTG GGTGCTGGGT TTTCACGGAG TATGCCTGCC +106
             CTGCCCCGGG AGGACTGTGT TTGAGCTTGG ACTCGCTCGC TGCTGAATTC +156
             CTGTTTGTTA TTCTTTTTTC TTCCAGGCTA GCTGGAAGGA CCAGCTAGCC +206
             TGGACTTTGG CTTCTTTGCC CGGGAGCCCT GGCATGATGG GGTGAGGGCT +256
Exon 1B      CTTGGTGGGT TAGTGGAGAG CTGGGGGCTG GCCCTTCCCC ATCTTCTTTT +306
             CTACCCACCC CCTCCCCGCC CCCCGCCAGG CCCAGCCACC ATCAGTCACG +356
             TCACTCCTGG GACTGAGGAG GCAGGGGAGG GATAAGGGGC AGAGATGGAG +406
             GCCCCACTCC CCGAGGTTGC CTAGACAACA TGAGAAATCG TGGCCAGGGC +456
             CTCTTCCGCC TGCGGAGCCG CTGCTTCCTG CATCAGTCAC TCCCGCTGGG +506
             GGCGGGGCGG AGGAAGGGGT TGGATGTGGC AGAGCCAGGC CCCAGCCGGT +556
             GCCGCTCAGA CTCCCCCGCT GTCGCCGCCG TGGTCCCAGC CATGGCATCC +606
             TATCCATCTG GCTCTGGCAA GCCCAAGGCC AAATATCCCT TTAAGAAGCG +656
             GGCCAGCCTG CAGGCCTCCA CTGCAGCTCC AGgtgatgtg tgtgcttcct +688
             ggcctcagga tagatgctgg .. ~4kb -> Exon 2

             ttcaggggct gcccctcctc cctttgaccc tggcatcact gtctctgcag 1B-2
        +474 AGGCTCGGGG TGGTCTGGGG GCCCCTCCGC TGCAGTCTGC CCGATCCCTG +738
Exon 2  +524 CCGGGCCCCG CCCCCTGCCT CAAGCACTTC CCGCTCGACC TGCGCACGTC +788
        +574 TATGGATGGC AAATGCAAGG AGATCGCCGA Ggtatcacct ggcaccaccc +819
             gcaccctcac cccgtgtctc catgccctgc ctctgctccc cacacccctc
             acctcaagct gtcccctcac ctcacgcttg gctgtctcct gatcctcagc
             ctctcccagg tacccctggt cctgctgccc tcaccccatc cccagactct      1B-3
             gtaggagagt gcccgagggc ggagggccag ccatgctgac cttccttccc
        +605 tccccccagG AGCTGTTCAC CCGCTCACTG GCTGAGAGCG AGCTCCGTAG +860 +729
Exon 3  +646 TGCCCCGTAT GAGTTCCCCG AGGAGAGCCC CATTGAACAG CTGGAGGAGC +910 +779
        +696 GGCGGCAGCG GCTGGAGCGG CAGATCAGCC AGGATGTCAA gtgagcccgg +950 +839
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Figure 4 The 5′-end of the human AMPD2 gene

(**a**) A physical map of the 3′-end of the AMPD2 genomic phage clone, RPMI-2A, which contains the entire 5′-end of the gene. Using complimentary oligonucleotides, designed from the cDNA sequence as primers in sequencing reactions of genomic DNA subclones, four exons are found within 7 kb upstream of the 3′-end (*Sau*3A1 restriction endonuclease site) of this clone. Exon 3 (■) contains 131 bp encompassing nucleotides +28 to +158 in the published HuPl 10B/16 cDNA sequence [7] and is found in all AMPD2 cDNA clones. Exon 2 (□) contains 131 bp that

