

## Alternative Splicing: A Mechanism for Phenotypic Rescue of a Common Inherited Defect

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### Abstract

Approximately 2% of Caucasians and African-Americans are homozygous for a nonsense mutation in exon 2 of the AMPD1 (AMP deaminase) gene. These individuals have a high grade deficiency of AMPD activity in their skeletal muscle. More than 100 patients with AMPD1 deficiency have been reported to have symptoms of a metabolic myopathy, but it is apparent many individuals with this inherited defect are asymptomatic given the prevalence of this mutant. Results of the present study provide a potential molecular explanation for "correction" of this genetic defect. Alternative splicing eliminates exon 2 in 0.6–2% of AMPD1 mRNA transcripts in adult skeletal muscle. Expression studies document that AMPD1 mRNA, which has exon 2 deleted, encodes a functional AMPD peptide. A much higher percentage of alternatively spliced transcripts are found during differentiation of human myocytes *in vitro*. Transfection studies with human minigene constructs demonstrate that alternative splicing of the primary transcript of human AMPD1 is controlled by tissue-specific and stage-specific signals. Alternative splicing of exon 2 in individuals who have inherited this defect provides a mechanism for phenotypic rescue and variations in splicing patterns may contribute to the variability in clinical symptoms. (*J. Clin. Invest.* 1993. 91:2275–2280.) Key words: adenosine monophosphate deaminase • cell differentiation • myopathy • nonsense mutation • RNA splicing

### Introduction

AMP deaminase (AMPD<sup>1</sup>; EC 3.5.4.6.) deficiency of skeletal muscle is one of the most common inherited defects in the Caucasian and African-American populations (1, 2). Approximately 20% of Caucasians and African Americans are heterozygous for a mutant allele in the AMPD1 gene, ~ 2% of individ-

uals are homozygous for this mutant allele (2), and 1–3% of randomly sampled muscle biopsies exhibit reduced (~ 2% of control) AMPD activity (1, 3, 4). This mutant allele specifies a nonsense mutation in the second exon of the AMPD1 gene, and mRNA transcripts containing this exon encode a severely truncated AMPD peptide which cannot be detected enzymatically or by Western blotting (2).

AMPD, which catalyzes deamination of AMP to IMP and plays a central role in purine nucleotide interconversion, is found in all eukaryotic cells. This enzyme activity is encoded by a multigene family in mammals (5), and high levels of expression of the AMPD1 gene are restricted to adult skeletal muscle (5).

More than 100 individuals with symptoms of a metabolic myopathy, characterized by easy fatigability, myalgia and rarely myoglobinuria, and coincidental AMPD1 deficiency have been reported in the literature (6). Because of the central role that AMPD plays in purine nucleotide interconversion and energy metabolism (6), it has been hypothesized that AMPD1 deficiency is a causative factor in the muscle symptoms exhibited by patients harboring this enzyme defect. However, a much larger group of individuals with the same inherited defect in the AMPD1 gene are relatively less symptomatic, or asymptomatic, based on the population studies cited above which indicate that ~ 2% of Caucasians and African-Americans are homozygous for this mutant allele. The disparity in frequency of this mutant allele and the prevalence of myopathic symptoms suggests that either this defect is not related to the skeletal muscle dysfunction seen in the individuals with this inherited defect or some compensating mechanism protects some individuals from the harmful effects of this mutation.

A potential molecular explanation for amelioration of symptoms secondary to this nonsense mutation in exon 2 of the AMPD1 gene was suggested by several prior studies. In rat muscle and cultured rodent myocytes, the primary transcript of the AMPD1 gene is subject to alternative splicing, and the only exon which is alternatively removed is exon 2 (7, 8). In rat fetal skeletal muscle, or undifferentiated myoblasts, AMPD1 transcripts that have exon 2 deleted predominate, whereas, in adult skeletal muscle and in well-differentiated myotubes, AMPD1 transcripts that retain exon 2 predominate (7, 8). Thus, if a fraction of the AMPD1 transcripts in individuals with this inherited defect have exon 2 deleted and this transcript encodes a functional peptide, the deleterious consequences of this mutation could be partially corrected.

