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A Novel Pathway for Alternative Splicing: Identification of an RNA Intermediate That Generates an Alternative 5′ Splice Donor Site Not Present in the Primary Transcript of *AMPD1*

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AMP deaminase (AMPD) is a central enzyme in eucaryotic energy metabolism, and tissue-specific as well as stage-specific isoforms are found in many vertebrates. This study demonstrates the *AMPD1* gene product in rat is alternatively spliced. The second exon, a 12-base miniexon, was found to be excluded or included in a tissue-specific and stage-specific pattern. This example of cassette splicing utilizes a unique pathway through an RNA intermediate that generates an alternative 5′ splice donor site at the point where exon 2 is ligated to exon 1. In the analogous intermediate of human *AMPD1*, the potential 5′ splice donor site created at the boundary of exon 1 and exon 2 was a poor substrate for splicing because of differences in exon 2 sequences, and human *AMPD1* was not alternatively spliced. These results demonstrate that in some cases alternative splicing may proceed through an RNA intermediate that generates an alternative splice donor site not present in the primary transcript. Discrimination between alternative 5′ splice donor sites in the RNA intermediate of *AMPD1* is apparently controlled by tissue-specific and stage-specific signals.

AMP deaminase (AMP aminohydrolase; EC 3.5.4.6) (AMPD) activity has been found in all eucaryotic cells examined thus far, but it is not present in procaryotes, which suggests that this enzyme catalyzes a reaction that is important for energy metabolism in the specialized environment of the eucaryotic cell. Tissue-specific and stage-specific isoforms of AMPD have been reported in many vertebrates (14, 22, 23). In human and rats, two functional AMPD genes have been identified (16a). *AMPD1* is expressed at high levels in skeletal muscle; *AMPD2* is expressed in many nonmuscle tissues of the adult animal as well as in embryonic muscle, cardiac muscle, and smooth muscle. Two clinical phenotypes of inherited AMPD deficiency have been described in humans. Homozygous deficiency of AMPD activity restricted to erythrocytes has been estimated to have a frequency of ≈0.03% in Japanese, Chinese, and Koreans (21). Deficiency of AMPD restricted to skeletal muscle is a common cause of inherited metabolic myopathy in the United States and Europe (30), and the latter phenotype is presumably a consequence of mutations in the *AMPD1* gene.

Alternative splicing is used in many tissues, including skeletal muscle, to generate protein diversity (2). Prior studies suggest that more than one transcript may be produced from the *AMPD1* gene in rat skeletal muscle. RNase mapping experiments performed with an *AMPD1* probe demonstrated the presence of two transcripts that have identical or closely related 5′ and 3′ termini, but the internal sequences of these transcripts diverge at one or more points (29). The study described here demonstrates that two mRNAs are produced from the rat *AMPD1* gene and that the only difference between these two transcripts is the inclusion or exclusion of exon 2, a 12-base miniexon. The relative abundance of these two AMPD1 transcripts is regulated by tissue-specific and stage-specific signals.

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This pattern of alternative splicing exhibited by rat *AMPD1* has been referred to as cassette splicing. It has been assumed in this type of splicing, the variably retained exon is removed as a part of a larger intron that contains the exon as well as both of the flanking introns (2). The study presented here demonstrates that this is not the only mechanism for cassette splicing. In the case of *AMPD1*, a novel pathway for alternative splicing has been identified. This pathway requires the formation of an RNA intermediate that generates a functional, alternative 5′ splice donor site at the point where exon 1 is ligated to exon 2. The analogous RNA intermediate of the human *AMPD1* transcript generates a potential 5′ splice donor site at the boundary of exon 1 and exon 2 that is a poor substrate for splicing because of differences in exon 2 sequences, and human *AMPD1* is not alternatively spliced. These differences in splicing patterns between rat and human *AMPD1* indicate that the RNA intermediate identified in this study is a requisite for alternative splicing of *AMPD1*.

MATERIALS AND METHODS

Isolation and sequencing of cDNAs. The cDNA clones described in this report were isolated from a λgt10 library prepared from adult rat soleus muscle (27). The cDNA probe used to isolate these clones was labeled by using multiprimer extension (5). Oligonucleotide probes were end labeled by T4 polynucleotide kinase and purified by electrophoresis in a 5% polyacrylamide gel (27). Hybridization and washing conditions of Nytran filters were the same as described previously (27) except that filters were hybridized with the cDNA probe at 55°C and washed four times in 1× to 2× SET (1× SET is 30 mM Tris chloride [pH 8.0], 150 mM NaCl, and 2 mM EDTA)–0.1% sodium dodecyl sulfate–0.1% sodium pyrophosphate at 55°C. cDNAs were subcloned into pBS (Bluescribe vector; Stratagene Cloning Systems, San Diego, Calif.) for double-stranded DNA sequencing by the dideoxy-chain termination method (31).

RNA isolation and analyses. Total cellular RNA was extracted from various tissues of adult (200- to 400-g) Sprague-

Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), human muscle biopsy specimens, mouse NIH 3T3 fibroblasts, and mouse C2C12 myoblasts with guanidine isothionate and isolated by cesium chloride gradient centrifugation (13).

RNase protection analysis was performed essentially as described by Krieg and Melton (10). Restriction fragments of *AMPD1* were ligated into the multiple cloning site of pBS, and complementary RNA (cRNA) probes were synthesized with T3 or T7 RNA polymerase and purified by electrophoresis in a urea-acrylamide denaturing gel. Total RNA (5 to 100 μg) was hybridized with an excess of cRNA probe (1×10^5 to 3×10^5 cpm per reaction) at 50°C overnight. The fragments protected from digestion with RNases A and T_1 were displayed on an 8% acrylamide gel containing 8 mM urea. After autoradiography, relative quantities of the protected fragments were estimated with a Bio-Rad model 620 video densitometer (Bio-Rad Laboratories, Richmond, Calif.).