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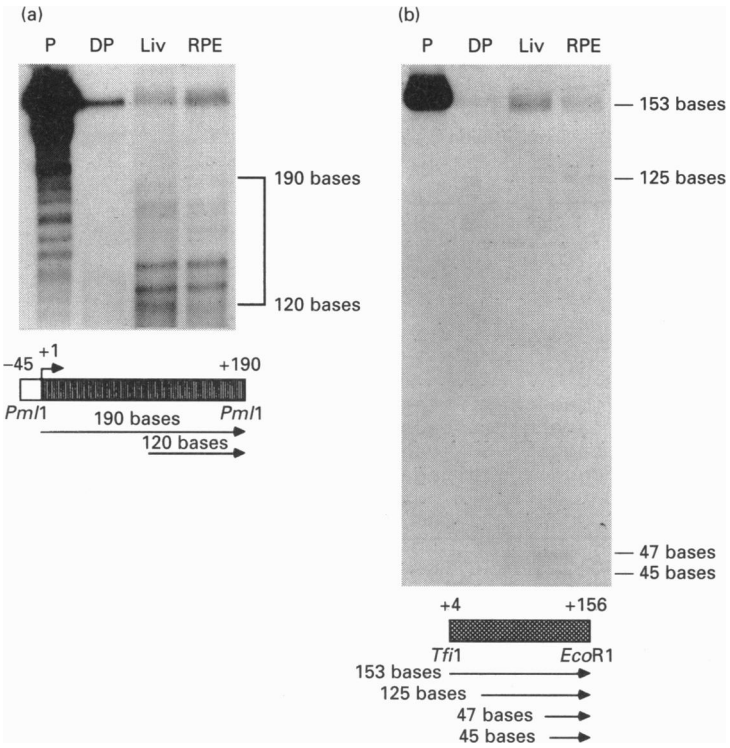

Figure 5 RNase protection mapping of transcription-initiation sites in exons 1A and 1B

Two AMPD2 genomic cRNA probes were synthesized for use in protection assays with total cellular RNAs isolated from human adult liver (Liv, 50 μg) and RPE cells (20 μg). (**a**) A recombinant plasmid containing a *Pml*1 insert, isolated from the AMPD2 genomic phage clone RPMI-2A, was used to generate a cRNA probe containing 235 bases complementary to exon 1A and the proximal 5′ flanking sequence. P, undigested probe (contains 22 bases and 12 bases of polycloning and/or pBS sequence at its 5′- and 3′-ends respectively); DP, digested probe (tRNA control). The size range of multiple protected fragments is denoted in bases. (**b**) A recombinant plasmid containing a *Pvu*II–*Eco*R1 insert, isolated from the AMPD2 genomic phage clone RPMI-2A, was linearized with *Tfi*1 and used to generate a cRNA probe containing 153 bases complementary to exon 1B. P, Undigested probe (contains four bases of pBS sequence at its 5′-end); DP, digested probe (tRNA control). Protected fragment sizes are denoted in bases.

Two other exons, labelled 1A and 1B, are remotely located approx. 5 kb upstream of exon 2 and are also bordered on their 3′-ends by GT dinucleotides (see Figures 4a and 4b). Exon 1A contains the entire sequence located at the 5′-end of the HuCe 11 cDNA clone and confirms the ambiguous nature of the sequence at the 5′-end of the HuCe 7A clone. Exon 1B contains the sequence at the 5′-ends of the Huce 3B, 7B and 10A cDNA clones, including the *Eco*R1 restriction endonuclease site that joins this sequence with the additional 75 bp *Eco*R1 fragment isolated from the HuCe 10A phage DNA.

Mapping the 5′-ends of alternative human AMPD2 transcripts

The preceding experiments establish that multiple human AMPD2 mRNAs are produced through shuffling of three exon sequences at, or near, the 5′-end of the gene. Furthermore, either the exon 1A or 1B sequence is found at the 5′-terminus of all isolated cerebellum cDNAs extending upstream from the previously reported HuPl 10B/16 cDNA. These data indicate that exons 1A and 1B may be mutually exclusive 5′-terminal sequences in human AMPD2 transcripts. RT-PCR analysis was performed to examine whether transcription of alternative AMPD2 transcripts initiates in exons 1A and 1B. Nested antisense oligonucleotide primers designed from exon 1A and 1B sequence (see Figure 4b) were used to generate additional cDNAs from poly(A)$^+$ mRNAs isolated from human RPE cells and placenta. In total, 23 new cDNAs (eight exon 1A and 15 exon 1B) were isolated and sequenced. Sixteen originated from RPE mRNA and seven from placenta. All are continuous with AMPD2 genomic DNA sequences upstream from the 5′-ends of the longest cerebellum cDNAs. The 5′-ends of exon 1A cDNAs begin within a stretch of 30 nucleotides (see Figure 4b). Similarly, the 5′-ends of exon 1B cDNAs begin within a stretch of 50 nucleotides, although ten of the 15 initiate within 7 bp of each other (see Figure 4b). These results support the contention that exons 1A and 1B are 5′-terminal sequences in human AMPD2 mRNAs, and further indicate that transcription may initiate at multiple sites within both of these exons.