Even a small fraction of AMPD1 transcripts which have exon 2 deleted could be physiologically important for pheno-

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1. Abbreviations used in this paper: AMPD, AMP deaminase; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; RT, reverse transcription; Sol8, soleus 8 cells; SSCP, single-stranded conformation polymorphism.

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typic rescue. Consequently, the present study has utilized a sensitive and specific reverse transcription-polymerase chain reaction (RT-PCR) assay to assess alternative splicing of AMPD1 transcripts in normal and deficient adult skeletal muscle, nonmuscle tissue, cultured human myocytes, and myocytes and nonmuscle cells transfected with human minigene constructs. The results demonstrate that exon 2 in the wild-type and mutant human AMPD1 primary transcript is subject to alternative splicing and the splicing pattern is responsive to stage-specific and tissue-specific signals. Although full-length cDNAs for both forms of human AMPD1 are not available, expression studies with the closely related rat cDNAs (92% amino acid identity with human AMPD1) demonstrate that the AMPD1 transcript that has exon 2 deleted encodes an active enzyme which reacts with antisera specific for the skeletal muscle isoform of AMPD.

## Methods

**Study subjects.** Skeletal muscle samples were obtained from a normal control and a previously described patient with AMPD deficiency (2).

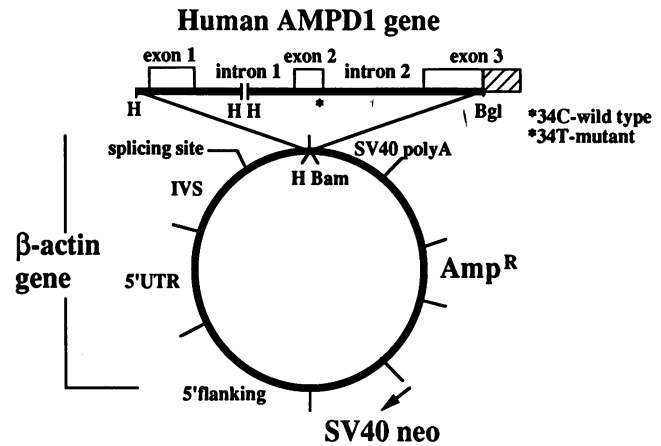
**Cell culture.** A primary culture of human myoblasts from an individual without any clinical symptoms was kindly provided by Dr. Helen Blau of Stanford University. These cells were maintained as myoblasts and induced to differentiate to myotubes as described before (9). Murine fibroblasts, BALB/c 3T3 cells (3T3), were maintained in Dulbecco's modified Eagle media supplemented with 10% calf serum (10). Murine myocytes, soleus 8 cells (Sol8), were maintained as myoblasts and induced to differentiate to myotubes as described before (11). Differentiated human or murine myotubes were harvested at the point of maximal myotube formation for RNA extraction.

**Human AMPD1 minigene constructs.** Fig. 1 shows the human AMPD1 minigene construct which contains a fragment of the human AMPD1 gene promoter element, exon 1, part of intron 2 (1 kb internal deletion), exon 2, intron 2, and part of exon 3 (12). Two minigenes, differing only in a C residue or a T residue at the last nucleotide of exon 2 (position 34 in the mRNA), were constructed to mimic the normal or mutant allele of the AMPD1 gene, respectively. These minigenes were introduced into 3T3 fibroblasts or Sol8 myoblasts by cationic liposome-mediated transfection (13). Cells containing integrated copies of the transfected DNAs were selected by adding Geneticin (G418) to the medium (0.8 mg/ml).

**RNA analyses.** Total cellular RNA was extracted with guanidine isothiocyanate and isolated by centrifugation through cesium chloride (14). RNA from human thymus was kindly provided by Dr. Barton Haynes of Duke University.

