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The *spf*^{ash} mouse: A missense mutation in the ornithine transcarbamylase gene also causes aberrant mRNA splicing

(polymerase chain reaction/mitochondrial protein transport/protein assembly/ornithine carbamoyltransferase)

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ABSTRACT **Ornithine transcarbamylase (ornithine carbamoyltransferase; carbamoyl-phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) is a mitochondrial matrix enzyme of the mammalian urea cycle. The X chromosome-linked *spf*^{ash} mutation in the mouse causes partial ornithine transcarbamylase deficiency and has served as a model for the human disease. We show here that the *spf*^{ash} mutation is a guanine to adenine transition of the last nucleotide of the fourth exon of the ornithine transcarbamylase gene. This nucleotide change produces two remarkably different effects. First, this transition causes ornithine transcarbamylase mRNA deficiency because the involved exon nucleotide plays a part in the recognition of the adjacent splice donor site. As a result of the mutation, ornithine transcarbamylase pre-mRNA is spliced inefficiently both at this site and at a cryptic splice donor site 48 bases into the adjacent intron. Second, two mutant proteins are translated from these mRNAs. From the correctly spliced mRNA, the transition results in a change of amino acid 129 from arginine to histidine. This missense substitution has no discernable effect on mitochondrial import, subunit assembly, or enzyme activity. On the other hand, the elongated mRNA resulting from mis-splicing is translated into a dysfunctional ornithine transcarbamylase subunit elongated by the insertion of 16 amino acid residues.**

Ornithine transcarbamylase (ornithine carbamoyltransferase; OTCase; carbamoyl-phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) is a mitochondrial matrix enzyme of the mammalian urea cycle. In both humans and mice, the OTCase gene is located on the X chromosome and is composed of 10 exons spread over 70–80 kilobase pairs (kbp) (1, 2). The gene is expressed primarily in the liver and directs the formation of a 40-kDa precursor protein (pOTCase). The pOTCase is imported into the mitochondrial matrix and cleaved of its N-terminal leader peptide, and the 36-kDa mature subunits assemble into an enzymatically active homotrimer.

In humans, hereditary OTCase deficiency is an X chromosome-linked disorder often leading to lethal neonatal hyperammonemia (3). Two mouse strains with partial OTCase deficiency serve as useful animal models: sparse fur (*spf*) (4); and sparse fur–abnormal skin and hair (*spf*^{ash}) (5). These mice have permitted the study of various clinical aspects of OTCase deficiency (6); they also have served as experimental targets for gene therapy (7). The *spf* mouse phenotype is caused by a missense mutation, changing histidine to asparagine at amino acid 117 (8). Although OTCase is produced in normal amounts, the mutation impairs enzymatic activity (9–11).

The *spf*^{ash} mutation has a unique effect on OTCase biogenesis (12, 13). From the single mutant gene in males, two OTCase precursors are produced, which together amount to only 10% of wild-type pOTCase levels. One pOTCase is normal in all characteristics of its structure and biogenesis except quantity and gives rise to the residual (5–10%) hepatic OTCase activity in the *spf*^{ash} mice. The other pOTCase is elongated, based on its apparent molecular weight. It is capable of uptake into mitochondria, where it is cleaved to an elongated mature subunit. It cannot assemble into an active trimeric structure, however, and appears to degrade rapidly within the mitochondrion without contributing to the detectable steady-state protein level or enzymatic activity. To answer the question of how one mutant OTCase gene produces two discrete translation products, we have studied the structure of the OTCase gene and its transcripts in *spf*^{ash} male mice and normal male littermates.

MATERIALS AND METHODS

Animals and Biological Samples. A colony of *spf*^{ash} mice has been maintained at the Yale School of Medicine from breeding pairs kindly provided by Donald Doolittle. All animals used for this study were male mice, either *spf*^{ash} hemizygotes or normal littermates. The genotype was assessed by the phenotypic appearance of the animals at about 1 week of age and was confirmed in every case by assay of hepatic OTCase enzyme activity (12). Hepatic mRNA was isolated (14) and enriched for polyadenylylated mRNA (15). RNA was separated on a 1.75% agarose gel in formaldehyde, transferred to nitrocellulose, and probed (15) first with a rat OTCase cDNA (16) and then with a mouse serum albumin cDNA isolated in this laboratory (P.E.H., unpublished data). Genomic DNA was prepared from brain (15).