In order to confirm the results of RT-PCR analysis, RNase protection analysis was also employed to map the 5′-ends of alternative AMPD2 mRNAs. Two cRNA probes, designed from the AMPD2 genomic DNA sequence, were generated from linearized subclones that spanned presumed regions of transcription initiation in exons 1A and 1B. The exon 1A cRNA probe was produced from a 233 bp *Pml*1 fragment (see Figure 4b) isolated from an AMPD2 genomic phage clone, RPMI-2A, and subcloned into the *Sma*1 polycloning site of pBS. The recombinant plasmid was sequenced to establish insert orientation and subsequently linearized at an *Xba*1 restriction endonuclease polycloning site located adjacent to the 5′-end of the insert. Using T7 RNA polymerase, a 269 base cRNA was produced that contained 235 bases (one base of each flanking, partial *Sma*1 site was identical with the corresponding base in the AMPD2 genomic DNA) complementary to the 5′-end of exon 1A and the proximal flanking sequence. Also included were 22 and 12 bases of a pBS polycloning sequence at the 5′- and 3′-ends of the probe respectively. The exon 1B cRNA probe was produced from a 637 bp *Pvu*II(5′)–*Eco*R1(3′) fragment isolated from AMPD2 genomic phage clone, RPMI-2A, subcloned into the *Sma*1(5′)–*Eco*R1(3′) polycloning sites of pBS. The recombinant

(*Figure 4 legend contd.*)

are variably found in AMPD2 cDNA clones. Exons 2 and 3 are separated by a 228 bp intron which is retained in the HuPl 10B/16 cDNA. Exons 1A (▥) and 1B (▩) are remotely located approx. 4 kb upstream of exon 2. Also shown above the physical map are identified patterns of exon shuffling in the 5′-end of the gene. (**b**) Partial sequence. All exons (upper case letters) are bordered on their 3′-ends by gt dinucleotides. Exons 2 and 3 are bordered on their 5′-ends by ag dinucleotides. In both cases, the 5′-ends of exons 1A and 1B are defined by the most upstream site of transcription initiation, which are labelled as nucleotide +1. Multiple transcription-initiation sites are used in both exon 1A and 1B. These were identified by sequencing AMPD2 cDNAs generated in RT-PCR analyses (5′-ends are denoted by closed circles above bases; stacked circles indicate multiple clones with identical 5′-ends) and by the sizes of partially-protected fragments of AMPD2 genomic cRNA probes (exon 1A, bidirectional arrow flanked by open circles denotes multiple fragments representing numerous initiation sites spread over this entire stretch of nucleotides; exon 1B, open circles above individual nucleotides denote discrete sites). Bases in exons 1A, 1B, 2 and 3 are numbered relative to nucleotide +1 in each alternative mRNA. The 5′ flanking regions to exons 1A and 1B are also numbered relative to nucleotide +1. Predicted start codons (ATG) in exons 1A and 1B are boxed (nucleotides +464–466, exon 1A-2 configuration; nucleotides +436–438, exon 1B-2 configuration; nucleotides +530–532, exon 1B-3 configuration). The 5′-ends of the AMPD2 cDNA sequence in HuCe clones 7A and 6A and HuPl 10B/16 are denoted by circled bases in exons 1A (nucleotide +314), 1B (nucleotide +660) and 2 (nucleotide +598, exon 1A-2 configuration) respectively. Relevant restriction endonuclease sites (see text) are heavily underlined: CACGTG, *Pml*1; CCCGGG, Sma1; GATTC, Tfi1; GAATTC, *Eco*R1; CCATGG, *Nco*1; TGCGCA, *Fsp*1; CAGCTG, *Pvu*II. Finally, sequences complementary to nested oligonucleotide primers used in RT-PCR analyses are underlined in exons 1A and 1B.

plasmid was linearized at a *Tfi*1 restriction endonuclease site (GATTC; see Figure 4b) and T7 RNA polymerase was used to generate a 157 base cRNA that included 153 bases complementary to the 5′-end of exon 1B and four bases of pBS sequence at its 5′-end.