**RT-PCR analysis.** First-strand cDNAs were synthesized from 0.5–3  $\mu$ g of total RNA by RT (8, 15) with oligonucleotides corresponding to exon 3 (5'-GTCTGGATCTCATCCACATC-3') or exon 7 (5'-CACCTTCCTGCAGTTCTAAA-3') of human AMPD1. cDNAs were amplified by PCR (16, 17) using several sets of oligonucleotide primers corresponding to exon 1 (5'-AACATGCCTCTGTTC AAC-3') and exon 3 (5'-GTCTGGATCTCATCCACATC-3' or 5'-TCAGGAGATTTCCCCCTTTG-3'). These oligonucleotides were designed to eliminate nonspecific amplification or possible cross-reactivity with endogenous mouse AMPD1 gene products.

To facilitate direct quantitation of PCR products, one of the oligonucleotides was end-labeled with [ $\gamma$ - $^{32}$ P]ATP by the T4-polynucleotide kinase reaction (18). After the PCR, products were displayed on a nondenaturing 6% acrylamide gel and visualized by autoradiography. Electrophoresis was performed under denaturing conditions according to the method of single-stranded conformational polymorphism (SSCP) analysis (19) in that preliminary studies revealed this method was superior to non-denaturing conditions for elimination of nonspecific PCR products.



**Figure 1.** Human wild-type and mutant AMPD1 minigene constructs. Fragments of the human wild-type (34C) or mutant (34T) AMPD1 genes, identical in all respects except for the point mutation in the last base of exon 2 (indicated by \*), were ligated into a human  $\beta$ -actin expression vector (12). The essential features of this vector include an SV40 promoter to direct synthesis of the selectable prokaryotic neomycin phosphotransferase (Neo) gene product and a human  $\beta$ -actin gene fragment for transcription of the AMPD1 gene product. Both constructs include the original muscle-specific promoter of the AMPD1 gene, exon 1, part of intron 1 (1 kb of internal sequence was deleted), exon 2, intron 2, and part of exon 3. *H*, HindIII; *Bam*, BamHI; *Bgl*, BglII restriction sites used in making these constructs are noted in the figure. These expression plasmids were introduced into 3T3 fibroblasts or Sol8 myoblasts by cationic liposome-mediated transfection (13) and stable transfectants selected in G418 (0.8 mg/ml).

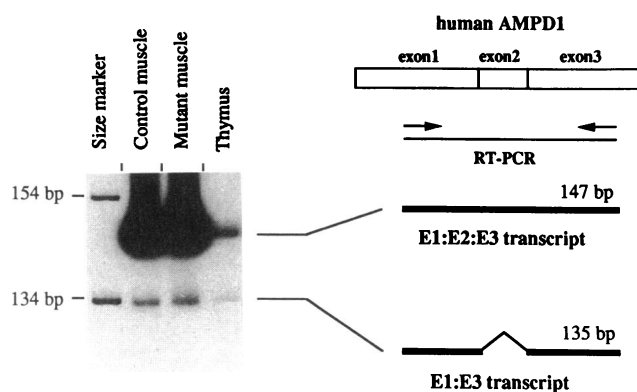
**Direct sequencing of RT-PCR products.** RT-PCR products were separated on 2% low-melting-temperature agarose. Each fragment was removed from the gel slice by the method described before (20) and reamplified by PCR using the same primers. The final product was sequenced directly by dideoxy chain termination method with a [ $\gamma$ - $^{32}$ P]ATP end-labeled primer (21).