Primer extension analysis was performed as follows. A 10-μg sample of total RNA was mixed with 5×10^5 cpm of end-labeled oligonucleotide primer in 5 μl of RNase-free water and heated to 55°C for 20 min. After addition of 2 μl of buffer (250 mM Tris chloride [pH 8.0], 25 mM $MgCl_2$, 250 mM KCl, 25 mM dithiothreitol), the reaction was allowed to gradually cool to 42°C. Extension was initiated by adding 1.5 μl of a 10 mM deoxynucleoside triphosphate mix, 1 μl of 1-mg/ml dactinomycin, and 20 U of reverse transcriptase, followed by incubation at 42°C for 60 min. The reaction was terminated by adding 2.5 μl of 500 mM EDTA. The products were extracted with phenol-chloroform, ethanol precipitated, and fractionated on an 8% acrylamide gel containing 8 mM urea.

cDNA copies of alternatively spliced *AMPD* transcripts were synthesized by using a 3′ primer (5′-CCTCCCTC ATCTTTGACTTC) for the reverse transcriptase reaction, and cDNAs were amplified by the polymerase chain reaction (PCR) with a 5′ primer (5′-GGATCCCAGTCACAATGCCT) together with the same 3′ primer (11). The 5′ and 3′ primers are complementary to exons 1 and 3, respectively, of the rat *AMPD1* gene. A 10-μg sample of total cellular RNA was reverse transcribed by using 100 ng of the 3′ primer in a 10-μl reaction mixture. After reverse transcription, 60 μl of water was added, and the tube was incubated in a boiling water bath for 5 min. PCR was performed with the cDNA product in 50 mM KCl–10 mM Tris chloride [pH 8.4]–1.5 mM $MgCl_2$–10 μg of gelatin per ml–0.2 mM each deoxynucleoside triphosphate–1 μM each 5′ and 3′ primer–2.5 U of *Thermus aquaticus* DNA polymerase (*Taq* polymerase; Perkin-Elmer/Cetus) in a total volume of 100 μl. The mixture was overlayed with mineral oil and amplified with the Programmable Cyclic Reactor (Ericomp Inc., San Diego, Calif.) for 40 cycles. Each cycle consisted of denaturation at 95°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 3 min. PCR products (10 μl of PCR mixture) were size fractionated in a 3% agarose gel in Tris-borate–EDTA buffer and transferred to a Nytran filter as described previously (27). Blots were hybridized with a ^{32}P-labeled oligonucleotide (5′-CTTTTTCAGCAAAGTT GCGCATTGCATCAT) complementary to an internal sequence in exon 3.

Construction of AMPD1 splicing intermediate and expression in cultured cells. A *Bam*HI-*Eco*RI fragment of *AMPD1* containing exon 1, exon 2, and part of intron 2 was obtained from a cDNA clone isolated in this study. An *Eco*RI-*Nco*I fragment of the rat *AMPD1* gene containing the remainder of intron 2 and exon 3 was isolated from the genomic DNA (28). These two DNA fragments ligated at the *Eco*RI sites were subcloned into a human β-actin expression vector (8) (Fig. 1). The resultant construct contains the entire sequence of exon 2 and intron 2, but the 5′ and 3′ regions of exons 1 and 3, respectively, have been truncated. NIH 3T3 fibroblasts and C2C12 myoblasts were transfected with 10 μg of this plasmid by using calcium phosphate coprecipitation (7). Cells containing integrated copies of the transfected DNA were selected by adding geneticin (G418) to the medium at a concentration of 0.8 mg/ml. Colonies resistant to G418 were collected with a cloning cylinder and expanded for RNA analyses as described above.

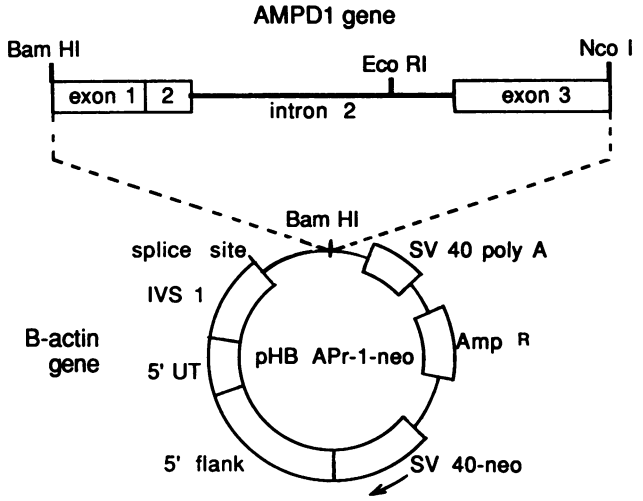

FIG. 1. β-Actin expression vector containing *AMPD1* splicing intermediate. A *Bam*HI-*Eco*RI fragment of rat *AMPD1* containing a portion of exon 1, all of exon 2, and a portion of intron 2 was ligated to an *Eco*RI-*Nco*I fragment of *AMPD1* containing the remainder of intron 2 and a portion of exon 3. This construct was inserted in the *Bam*HI site of the β-actin expression vector (pHβ APr-1-neo) developed by Gunning et al. (8). 5′ UT, 5′ untranslated sequences of β-actin; IVS 1, intervening sequence 1 of β-actin; SV 40 poly A, polyadenylation site of simian virus 40; Amp^R, ampicillin resistance gene; and SV 40-neo, neomycin resistance gene under the control of the simian virus 40 early promoter.