Figure 5 illustrates the results of analyses using total cellular RNAs isolated from human adult liver (50 μg) and RPE cells (20 μg) to protect these probes. Figure 5(a) shows multiple protected fragments of the exon 1A probe ranging in size from 120–190 bases. Moreover, similar protection patterns are evident with adult liver and RPE cell RNAs. Figure 5(b) reveals that while both sources of RNA predominantly protect the entire 153 bases of the AMPD2 sequence in the exon 1B probe, patterns of partial protection differ. Adult liver RNA protects 45 and 47 base fragments and RPE cell RNA protects a 125 base fragment.

These combined RNase protection results complement those derived from the RT-PCR analyses. Protected fragment sizes of both probes indicate that AMPD2 mRNAs extend upstream across regions defined by the variable 5′-ends of exon 1A and exon 1B cDNAs generated by RT-PCR analysis (see Figure 4b). Taken together, these data establish that AMPD2 gene transcription initiates at multiple sites in both exons 1A and 1B.

RNase protection analyses of AMPD2 transcript expression

Four cRNA probes were designed for use in additional RNase protection analyses to estimate alternative AMPD2 transcript abundances in human tissues. The first probe was designed from the 3′-end of the HuCe 1 cDNA and was used to examine heterogeneity at the 3′-end of AMPD2 transcripts. This probe extended 187 bases upstream to the middle of a *Pvu*II restriction endonuclease site (nucleotide +3077; see Figure 6). AMPD2 transcripts terminating at nucleotide +3178 were expected to protect only 102 bases, i.e. nucleotides +3077 to +3178. Conversely, AMPD2 transcripts extending beyond this site were expected to fully protect the 187 bases of the AMPD2 sequence in this cRNA probe. Figure 6 shows that total cellular RNA isolated from HuCe predominantly protects a 102 base fragment. Nevertheless, detection of a 187 base fragment shows that a small percentage of AMPD2 transcripts in HuCe extend beyond nucleotide +3178.

Three additional cRNA probes were designed to distinguish AMPD2 transcripts differing at, or near, their 5′-ends. The HuCe 11 (exon 1A-2 configuration), 3B (exon 1B-2 configuration) and 6A (exon 1B-3 configuration) cDNAs were chosen to represent different patterns of exon shuffling, as illustrated in Figure 2. The first two of these three cRNA probes were composed of an antisense sequence located between an *Fsp*1 restriction endonuclease site in exon 2 (TGCGCA; see Figure 4b) and the 5′-ends of either the HuCe 11 or 3B cDNA inserts. The third cRNA probe was composed of an antisense sequence located between a *Pvu*II restriction endonuclease site in exon 3 (CAGCTG; see Figure 4b) and the 5′-end of the HuCe 6A insert. The three AMPD2 cDNAs were digested with *Eco*R1 (5′-end) and either *Fsp*1 (HuCe 11 and 3B) or *Pvu*II (HuCe 6A), and fragments of the expected sizes were isolated and subcloned into the *Eco*R1 and *Sma*1 (blunt-end) polycloning sites of pBS.