**Prokaryotic expression studies.** The expression plasmid pKK233-2 (pKK; Pharmacia Biosystems, Piscataway, NJ) which has a *trc* promoter inducible by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and an ATG initiation codon located within a unique *Nco* I site was employed for these studies. A prokaryotic expression system was selected for these studies since bacteria do not contain an endogenous AMPD activity. Rat AMPD1 cDNAs, with and without exon 2, were isolated previously (8). Full-length AMPD1 cDNAs were subcloned into the *Nco* I site of the pKK vector by site-directed mutagenesis (16) of the 5' termini of the respective cDNAs to create a compatible restriction site (*Stu* I) that preserves the initiation codon of AMPD1 in an appropriate position with respect to the Shine-Dalgarno sequence in pKK. The resulting AMPD vectors (plasmid pKKrAMPD, corresponding to the transcript with exon 2; plasmid pKKrAMPD $\Delta$ exon2, corresponding to the transcript without exon 2) were used for transforming *Escherichia coli* strain JM105. IPTG-induced cultures were pelleted, resuspended in 0.5 ml of extraction buffer (50 mM imidazole, pH 6.5, containing 100 mM potassium chloride and 1 mM dithiothreitol) and sonicated on ice. Extracts were centrifuged to remove debris, and the supernatant was dialyzed against extraction buffer. AMPD activity was assayed radiochemically ([ $^{14}$ C]AMP from Amersham International, Amersham, UK) at 37°C in a 25 mM imidazole/HCl, pH 6.5 with 150 mM KCl, 0.2 mg/ml bovine serum albumin at AMP concentrations up to 10 mM (7). Immunoreactivity of AMPD activity in the extract was evaluated by a previously described solution hybridization assay using available isoform-specific antisera (7).

## Results

**Alternative splicing of AMPD1 in adult human tissues.** A sensitive and specific RT-PCR assay employing radioactive primers from exon 1 or exon 3 sequences was used to assess the relative abundance of AMPD1 mRNA transcripts that retain or delete exon 2 in two human tissues (Fig. 2). The RT-PCR product from the transcript that retains exon 2 is 147 bp in length and it is referred to as the exon 1:exon 2:exon 3 (E1:E2:E3) transcript; the RT-PCR product from the alternative transcript, which has exon 2 deleted, is 135 bp in length and it is referred to as the E1:E3 transcript. The identity of both PCR products was confirmed by DNA sequencing of the PCR products (data not shown).

Skeletal muscle of a normal adult subject and an individual homozygous for the exon 2 mutant allele contains both the E1:E2:E3 and E1:E3 AMPD1 transcripts, but the former transcript is much more prevalent in both muscle samples. Scintillation counting of the radiolabeled PCR products excised from gels similar to those shown in Fig. 2 indicates that 0.6–2% of the AMPD1 transcripts in skeletal muscle from the control subject and the individual homozygous for the mutant allele have exon 2 deleted, i.e., the E1:E3 transcript. RNase protection assays on the RNA isolated from these same muscle samples using a protocol described previously (8) yielded results consistent with the RT-PCR findings (data not presented). However, the



**Figure 2.** RT-PCR analysis of RNA isolated from adult skeletal muscle of a normal control and a homozygote for the AMPD1 mutant allele. Total cellular RNA was extracted from skeletal muscle with guanidine isothiocyanate and isolated by centrifugation through cesium chloride (14). RNA from human thymus was kindly provided by Dr. Barton Haynes of Duke University. First-strand cDNAs were synthesized from 0.5–3  $\mu$ g of total RNA by the RT using a primer (5'-CACCTTCTGCAGTTATAAA-3') which is complementary to exon 7 of human AMPD1 cDNA; PCR was performed on this cDNA using primers corresponding to exon 1 and exon 3 (the exon 3 primer was radiolabeled with [ $\gamma$ - $^{32}$ P]ATP by T4 polynucleotide kinase [18]). **Left panel:** After 30 cycles of PCR, the products were displayed on a 6% nondenaturing acrylamide gel and visualized by autoradiography. **Size markers**, 154 bp and 134 bp; **Control muscle**, RNA from skeletal muscle of a normal control; **Mutant muscle**, RNA from skeletal muscle of an individual homozygous for the nonsense mutation at nucleotide 34 in exon 2; **Thymus**, RNA from human thymus. The diagram on the right illustrates how the PCR reaction was performed schematically, and it illustrates the predicted sizes of the two PCR products. The larger, 147-bp fragment corresponds to the exon 1:exon 2:exon 3 transcript (E1:E2:E3) and the smaller, 135-bp fragment corresponds to the exon 1:exon 3 transcript (E1:E3).