RESULTS

Cloning and characterization of rat AMPD1 cDNAs. As outlined in the introduction, previous studies suggest that there may be two transcripts derived from the *AMPD1* gene in rats (29). Both of these transcripts hybridize to a cRNA probe synthesized from the 5′ terminus of an *AMPD1* cDNA. Consequently, the adult rat soleus muscle λgt10 cDNA library used to isolate the original *AMPD1* cDNA (27) was rescreened with a 215-base cDNA probe containing sequences corresponding to the 5′ termini of both *AMPD1* transcripts (29). Twenty-eight clones that hybridized to this probe, presumably a mixture of both types of *AMPD1* transcripts, were isolated.

Comparison of the recently elucidated structure of the rat *AMPD1* gene (28) and the results of previous RNase mapping studies (29) suggest that the sequences of the two *AMPD1* transcripts may diverge after exon 1. Thus, each of the 28 positive clones was tested for hybridization to an oligonucleotide specific for exon 1 (5′-GCTGTCCTAGA ATC CAG) or exon 2 (5′-AGGTCAAGGAAAAC; two bases

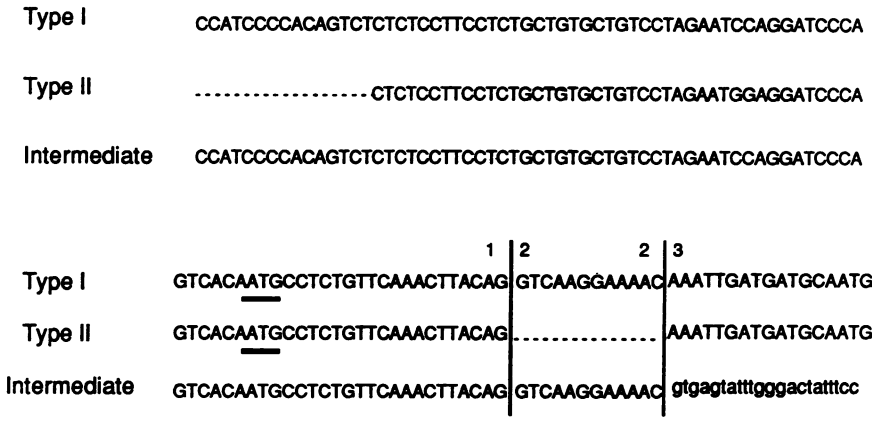

FIG. 2. Partial sequence of rat *AMPD1* cDNAs. cDNAs were cloned from a rat soleus λgt10 library as described in the text. The sequence of only the 5′ region of the three cDNAs cloned from this library is shown. The cDNA indicated as Intermediate is 2.6 kilobases in length, and it terminates in intron 2 sequences. The boundaries of exon 1, exon 2, and exon 3 are identified by vertical lines and numerical superscripts. -----, Nucleotides present in type I but not type II cDNA. Lowercase letters indicate nucleotides identical to the sequence of intron 2 in the rat *AMPD1* gene (28). The presumed initiation codon is underlined.

were added to the 5′ end of the sequence of exon 2 to enhance hybridization efficiency). All 28 clones hybridized to the exon 1 oligonucleotide, but 15 of these 28 clones did not hybridize to the exon 2 oligonucleotide. The latter represent potential cDNAs that do not contain exon 2, and several of these recombinants, as well as ones that hybridized to the exon 2-specific probe, were subcloned in pBS for sequence analysis.

Two types of *AMPD1* cDNAs were detected (Fig. 2), and they differed by only 12 bases deleted from the 5′ coding region of one of the cDNAs. This deletion does not change the reading frame, but it does eliminate four codons and changes the amino acid specified by a fifth codon. In the remainder of the sequences, these two cDNAs are identical (not shown). The 12 bases that are deleted correspond to the entire sequence of the second exon of the rat *AMPD1* gene (28).

Although comparison of these cDNA sequences did not indicate any other difference between these two *AMPD1* transcripts, cRNA probes spanning the entire cDNA sequence were used in RNase protection analyses to determine whether there are additional *AMPD1* transcripts in RNA isolated from two skeletal muscle fiber types (Fig. 3 and 4). (RNase mapping of the 3′ terminus [exons 15 and 16] was presented in a previous publication [29].) Other than the inclusion or removal of exon 2 sequences, there was no evidence for heterogeneity in the *AMPD1* transcript in these different muscle fiber types. For convenience, the *AMPD1* mRNA that contains exon 2 sequences will be referred to as the type I transcript, and the mRNA from which exon 2 is deleted will be referred to as the type II transcript.

Relative abundance of type I and type II *AMPD1* transcripts in rat tissues. A cRNA probe complementary to the 5′ region of the rat type I transcript, containing a portion of exon 1, all of exon 2, and a portion of exon 3, was used in RNase protection assays to assess the relative abundance of type I and type II mRNAs in different muscle fiber types and nonmuscle tissues of the adult rat (Fig. 4). The 5′ cRNA probe used in these studies was fully protected by the type I transcript (215-base fragment); the type II transcript protected 170 bases of exon 3 and a 33-base fragment of exon 1 (the latter was too small to be visualized on the gel shown in Fig. 4). In studies not illustrated, it was shown that the 170-base and 33-base partially protected fragments were