The HuCe 11 subclone was linearized with *Sma*1, employing a unique site located in exon 1A (CCCGGG; see Figure 4b). The cRNA produced from this construct contained 93 bases of exon 2 and ended at the middle of the *Sma*1 site 67 bases into exon 1A. AMPD2 transcripts containing the exon 1A-2 configuration were expected to protect the entire 160 bases of the AMPD2 sequence in this cRNA probe. The HuCe 3B subclone was linearized with *Nco*1, employing a unique site located in exon 1B

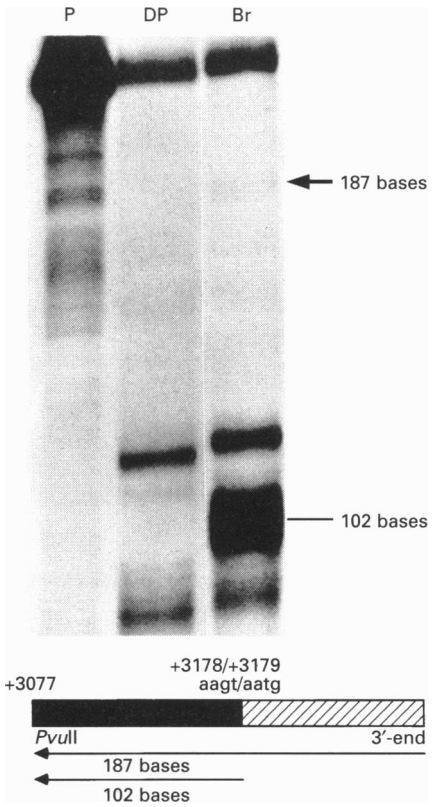

Figure 6 RNase protection analysis of alternative AMPD2 transcripts-I

Variation at the 3′-end of mRNAs. A 187 base cRNA probe was designed from the 3′-end of the HuCe 1 cDNA that extended upstream and ended at nucleotide +3077 in the middle of a *Pvu*II restriction endonuclease site. As depicted in the schematic diagram, AMPD2 transcripts utilizing the predicted downstream termination site would be expected to protect the entire 187 bases of the AMPD2 sequence in this probe, whereas those using the previously identified site at nucleotide +3178 [7] would be expected to protect only 102 bases. P, Undigested probe (full-length probe is 202 bases and includes 15 bases of polycloning and linker sequence); DP, digested probe (tRNA control); Br, 15 μg of HuCe (brain) total cellular RNA.

(CCATGG; see Figure 4b). The cRNA probe produced from this construct also contained 93 bases of exon 2, but then extended 92 bases into exon 1B where it ended at the 5′-overhang of the *Nco*1 site. AMPD2 transcripts containing the exon 1B-2 configuration were expected to fully protect the 185 bases of the AMPD2 sequence in this cRNA probe. The HuCe 6A subclone was linearized with *Eco*R1, employing the created site at the 5′-end of this clone. The cRNA probe produced from this construct, which was missing exon 2, contained 84 bases of exon 3 and 29 bases of exon 1B. AMPD2 transcripts containing the exon 1B-3 configuration were expected to fully protect the 113 bases of the AMPD2 sequence in this probe.

Figure 7 shows the results of parallel RNase protection analyses using total cellular RNAs isolated from human liver (50 μg) and cerebellum (20 μg) to protect each cRNA probe. From these data, it is apparent that AMPD2 gene expression is dramatically different in human liver and brain. Human liver predominantly contains the AMPD2 transcript with the exon 1A-2 configuration (Figure 7a) and relatively less with either the exon 1B-2 (Figure 7b) or exon 1B-3 (Figure 7c) configurations. Conversely, HuCe predominantly contains AMPD2 transcript initiating in exon 1B, both in the exon 1B-2 and exon 1B-3