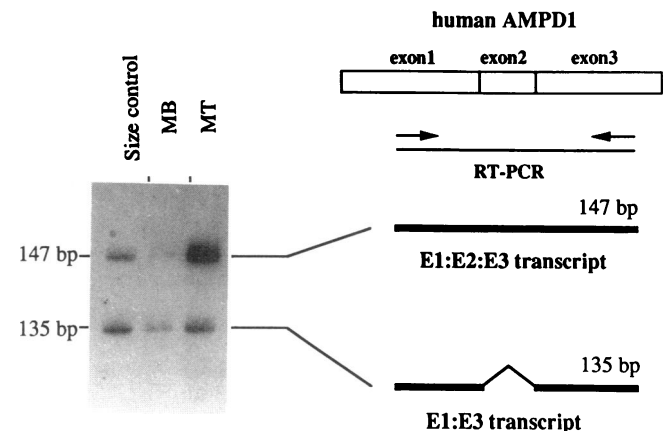
RNase protection results are less reliable than the RT-PCR results in that the E1:E3 transcript cannot be distinguished from the primary transcript in the RNase protection assay (8).

RNA isolated from a human thymus sample has a more equal distribution of the AMPD1 E1:E2:E3 and E1:E3 transcripts (Fig. 2). The ratio of these two AMPD1 transcripts in human thymus is similar to that found in rat thymus (8).

**Alternative splicing of AMPD1 in cultured human myocytes.** RNA isolated from a primary culture of human myoblasts and the myotubes subsequently derived from these myoblasts was analyzed for the relative abundance of the AMPD1 E1:E2:E3 and E1:E3 transcripts using the RT-PCR assay described above (Fig. 3). When the RT-PCR products were sequenced to confirm their identity, it was discovered that these myocytes had been serendipitously derived from an individual who was homozygous for the AMPD1 mutant allele.

As observed previously with rodent myocyte culture systems (7), human myoblasts express predominantly the E1:E3 AMPD1 transcript and following differentiation of these cells into myotubes the E1:E2:E3 transcript predominates. Apparently none of these culture systems, rat or human, achieve the same level of maturity as adult skeletal muscle based on the relative ratios of E1:E3 to E1:E2:E3 transcripts in myoblasts vs. myotubes vs. adult skeletal muscle. The ratio of E1:E3 to E1:E2:E3 transcripts in human and rat myotubes is more similar to the ratio observed in fetal rat skeletal muscle (7).

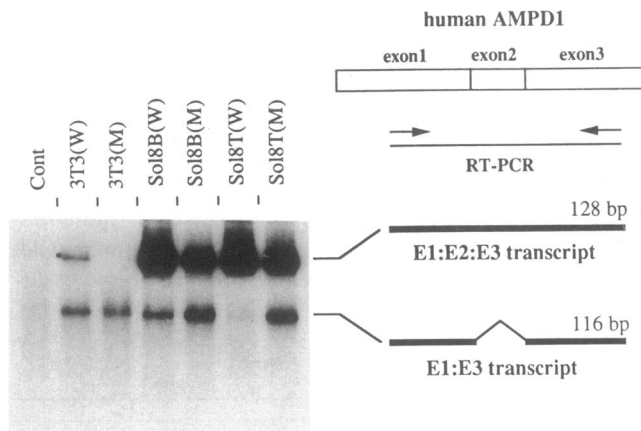
**Alternative splicing of human AMPD1 minigenes transfected into different cell types.** Fragments of the human wild-type and mutant AMPD1 genes containing the promoter ele-