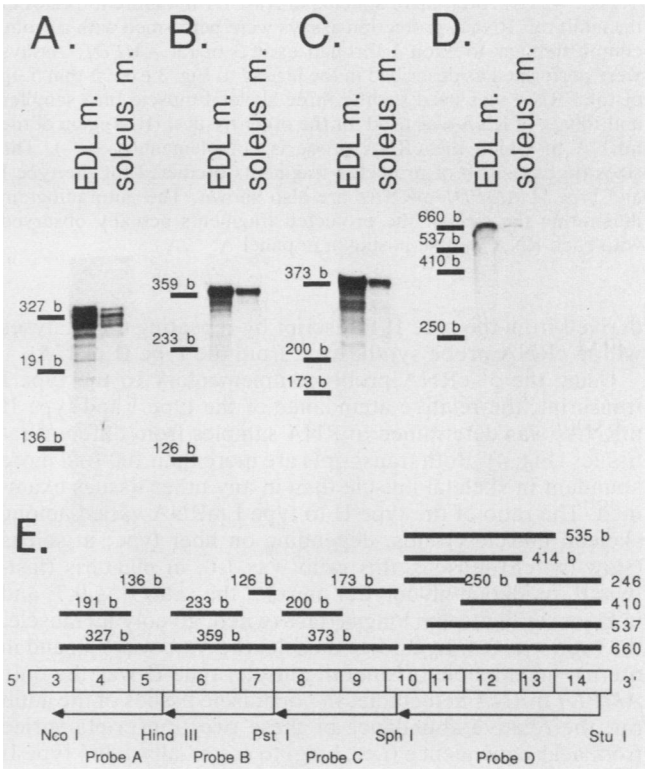

FIG. 3. (A to D) RNase mapping of rat *AMPD1* transcripts. RNase protection assays were performed with probes complementary to exons 3 through 14 of rat *AMPD1*. Total RNA (10 μg) from extensor digitorum longus muscle (EDL m.) or soleus muscle (soleus m.) was hybridized to each probe; after digestion with RNases A and T_1, the protected fragments were displayed on a 8% denaturing polyacrylamide gel (10). Sizes are indicated in bases (b). (E) Regions of the mRNA to which the probes are complementary (←—). The sizes (in bases [b]) of the protected fragments predicted for the different potential patterns of alternative splicing are illustrated by solid bars above the probes. The autoradiogram demonstrating the size of the protected fragments actually observed with each probe is shown in panels A to D, corresponding to the probe of the same letter illustrated in panel E.

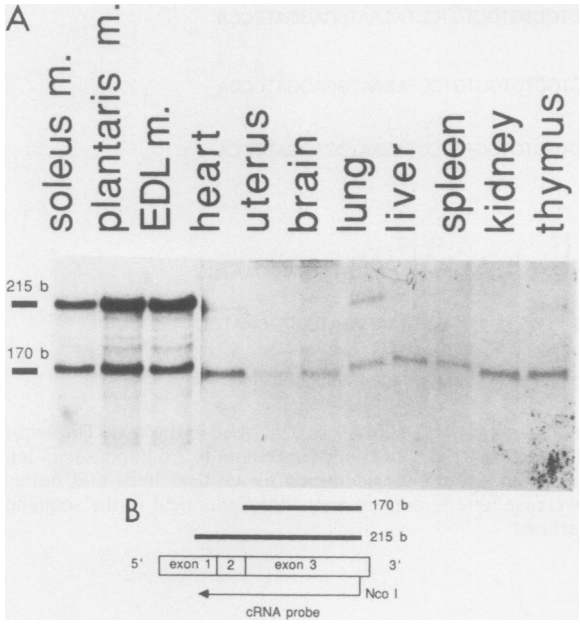

FIG. 4. (A) Alternative splicing of *AMPD1* in different tissues of the adult rat. RNase protection assays were performed with a probe complementary to exon 1 through exon 3 of rat *AMPD1*. Assays were performed as described in the legend to Fig. 3 except that 5 μg of total RNA was used for the three skeletal muscle (m.) samples and 100 μg of RNA was used for the other tissues. (B) Region of the mRNA to which the cRNA probe is complementary (←—). The sizes (in bases [b]) of protected fragments predicted for the type I and type II *AMPD1* mRNAs are also shown. The autoradiogram illustrating the size of the protected fragments actually observed with each RNA sample is shown in panel A.

derived from the type II transcript by repeating the analyses with a cRNA probe synthesized from the type II cDNA.

Using the 5′ cRNA probe complementary to the type I transcript, the relative abundance of the type I and type II mRNAs was determined in RNA samples from different rat tissues (Fig. 4). Both transcripts are more than 100-fold more abundant in skeletal muscle than in any other tissues examined. The ratio of the type II to type I mRNA varied among skeletal muscle groups, depending on fiber type; in soleus (slow-twitch) muscle, this ratio was 1.0; in plantaris (fast-twitch, oxidative-glycolytic) muscle, the ratio was 0.7; and in extensor digitorum longus (fast-twitch, glycolytic) muscle, the ratio was 0.4. In cardiac muscle, the ratio was 9.0, and in uterus, an example of smooth muscle, type II was the only *AMPD1* mRNA detectable. In nonmuscle tissues of the adult rat, the relative abundance of these two transcripts varied from near equivalence (i.e., lung) to essentially 100% type II transcript (i.e., brain, liver, spleen, and kidney) (Fig. 4). Prior studies using this same cRNA probe indicate that the type II transcript predominates in perinatal skeletal muscle of the fetal rat, and it appears several days before the type I transcript during in vitro differentiation of rat L6 myoblasts into myotubes (29).

Identification of an RNA intermediate that generates alternative 5′ splice donor sites in rat *AMPD1*. In the course of screening the rat skeletal muscle cDNA library described above, a cDNA of composition exon 1-exon 2-intron 2 was isolated (Fig. 2). Others have reported that incompletely processed transcripts accumulate in association with alternative splicing (1, 3, 4, 9, 24), and the composition of these intermediates suggests that the splicing machinery may pause at key introns (4). Initial inspection of the *AMPD1* intermediate described above suggested that it might give rise to a type I transcript only. However, careful examination of the sequence of this cDNA suggests a mechanism whereby exon 2 can also be recognized as an intron, and this could account for stalling of the splicing apparatus at this key intron. As a consequence of ligation of exon 1 to exon 2, a potential 5′ splice donor site is created at the boundary of exon 1 and exon 2 (CAG-GTCAAG). This sequence is in good agreement with the consensus sequence for a 5′ splice donor site in higher eucaryotes, i.e., (c/a)AG-GT(g/a)AGT (17). If the spliceosome recognizes this sequence as a 5′ splice donor site, exon 2 will be deleted; on the other hand, if the spliceosome recognizes the 5′ splice donor site 12 bases away at the junction of exon 2 and intron 2, exon 2 will be retained in the mature mRNA. This proposed mechanism for alternative splicing of *AMPD1* is consistent with observations demonstrating that primary transcripts with contiguous 5′ splice donor sites can be substrates for alternative splicing (2).