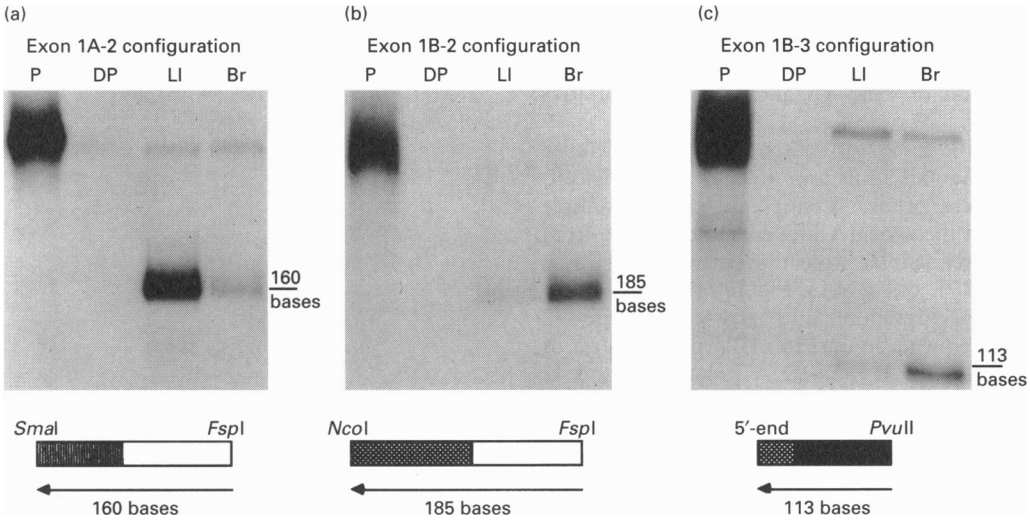

Figure 7 RNase protection analysis of alternative AMPD2 transcripts-II

Variation at the 5′-end of mRNAs. (**a**) Using a subclone of the HuCe 11 cDNA insert as the template, a 160 base AMPD2 cRNA probe was synthesized from an *Fsp*I restriction endonuclease site in exon 2 and extended upstream where it ended in the middle of an *Sma*I restriction endonuclease site in exon 1A (see Figure 4b). As depicted in the schematic diagram, AMPD2 transcripts containing the exon 1A-2 configuration would be expected to protect the entire 160 bases of the AMPD2 sequence in this probe. (**b**) Using a subclone of the HuCe 3B cDNA insert as the template, a 185 base AMPD2 cRNA probe was synthesized from the same *Fsp*I restriction endonuclease site in exon 2 and extended upstream where it ended at the 5′-overhang of an *Nco*I restriction endonuclease site in exon 1B (see Figure 4b). As depicted in the schematic diagram, AMPD2 transcripts containing the exon 1B-2 configuration would be expected to protect the entire 185 bases of the AMPD2 sequence in this probe. (**c**) Using a subclone of the HuCe 6A cDNA insert as the template, a 113 base AMPD2 cRNA probe was synthesized from a *Pvu*II restriction endonuclease site in exon 3 and extended upstream into exon 1B where it ended at the 5′-overhang of an engineered *Eco*R1 restriction endonuclease site at the 5′-end of the cDNA insert (see Figure 4b). As depicted in the schematic diagram, AMPD2 transcripts containing the exon 1B-3 configuration would be expected to protect the entire 113 bases of the AMPD2 sequence in this probe. P, Undigested probes (all three undigested cRNA probes include 23 bases of pBS polycloning sequence at their 5′-ends; the undigested HuCe 6A cRNA probe also includes eight bases of pBS polycloning and phage linker sequence at its 3′-end); DP, digested probes (tRNA control); Li, 50 μg of total cellular RNA isolated from human liver; Br, 30 μg of total cellular RNA isolated from HuCe (brain). All three protection experiments were performed simultaneously and identical exposure times of 72 h are shown.

configurations, and relatively less transcript initiating in exon 1A.

DISCUSSION

A previous report from this laboratory represents the only available information on the human AMPD2 gene [7]. Data presented in this study clarify and extend knowledge of AMPD2 gene structure and its expression in human tissues. An intron has been identified in the original cDNA sequence and its location determined in the AMPD2 gene. Additional cDNA and genomic clones were used to identify four exons at the 5′-end of the AMPD2 gene, three of which are variably found in alternative AMPD2 mRNAs. RT-PCR and RNase protection analyses demonstrate that two of these exons, labeled 1A and 1B, are 5′-terminal sequences in alternative AMPD2 mRNAs and that transcription can initiate at multiple sites in both. Further RNase protection analyses show that alternative AMPD2 mRNAs differ in their relative patterns of expression in human liver and cerebellum. Finally, another cDNA was isolated with an extended 3′-untranslated sequence. Using this cDNA as a template in additional RNase protection analyses demonstrates heterogeneity at the 3′-end of human AMPD2 transcripts as well. How the use of a variable-length 3′ untranslated sequence relates to the shuffling of exons at the 5′-end of the AMPD2 gene is unknown, but taken together, the data permit a combinatorial possibility for at least six different mRNAs.