**Figure 3.** RT-PCR analysis of RNA isolated from a primary culture of myoblasts (MB) and myotubes (MT) from an individual homozygous for the exon 2 C  $\rightarrow$  T mutation. The primary culture of human myoblasts was kindly provided by Dr. Helen Blau of Stanford University. Myotubes were obtained by induction of differentiation after changing the media from Ham's F10 media with 20% fetal bovine serum and 0.5% chick embryo extract to Dulbecco's modified Eagle media with 2% horse serum supplemented with insulin and dexamethazone (9). RNA preparations and RT-PCR were performed as described in the legend to Fig. 1. After 30 cycles of PCR, the products were displayed on a 6% nondenaturing acrylamide gel and visualized by autoradiography (panel on the left). **Size control**, 147 bp and 135 bp; **MB**, RNA from undifferentiated human myoblasts; **MT**, RNA from differentiated human myotubes. The diagram on the right illustrates the PCR reaction schematically and the predicted sizes of the two products. The larger, 147-bp fragment corresponds to the exon 1:exon 2:exon 3 transcript (E1:E2:E3) and the smaller, 135-bp fragment corresponds to the exon 1:exon 3 transcript (E1:E3).

ment, exon 1, part of intron 1, exon 2, intron 2, and part of exon 3 were used to construct reporter minigenes in a eukaryotic expression vector (Fig. 1). These minigenes were used to assess the responsiveness of the wild-type and mutant AMPD1 primary transcripts to splicing signals elaborated in different cell types or the same cell type at different stages of differentiation (Fig. 4). The RT-PCR assay used to quantitate the relative abundance of E1:E2:E3 and E1:E3 transcripts produced from the endogenous AMPD1 gene in human cells was employed to assess the relative abundance of E1:E2:E3 versus E1:E3 transcripts in cells transfected with the two human minigene constructs. The oligonucleotide primers used for these RT-PCR assays did not detect the endogenous murine AMPD1 gene products in any of the cell types used in these transfection studies.

For both the wild-type and mutant minigene constructs a qualitatively similar pattern of alternative splicing is observed



**Figure 4.** RT-PCR analysis of RNA isolated from murine cells transfected with human AMPD1 minigene constructs. Total cellular RNA was extracted from stably transfected cultured cells as described in the legend to Fig. 1. First-strand cDNA was synthesized with an oligonucleotide complementary to exon 3 (5'-GTCTGGATCTCATCCACATC-3'). These cDNAs were amplified by PCR using oligonucleotide primers corresponding to exon 1 (5'-AACATGCCTCTGTTC AAC-3') and exon 3 (5'-TCAGGAGATTTCCCTTTG-3'). These oligonucleotides were designed to eliminate cross-reactivity with the endogenous murine AMPD1 gene products. To facilitate quantitative comparison of the two PCR products, one of the oligonucleotides was end-labeled with [ $\gamma$ - $^{32}$ P]ATP by the T4-polynucleotide kinase reaction (18). After 30 cycles of PCR, the products were displayed on a 6% nondenaturing acrylamide gel and visualized by autoradiography (panel on the left). *Cont*, untransfected Sol8 myotubes; *3T3(W)*, BALB/c 3T3 fibroblasts transfected with the wild-type (34C) minigene construct; *3T3(M)*, BALB/c 3T3 fibroblasts transfected with the mutant (34T) minigene construct; *Sol8B(W)*, Soleus8 myoblasts transfected with the wild-type (34C) minigene construct; *Sol8B(M)*, Soleus8 myoblasts transfected with the mutant (34T) minigene construct; *Sol8T(W)*, Soleus8 myotubes transfected with the wild-type (34C) minigene construct; *Sol8T(M)*, Soleus8 myotubes transfected with the mutant (34T) minigene construct. The diagram on the right illustrates the PCR reaction schematically and the sizes of the two predicted PCR products are also illustrated. The mature transcript containing exon 1:exon 2:exon 3 (E1:E2:E3) gives a 128-bp PCR product, while the alternatively spliced transcript contains only exon 1 and exon 3 (E1:E3) and it gives a 116-bp PCR product. PCR was performed using several different primers and identical results were observed in all experiments.