As a first step toward determining whether an RNA of the structure illustrated in Fig. 1 is an intermediate in the alternative splicing pathway of *AMPD1*, experiments were undertaken to demonstrate the presence of this RNA in vivo. For these studies, RNase protection assays were performed by using two types of cRNA probes: probe A, complementary to exon 1-exon 2-intron 2 sequences, and probe B, complementary to intron 1-exon 2-intron 2 sequences (Fig. 5). The RNA intermediate of the structure illustrated in Fig. 1 is expected to protect 274- and 180-base fragments of probes A and B, respectively. These fragments were easily detectable in RNA from skeletal muscle (Fig. 5). An alternative RNA intermediate of the structure exon 1-intron 2-exon 2-exon 3 is also a possible product of the *AMPD1* gene, and it is expected to protect a 191-base fragment of probe B. This intermediate, if formed, does not accumulate to sufficient levels in skeletal muscle to permit detection by this technique. The *AMPD1* primary transcript containing both introns 1 and 2 was readily detectable with both probes (180- and 359-base protected fragments of probes A and B, respectively). Thus, these results demonstrate that an RNA intermediate with the structure exon 1-exon 2-intron 2 is formed and accumulates to significant levels in vivo. Although the RNAs that gave rise to the protected fragments shown in Fig. 5 may be heterogeneous downstream of exon 3, for the purposes of this discussion we will refer to them simply as the intermediate and primary transcripts.

To determine whether an *AMPD1* transcript of the structure exon 1-exon 2-intron 2-exon 3 is an intermediate capable of being alternatively spliced to either a type I or a type II mRNA, a construct containing these sequences in an expression vector (Fig. 1) was transfected into 3T3 fibroblasts and C2C12 myoblasts. RNA from these cells was analyzed by two techniques. RNase protection assays using a cRNA probe like the one described in Fig. 5A illustrate that the transcript produced by this expression vector, which was similar to the RNA intermediate produced from the rat *AMPD1* primary transcript, could be processed to a transcript analogous to the type I mRNA (80-base protected fragment) and the type II mRNA (68-base protected fragment) (Fig. 6). The ratio of type II to type I transcripts was considerably greater in C2C12 myoblasts than in 3T3 fibroblasts. In neither cell line was the ratio similar to that observed in any tissue in vivo (Fig. 4). This finding may reflect differences in *trans*-acting factors in the various cell

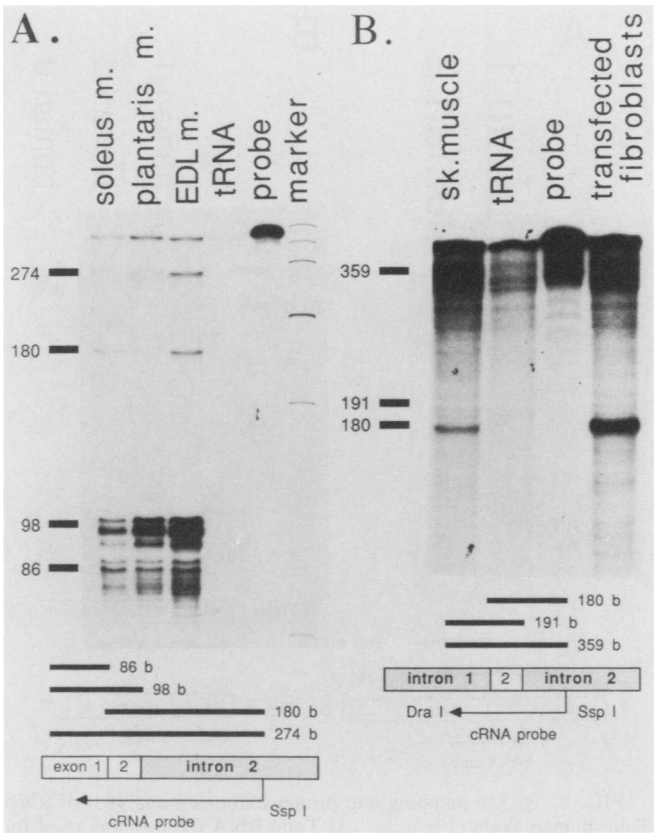

FIG. 5. Identification of RNA splicing intermediate in rat skeletal muscle. RNase protection assays were performed with 25 μg of total RNA from the various fiber types of skeletal (sk.) muscle and from 3T3 cells transfected with the minigene construct shown in Fig. 1. The compositions of cRNA probe A (complementary to exon 1-exon 2-intron 2) and probe B (complementary to intron 1-exon 2-intron 2) are shown below panels A and B, respectively. The sizes (in bases [b]) of the protected fragments predicted for the different types of transcripts are shown by the solid bars above the probes. Autoradiograms illustrating the protected fragments actually observed with each probe are shown in panels A and B, and the source of cellular RNA to which the probe was hybridized is shown above each lane. The tRNA lane is the digestion product of the probe plus 40 μg of tRNA; the undigested probe is illustrated in the lane labeled probe, and size markers (396, 344, 298, 220, 154, and 75 nucleotides) are shown in the lane labeled marker. m., Muscle.