Shuffled exons at the 5′-end of the AMPD2 gene, labelled 1A, 1B and 2, exhibit different patterns of inclusion in mature transcripts (see Figure 4a). Exons 1A and 1B show a mutually exclusive pattern of expression, i.e., one or the other is found in all AMPD2 mRNAs, but never together. Conversely, exon 2 can either be retained or removed from mature AMPD2 transcripts. While these combined patterns of inclusion would predict four possibilities involving the three exons, no evidence was obtained for an AMPD2 transcript containing exon 1A but lacking exon 2, i.e. that with an exon 1A-3 configuration. However, the existence of an additional mature AMPD2 transcript with this pattern of exon usage remains a formal possibility.

Combining identified patterns of exon inclusion and new sequence with that of a previously published cDNA sequence (from +28 to +3178 [7]), the sizes of the three identified AMPD2 mRNAs may now be set at: 3755 nucleotides, exon 1A-2 configuration; 3970 nucleotides, exon 1B-2 configuration; and 3839 nucleotides, exon 1B-3 configuration. These sizes are based on usage of 5′-most transcription-initiation sites in exons 1A and 1B and do not take into account the use of downstream initiation sites or the extension of the 3′ untranslated sequence in alternative transcripts. Nevertheless, the addition of a poly(A)$^+$ tail to these updated sequences closely approximates the 4 kb Northern blot estimation of AMPD2 transcript size [7].

Molecular events leading to alternative AMPD2 transcript production may now be proposed. Based on its internal position and pattern of inclusion in mature transcripts, exon 2 appears to be subject to a cassette alternative splicing event [13]. Conversely, exons 1A and 1B are 5′-terminal sequences in alternative AMPD2 mRNAs. The sizes of these two exons, 473 and 688 bp respectively, are consistent with a survey performed across all mammalian genes showing that few internal exons exceed 300 nucleotides [14]. The molecular basis for the mutual exclusion of exon 1A and 1B sequences in AMPD2 mRNAs appears to be alternative usage of transcription-initiation sites, presumably

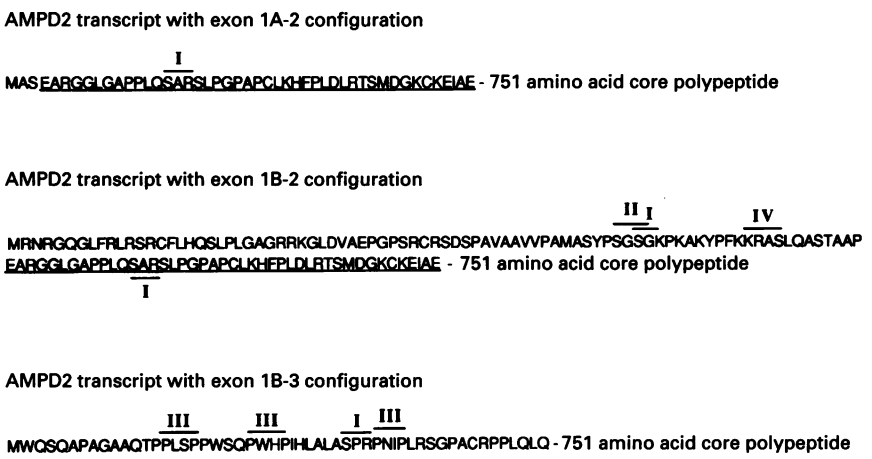

Figure 8 Extreme N-terminal diversity of AMPD isoform L encoded by alternative AMPD2 mRNAs

Each AMPD2 mRNA would be predicted to encode a 751 amino acid core polypeptide. N-Terminal extensions would be conferred by each of the three identified AMPD2 transcripts. Alternative AMPD2 mRNAs and their predicted N-terminal extensions would be: exon 1A-2 configuration, 47 amino acid residues; exon 1B-2 configuration, 128 amino acid residues; and exon 1B-3 configuration, 53 amino acid residues. The exon 2-encoded sequence is underlined. Potential sites for post-translational modifications and predicted binding motifs are denoted by Roman numerals as follows: I, protein kinase C-mediated phosphorylation ([S,T]X[R,K]); II, glycosaminoglycan attachment (SGXG); III, SH3-binding motif (PXXP); IV, cAMP-dependent phosphorylation ([R,K]XX[S,T]).