in the various transfected cell types (Fig. 4). In 3T3 fibroblasts the wild-type minigene primary transcript gives rise to roughly equal proportions of E1:E2:E3 and E1:E3 mature transcripts, similar to the ratio observed for the products of the native AMPD1 gene in normal human thymus. The mutant minigene primary transcript also gives rise to both E1:E2:E3 and E1:E3 mature transcripts, but the latter predominates in 3T3 cells. In undifferentiated Sol8 myoblasts the wild-type and mutant AMPD1 minigene primary transcripts are processed to both E1:E2:E3 and E1:E3 mature transcripts. In contrast to 3T3 cells, the myoblast background gives rise to a predominance of E1:E2:E3 versus E1:E3 mature transcripts. Again, the relative abundance of the E1:E3 RNA product is greater when the primary transcript harbors a mutant exon 2. In differentiated Sol8 myotubes the E1:E2:E3 transcript is even more predominant than the E1:E3 transcript, when compared to undifferentiated myoblasts. Once again, the E1:E3 transcript is relatively more abundant when the primary transcript harbors a mutant exon 2.

These results indicate that different cell types contain variable amounts of components of the splicing apparatus that determine whether exon 2 will be recognized in the AMPD1 primary transcript. In this model system, the C to T mutation in the 3' terminal base of exon 2 is associated with accumulation of a relatively greater proportion of mature transcripts which have exon 2 deleted, indicating the 5' donor site at the boundary of exon 2:intron 2 is one determinant for the recognition and retention of exon 2.

**Expression of AMPD1 cDNAs with and without exon 2.** Full-length cDNAs for both forms of human AMPD1, i.e. with and without exon 2, are not available for expression studies. However, the closely related rat AMPD1 cDNAs are available (8). Rat and human AMPD1 peptides are 92% identical in primary amino acid sequence, including the amino terminal region containing exon 2 (22). Exon 2 is twelve bases in length in human and rat AMPD1 (22) and deletion of this mini-exon preserves the same reading frame in both human and rat AMPD1. Since full length cDNAs for both forms of rat AMPD1 were available, these cDNAs were used for the expression studies reported here. A prokaryotic expression system was selected for these studies because bacteria do not contain an endogenous AMPD activity.

Extracts from *E. coli* transformed with the pKK vector alone have no detectable AMPD activity (< 0.1 mIU/mg protein) (Table I). AMPD activity determined at saturating concentration of AMP is 147 mIU/mg protein in extract isolated from *E. coli* transformed with pKKrAMPD, the expression vector containing the cDNA with exon 2. Extract from *E. coli* transformed with pKKrAMPD $\Delta$ exon2, the expression vector containing the AMPD1 cDNA which has exon 2 deleted, has an AMPD activity of 108 mIU/mg protein. The apparent  $K_m$  for AMP is similar for the AMPD activity in both of these extracts, and the AMPD activity in both extracts reacts with antisera specific for the skeletal muscle isoform of AMPD (Table I).

## Discussion

Results of the present study demonstrate that the primary transcript of the human AMPD1 gene is subject to alternative splicing. The second exon is deleted from or retained in mature

Table I. Properties of Recombinant AMPD1 Peptides Encoded by cDNAs with and without Exon 2

Expression plasmid*	AMPD activity <i>mIU/mg protein</i>	$K_m$ for AMP <i>mM</i>	Reactivity with AMPD antisera <sup>†</sup>	
			Skeletal muscle %	Heart
pKK	<0.1	—	—	—
pKKrAMPD	147	3.1	88	<10
pKKrAMPD $\Delta$ exon2	108	3.0	93	<10

\* pKK = expression vector with no cDNA insert; pKKrAMPD = vector with rat AMPD1 cDNA containing exon 2; pKKrAMPD- $\Delta$ exon2 = vector with rat AMPD1 which has exon 2 deleted.

<sup>†</sup> Antisera were raised to the AMPD isoform purified from the indicated organ (7), and this table reports the percentage of AMPD activity in the extract that can be immunoprecipitated with the respective antisera.

mRNA in response to tissue-specific and myocyte stage-specific signals.