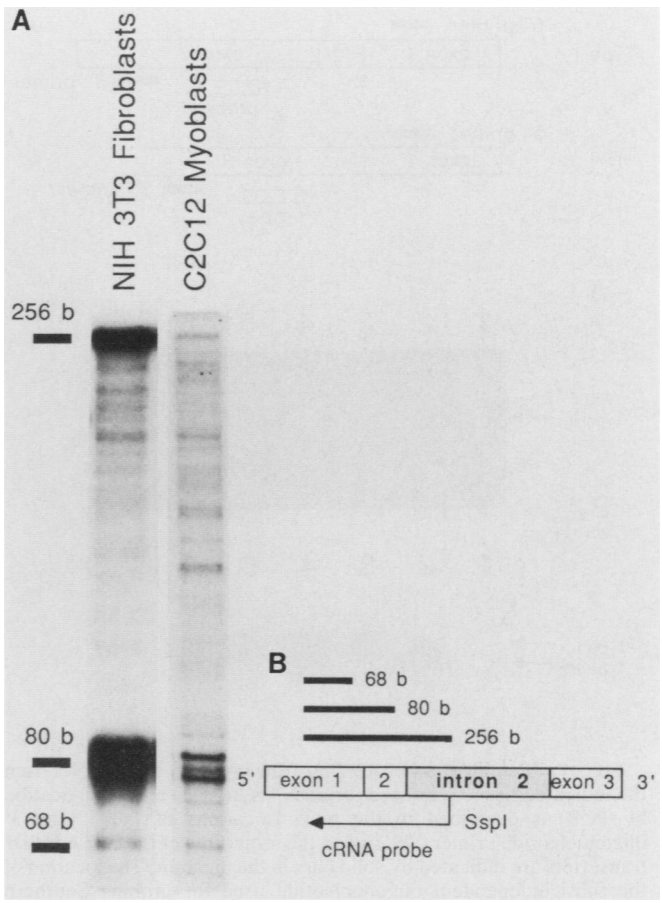

FIG. 6. RNase map of transcripts produced by the exon 1-exon 2-intron 2-exon 3 expression vector. 3T3 fibroblasts and C2C12 myoblasts were transfected as described in the text with the expression vector illustrated in Fig. 1. RNase protection assays were performed with 20 μg of total RNA as described in the legend to Fig. 3. (A) Autoradiogram of protected fragments; (B) cRNA probe and predicted protected fragments.

types or differences between the intermediate produced by the chimeric minigene and the RNA intermediate generated from the natural gene as a consequence of consecutive splicing reactions (6, 25, 26, 34).

As a second assay, the same RNA samples from transfected 3T3 cells, along with cDNAs for the type I and type II rat *AMPD1* transcripts, were used as templates for PCR reactions primed by oligonucleotides corresponding to exon 1 and exon 3 sequences. PCR products of 105 and 117 base pairs were obtained with RNA from 3T3 cells transfected with the exon 1-exon 2-intron 2-exon 3 expression vector, whereas RNA from control cells did not produce these DNA fragments (Fig. 7). Each PCR fragment produced by RNA from transfected cells was the same size as the PCR fragment produced from authentic type I or type II cDNA, and both of these DNA fragments hybridized to an *AMPD1* probe. These results demonstrate that the 5′ splice donor site created by ligation of exon 1 to exon 2 is functional in vivo and that an RNA intermediate of the composition exon 1-exon 2-intron 2-exon 3 is a substrate for alternative splicing of exon 2 from *AMPD1* transcripts.

Splicing of the human *AMPD1* transcript. The primary structures of the human and rat *AMPD1* genes have been highly conserved, including the location and sequence of the 5′ splice donor and 3′ branch-splice acceptor sites (28). However, the first base of exon 2, a 12-base miniexon in both genes, has not been conserved, and this change of a single base in human exon 2 has potential implications for the generation of an RNA splicing intermediate like the one described above for rat *AMPD1*. The sequence of the potential 5′ splice donor site created at the point where exon 1 is ligated to exon 2 in human *AMPD1* is CAG-CTGAAG, compared with CAG-GTCAAG in rat *AMPD1*. Although there is considerable tolerance for deviation from the consensus sequence of the 5′ splice donor site at some positions of the introns examined thus far, there has been only one exception to the apparent requirement for a G at position +1 in higher eucaryotes (35). Moreover, several studies have shown that mutation of the G at position +1 of the 5′ splice donor site results in failure of this site to be recognized by the spliceosome (39, 40). As a result of this difference in sequence between rat and human *AMPD1*, type II mRNA may not be produced in humans if alternative 5′ splice donor