Isolation of a Wild-Type Mouse OTCase cDNA. A cDNA library from wild-type mouse liver mRNA was prepared exactly as described for the rat (16). A partial 3′ OTCase cDNA was isolated from it by hybridization to a rat OTCase cDNA (16). Additional clones were created from the same cDNA by attachment of *Eco*RI linkers and ligation into plasmid pGEM-2 (Promega). From these clones, a partial 5′ OTCase cDNA was isolated. A full-length OTCase cDNA, spMO-3, was constructed by ligation of these two fragments at a common restriction site. This cDNA contains 40 bp of 5′ and 263 bp of 3′ untranslated sequences and the entire OTCase coding sequence inserted between the *Xba* I and *Pst* I sites of pSP65 (Promega). As constructed, *in vitro* transcription by SP6 RNA polymerase produces translatable mRNA. This cDNA was sequenced in its entirety by a combination of Maxam and Gilbert (17) and dideoxynucleotide sequencing with Sequenase (United States Biochemical). The sequence agrees with that published by others (8) with three exceptions: nucleotide 163 reported as T, we find

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Abbreviations: pOTCase, precursor of ornithine transcarbamylase; OTCase, ornithine transcarbamylase; PCR, polymerase chain reaction.

C; 451 reported as C, we find A; 1251 reported as T, we find C.

S1 Nuclease Protection Assays. Conditions for RNA·DNA hybridization and S1 nuclease digestion were adapted from Battey *et al.* (18). Double-stranded DNA probes were prepared from restriction fragments of the mouse OTCase cDNA eluted from acrylamide gels (probe A: 554 bp, pSP65 *Hha* I site to OTCase *Bgl* II site; probe B: 255 bp, *Bgl* II/*Bgl* II; probe C: 417 bp, *Xho* I/*Hin*dIII; probe D: 418 bp, *Hin*dIII/*Hin*dIII; probe E: 467 bp, *Pvu* II/*Pvu* II). The probes were end-labeled by 3′ fill-in reaction (probes B, C, and D) or 5′ kinase reaction (probes A and E) to 10^6 cpm/pmol end (15). The probe (10^5 cpm) and excess hybridizing RNA (each reaction mixture contained either 50 μg of *spf*^{ash} mouse liver mRNA, or 5 μg of wild-type mouse liver mRNA plus 45 μg of nonhomologous yeast RNA, or 50 μg of nonhomologous RNA as a control) were denatured and hybridized, then digested for 30 min at 37°C with a range of concentrations of S1 nuclease from 33 units/ml to 3300 units/ml (18). A single S1 nuclease concentration from each titration is shown in Fig. 2 (*Lower*) (*A*, 900 units/ml; *B*, 600 units/ml; *C* and *D*, 250 units/ml; *E*, 750 units/ml). Protected fragments were visualized by autoradiography after electrophoresis through 8 M urea/6% polyacrylamide gels.

Polymerase Chain Reaction (PCR) Amplification and Cloning. We performed PCR amplification (19) using *Thermus aquaticus* DNA polymerase (New England Biolabs) and the reagent concentrations recommended by the supplier. The cDNAs to be amplified (100 ng per 100 μl of reaction mixture), either double stranded or first stranded only, were prepared as described (16, 20).