In cultured human myoblasts the predominant AMPD1 mRNA has exon 2 deleted. If AMPD1 expression in human myocyte culture systems parallels expression of this gene during muscle development in vivo, as observed previously with AMPD1 expression in rat myocyte cultures and muscle development in vivo (7, 8), a large fraction of AMPD1 mRNA transcripts will have exon 2 deleted during human skeletal muscle development. Deletion of the second exon from the majority of primary transcripts in individuals with a nonsense mutation in this exon would be expected to preserve a significant level of AMPD activity in myocytes during skeletal muscle development, in that mRNA transcripts that do not contain exon 2 sequences encode a functional AMPD peptide. Similarly, adult nonmuscle tissues of individuals with this inherited defect might be spared to a large extent the potential consequences of AMPD1 deficiency because alternative splicing in nonmuscle tissues results in a large fraction of mRNA transcripts which have exon 2 deleted.

In adult human skeletal muscle > 95% of AMPD1 mRNA transcripts retain exon 2 explaining the reduction in AMPD activity observed in adults who have inherited the allele with a nonsense mutation in exon 2 (2). However, a small but clearly detectable fraction of AMPD1 mRNA in adult human skeletal muscle does not contain exon 2, providing a mechanism for synthesizing a low level of this AMPD isoform in adult myocytes. Because the abundance of total AMPD1 mRNA transcripts is two- to threefold greater in individuals with this inherited defect (2) and the mutation in exon 2 increases, if anything, the proportion of mRNA transcripts that have exon 2 deleted (results of present study with minigene constructs), skeletal muscle of individuals with this inherited defect may have more AMPD1 mRNA with exon 2 deleted than do normal subjects.

Is the extent of alternative splicing observed in adult human skeletal muscle sufficient to explain the absence of or variable symptoms in individuals with this inherited defect? There are examples of other inherited defects in purine metabolism where 1%, or less, of residual enzyme activity is sufficient to ameliorate or eliminate a clinical phenotype (23–26). The

fraction of AMPD1 mRNA transcripts that have been “corrected” through alternative splicing is within this range, and the level of residual enzyme activity in skeletal muscle is consistent with this degree of alternative splicing (6, 27). Moreover, a portion of this residual AMPD activity reacts with antisera specific for the skeletal muscle isoform of AMPD (27), an observation that also argues for production of an AMPD peptide encoded by the AMPD1 gene. Thus, it seems plausible that alternative splicing of the AMPD1 primary transcript could result in a partial or complete phenotypic rescue for this inherited enzyme defect.

Variability in the extent of alternative splicing could account for a partial correction of the phenotype and variability in clinical symptoms in some patients. As illustrated in this report, alternative splicing of AMPD1 varies in response to tissue-specific and stage-specific signals. As a result, the pattern of splicing might be expected to vary in different individuals or in the same individual at different times and in different muscle fiber types. Indirect data suggest that the extent of alternative splicing is likely to be variable in different individuals with AMPD deficiency. Individuals with this inherited defect exhibit a wide range of AMPD residual activity which reacts with antisera specific for the muscle isoform of AMPD; 0–53% of the residual AMPD activity reacts with muscle-specific antisera (27, unpublished observations from our laboratory). Because the mutant AMPD1 transcript that retains exon 2 does not encode a functional peptide or a peptide which reacts with this antisera (2), it follows that the source of this residual activity is the AMPD1 transcript that has exon 2 deleted. Thus, variation in the extent of alternative splicing of the AMPD1 primary transcript provides a plausible explanation for the observed variability in the fraction of residual AMPD activity which reacts with muscle-specific antisera in these different patients.

Based on these considerations we conclude that the extent and variability of alternative splicing of the AMPD1 transcript are in the range where alteration in AMPD enzymatic activity could influence the presence, or extent, of clinical symptoms. A definitive test of this alternative splicing hypothesis for phenotypic rescue, however, will require careful study of a number of patients and asymptomatic individuals to determine if the proportion of alternatively spliced AMPD1 transcripts in affected muscle groups correlates with the presence or absence of symptoms.

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