regulated by two tandem promoters. Notably, there are no apparent TATA or CAAT boxes in proximal sequence immediately upstream of either exon 1A or 1B (see Figure 4b). One of the proposed functions of TATA boxes in higher eukaryotic promoters is to direct the formation of a stable pre-initiation complex with RNA polymerase [15]. Lack of TATA boxes in putative promoter regions of the AMPD2 gene may relate to the observation of multiple transcription-initiation sites in exons 1A and 1B. While neither putative promoter region appears to exhibit features typical of higher eukaryotic genes, functional regulatory elements are likely to be present in these sequences. Planned studies are aimed at examining these upstream regions for promoter activities, which may overlap owing to their close proximity. Regarding the possibility of two tandem promoters in the human AMPD2 gene, relative transcript abundance data presented in this study indicate that the putative exon 1B promoter predominates in brain, whereas the putative exon 1A promoter is stronger in liver.

The AMPD2 gene is specific for AMPD isoform L, and a 760 amino acid polypeptide with a predicted molecular mass of 88.1 kDa had been proposed based on an identified 2280 bp open reading frame in the original HuPl 10B/16 cDNA [7]. However, it is now apparent that the first 27 nucleotides of the published open reading frame actually represented the 3′-terminal region of an intron. What impact does the corrected and additional sequence information presented in this study have on the predicted primary amino acid sequence of isoform L? Figure 8 shows three different predicted N-terminal sequences together with a 751 amino acid C-terminal core polypeptide, i.e. residues 10–760 of the published predicted amino acid sequence [7].

AMPD2 mRNA containing the exon 1A-2 configuration extends the N-terminus of this isoform L variant by 47 residues, resulting in a 798 amino acid polypeptide with a predicted subunit molecular mass of 92.1 kDa. Most of this N-terminal extension results from an exon 2-encoded sequence, with the initiator methionine codon predicted to be near the 3′-end of exon 1A (see Figure 4b). AMPD2 mRNA containing the exon 1B-2 configuration encodes the largest isoform L variant and extends the N-terminus by 128 residues, i.e. an 879 amino acid polypeptide with a predicted subunit molecular mass of 100.7 kDa. AMPD2 mRNA containing the exon 1B-3 configuration extends the N-terminus of this isoform L variant by 53 amino acids and encodes an 804 amino acid polypeptide with a predicted subunit molecular mass of 92.9 kDa. Compared with the one encoded by AMPD2 mRNA containing the exon 1B-2 configuration, the predicted polypeptide encoded by this transcript is smaller. This is due not only to the lack of the exon 2 sequence, but also because a different exon 1B open reading frame is employed.

The functional significance of N-terminal variability among the predicted isoform L variants is unknown. However, a computer-assisted examination of encoded N-terminal residues reveals that the extension encoded by the exon 1B-2 mRNA has several potential sites for post-translational modifications, including three sites for phosphorylation (two protein kinase C and one cyclic AMP-dependent) and one for the attachment of a glycosaminoglycan residue (see Figure 8). Comparisons among the entire three predicted isoform L sequences indicate that cAMP-dependent phosphorylation and glycosaminoglycan attachment sites are unique to the N-terminal region encoded by exon 1B-2 mRNA. Potential post-translational events can also occur in the N-terminal region encoded by the two other alternative AMPD2 transcripts, but are not unique modifications relative to the rest of the predicted polypeptides. However, the N-terminal extension encoded by exon 1B-3 mRNA is proline-rich and contains three consensus SH3-binding motifs [16].

Previous work has shown that the human AMPD2 gene and its encoded protein product, AMPD isoform L, are widely expressed in adult tissues and cells [3,4,7]. This study has identified alternative AMPD2 mRNAs and shown different patterns of their expression in liver and brain. Furthermore, alternative AMPD2 mRNAs are predicted to encode variant forms of isoform L, differing from each other in their extreme N-terminal regions. Additional studies will be required in order to determine the biological significance for this microdiversity in isoform L expression.

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