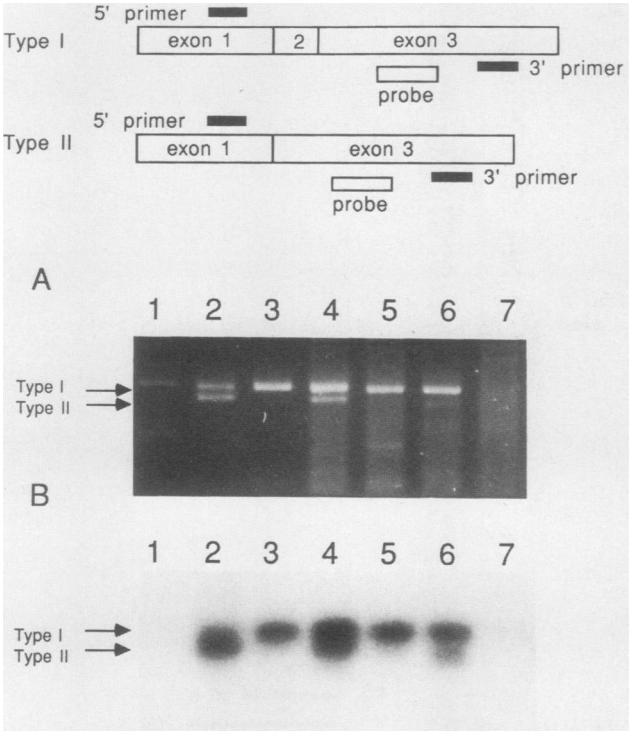

FIG. 7. Identification of *AMPD1* transcripts by PCR. RNA from the indicated tissue was used for cDNA synthesis and amplification by PCR as described in the text. Locations of the 5′ and 3′ oligonucleotide primers relative to the structures of the two *AMPD1* transcripts are indicated by solid bars in the diagram. The location of the third independent oligonucleotide used for probing Southern blots is indicated by the open bar. Products of the PCR reactions were displayed on 3% agarose gels, and the gel was stained with ethidium bromide (A) or transferred to Nytran paper for hybridization to the *AMPD1* oligonucleotide probe indicated by the open bar in the diagram (B). Lanes: 1, size markers, 118 and 78 base pairs; 2, PCR products of the type I and type II rat *AMPD1* cDNAs; 3, PCR product of human type I *AMPD1* cDNA; 4, PCR product of 1 μg of total RNA from rat skeletal muscle; 5, PCR product of 1 μg of total RNA from human skeletal muscle; 6, PCR product of 1 μg of total RNA from 3T3 cells transfected with the rat exon 1-exon 2-intron 2-exon 3 expression vector; 7, PCR product of 1 μg of RNA from nontransfected 3T3 cells. Arrows indicate locations of the type I (117-base-pair) and type II (105-base-pair) PCR products.

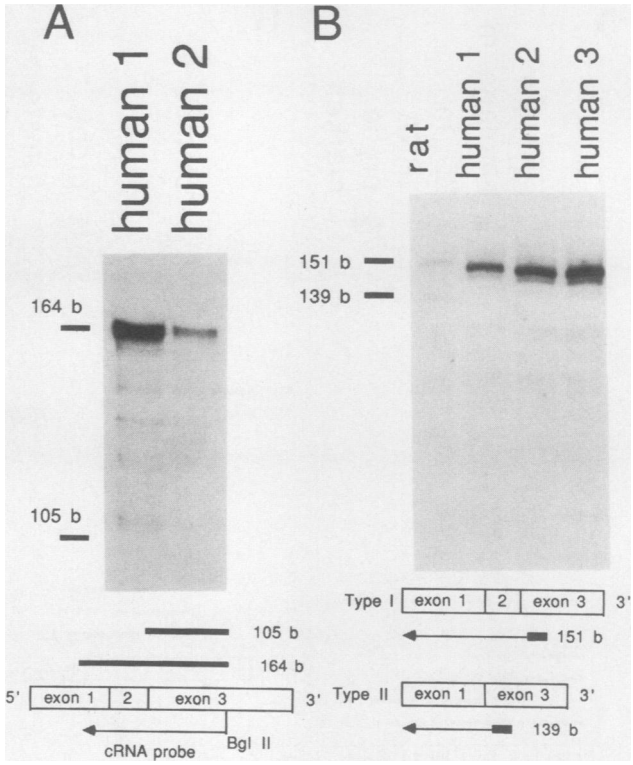

FIG. 8. RNase mapping and primer extension analyses of RNA from human skeletal muscle. (A) Total RNA (50 μg) was used for RNase protection as described in the legend to Fig. 3. The region of the mRNA to which the cRNA probe is complementary is illustrated by the inverted arrow in the diagram, and the solid bars indicate the sizes (in bases [b]) of the protected fragments predicted for the two *AMPD1* transcripts. The autoradiogram of the protected fragments actually observed is shown. (B) Primer extension was performed with 20 μg of total RNA from rat or human skeletal muscle. The oligonucleotide used for these studies is complementary to human exon 3 sequences, and consequently the signal intensity is stronger with human than with rat RNA. The sizes (in bases [b]) of the extension products expected for the two types of *AMPD1* transcripts are illustrated in the diagram.

site selection from an RNA intermediate is necessary for formation of type II mRNA. On the other hand, if the type II mRNA is produced by a mechanism that recognizes intron 1-exon 2-intron 2 as a single large intron in the primary transcript, the sequence of exon 2 may have little effect on alternative splicing. Three different assays, RNase protection, primer extension, and PCR, were used to assess alternative splicing of exon 2 in the *AMPD1* transcript from muscle of three normal individuals (Fig. 7 and 8). Type II mRNA was not detectable in human muscle with any of these assays.

DISCUSSION

This study confirms that the rat *AMPD1* primary transcript is alternatively spliced and that alternative splicing is limited to exon 2, a 12-base miniexon. Since transcription begins at the same point in both *AMPD1* transcripts (28) and an internal exon is either included in excluded, this alternative splicing pattern falls into the category that has been called cassette splicing (2). The relative abundance of the two types of *AMPD1* transcripts varies over a wide range in different tissues of the adult animal as well as in different skeletal muscle fiber types of the adult rat. During in vivo and in vitro myogenesis, the type II transcript is detectable several days before the type I transcript, and the ratio of type II to type I *AMPD1* mRNA continues to decline during myocyte development (29). Although we have not assessed the relative stability of these two *AMPD1* transcripts in different tissues and at different stages of development, we assume that the relative abundance of the two transcripts reflects differences in splicing. If this assumption is correct, cassette splicing of the rat *AMPD1* primary transcript is regulated by tissue-specific and stage-specific signals.

Results of this study have identified an RNA intermediate derived from the rat *AMPD1* gene that generates an internal, alternative 5′ splice donor site not present in the primary transcript. This intermediate accumulates in vivo in association with alternative splicing of rat *AMPD1*. Expression in cell lines of a chimeric gene that produces a transcript containing a portion of the *AMPD1* RNA intermediate

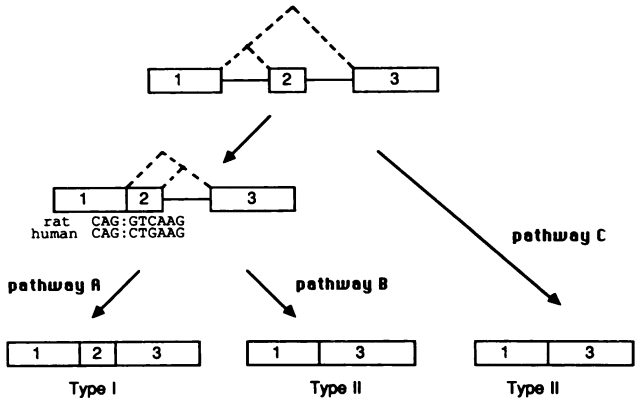

FIG. 9. Model of potential pathways leading to alternative splicing of exon 2 in *AMPD1*. The sequence of the potential 5′ splice donor sites created by ligation of exon 1 to exon 2 in rat and human *AMPD1* is indicated under the RNA intermediate.

demonstrates that the 5′ splice donor site created by ligation of exon 1 to exon 2 is functional. Moreover, this intermediate is capable of being alternatively spliced to transcripts analogous to the type I and type II *AMPD1* mRNAs (pathways A and B in the model of alternative splicing illustrated in Fig. 9).

Results of these experiments do not exclude the possibility that cassette splicing of the rat *AMPD1* transcript also takes place by excision of exon 2 as a part of a larger intron containing both flanking introns 1 and 2 (pathway C in the model of alternative splicing illustrated in Fig. 9). Although it is not possible to exclude a role for pathway C in alternative splicing of rat *AMPD1* by these studies, the following comparisons between rat and human *AMPD1* lead us to speculate that pathway C (Fig. 9) may not be the predominant route leading to formation of type II *AMPD1* mRNA. If pathway C contributes significantly to the production of type II mRNA in the rat, then it is not obvious why type II mRNA is not found in human skeletal muscle. The 5′ splice donor sites at the boundary of exon 1 and intron 1 and at the 3′ branch splice acceptor sites at intron 2-exon 3 have been conserved, and they are functional for constitutive splicing of both genes. Although there may be unappreciated differences in these sites or other sequences of the introns, it is not apparent how the minor differences in exon 2 sequences preclude pathway C in humans but not in rats. On the other hand, if pathway B is the predominant route leading to the formation of type II mRNA, the difference in sequence of exon 2 between these two species provides a good explanation for the failure to find type II mRNA in human skeletal muscle. The RNA intermediate essential to processing through pathway B does not provide a good alternative 5′ splice donor site in human *AMPD1*, whereas it does in rat *AMPD1*. On the basis of these comparisons, it seems more likely that pathway B rather than pathway C is the predominant route for production of type II mRNA.

This study does not exclude an additional pathway for synthesis of type I mRNA through an RNA intermediate of the composition exon 1-intron 1-exon 2-exon 3. However, there is no evidence for accumulation of such an RNA intermediate in vivo (Fig. 5).

Recognition that an internal, functional 5′ splice donor site can be generated subsequent to exon ligation raises the question of how common this mechanism is. By chance, a reasonable 5′ splice donor site consensus sequence will be generated frequently during constitutive splicing of many primary transcripts. If functional, many of these newly generated sites would be expected to have deleterious effects. Consequently, some mechanism must prevent this type of splicing from occurring randomly. An unusual property of exon 2 in the *AMPD1* gene is its small size, only 12 bases. As a consequence, the alternative 5′ splice donor site created at the boundary of exon 1 and exon 2 in this RNA intermediate is only 12 bases away from the 5′ splice donor site at the boundary of exon 2 and intron 2. In examples of internal, alternative 5′ splice donor sites in primary transcripts, a situation somewhat analogous to the RNA intermediate of the rat *AMPD1* transcript, the distance between alternative 5′ splice donor sites is rather short (6 to 27 bases) (18, 19, 24, 32, 33, 37). Thus, one factor that may operate to prevent random utilization of internal 5′ splice donor sites generated by exon ligation during constitutive splicing is exon size. Size alone, however, is not the only determinant of whether an internal 5′ splice donor site created by exon ligation will be functional, since in the case of rat *AMPD1*, the relative abundance of type I and type II mRNAs is regulated by stage- and tissue-specific signals. These results suggest that one or more *trans*-acting factors influence which of the alternative 5′ splice donor sites will be selected in this splicing reaction. The recognition that factors other than U1 small nuclear ribonucleoproteins bind to the 5′ splice donor site (36, 41) and that spliceosomes may dissociate and reassemble on RNA precursors (25) provides potential mechanisms by which the cell can regulate selection of alternative 5′ splice donor sites.

The physiological consequences of alternative splicing of the *AMPD1* transcript are not known. The amino-terminal 200 residues of this 727-amino-acid peptide, which include the region encoded by exon 2, have been found not to be conserved when the sequence of rat or human *AMPD1* gene product is compared with that of yeast AMPD (16). Deletion of the 5′-most 215 base pairs from rat *AMPD1* cDNA, which encompasses the area containing exon 2 sequences, does not eliminate catalytic activity of AMPD when this truncated cDNA is placed in a procaryotic expression vector (H. Morisaki et al., unpublished observations). If this alternative splicing reaction is functionally significant, it may prove to be important for some isoform-specific property, such as allosteric regulation of this enzyme by nucleotides or inorganic phosphate (12), posttranslational modification of the protein (38), binding of AMPD to myosin heavy chain (15), or interaction of this enzyme with other macromolecules in the cell (20).

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