The amplification primers were M2 (5′-GCCATGGTGTCCAGATCTGA) and M3 (5′-ATTGCAAGGGAAATCCTTAGGAATG). After initial denaturation at 98°C for 7 min, the 40 amplification cycles were at 90°C for 30 sec, 37°C for 30 sec, and 63°C for 90 sec; the final polymerization was extended for 15 min. Samples were desalted by Centricon centrifugal filtration (Amicon), digested with appropriate restriction enzymes, and ligated into similarly digested plasmid vectors, either pGEM-3Z (Promega) or the wild-type mouse OTCase cDNA plasmid, replacing the analogous region of spMO-3. In this way, spAMO-PM (containing the *spf*^{ash} mouse point-mutated OTCase cDNA) and spAMO-E (containing the *spf*^{ash} mouse elongated OTCase cDNA) were created.

PCR amplification of genomic DNA (1 μg, intact) was performed under identical conditions, using oligonucleotide primers M10 (5′-ATTTGGTTAACATTTTAGTT) and M11 (5′-GGCATTATCTAAGGAGAAGC). Products were ligated directly into *Hin*cII-digested pGEM-3Z (Promega). Sequences of PCR-derived clones were determined by dideoxynucleotide sequencing as described above.

***In Vitro* Reconstruction of OTCase Biogenesis.** The methods for *in vitro* SP6 RNA polymerase transcription, *in vitro* translation, immune precipitation, isolation of rat liver mitochondria, *in vitro* mitochondrial uptake, assay for OTCase assembly using N^{δ}-phosphonoacetyl-L-ornithinyl-Sepharose chromatography, and polyacrylamide gel electrophoresis have been described elsewhere (21, 22). To assay for intramitochondrial stability of newly imported OTCase polypeptides, samples containing translation mixtures and intact mitochondria were first incubated at the usual temperature (27°C) for 1 hr. The incubation temperature was then raised to 37°C, and serial samples were removed for an additional 8 hr.

RESULTS

Isolation of a Normal Mouse OTCase cDNA and RNA Blot Analysis of mRNA. For comparison with the structure of the *spf*^{ash} mouse OTCase mRNA, we isolated and analyzed cDNA clones encoding wild-type mouse OTCase. RNA blot analysis (Fig. 1) showed that hepatic OTCase mRNA from normal and *spf*^{ash} mice is ≈1700 nucleotides long and is heterogenous in size [due perhaps to multiple transcription initiation sites (8)]. Total *spf*^{ash} OTCase mRNA is reduced to ≈10% of control levels, consistent with estimations from *in vitro* translation (12) and results reported by others (23). No mRNA of abnormal size is detectable in *spf*^{ash} mouse liver, but these blots would not have resolved the small size difference corresponding to the observed elongation in the *spf*^{ash} pOTCase protein.

S1 Nuclease Protection Assays. We modified S1 nuclease protection assays specifically to detect small RNA insertions relative to the DNA probes. This detection involved nuclease "cut through" (24–26), digesting not only the single-stranded RNA loop but also cutting the probe at the site in the duplex opposite the resulting nick. Using titration of S1 nuclease to high enzyme concentrations and higher than usual temperatures, we were able to obtain uniform cleavage of the probe strand opposite as little as a 12-base RNA insertion (data not shown).

S1 nuclease protection experiments were carried out with wild-type and *spf*^{ash} male liver poly(A)$^+$ mRNA. Using 10 times the amount of RNA from the mutant mice, equal protection levels of the probes were obtained. The coding sequence of the OTCase cDNA was analyzed in four sections, using four end-labeled cDNA probes A, B, C, and D (Fig. 2). Probes A, C, and D were protected identically by normal and mutant mRNA. This protection extended over the entire length of the OTCase cDNA sequences, while residual plasmid sequences were accurately cleaved from probe A. No internal cleavages were detected with these probes; i.e., no shorter fragments of the probe were created by S1 nuclease digestion. Probe B, however, was cleaved by S1 nuclease internally when protected by *spf*^{ash} mRNA (Fig. 2 *Lower, B*, arrowhead). Only a portion of the *spf*^{ash} mRNA·cDNA hybrids allowed internal S1 nuclease cleavage. Most of the hybrids were protected along their entire length. This implied that there were two OTCase mRNA species in the *spf*^{ash} mice, one apparently normal in structure, and one with a detectable internal difference.

To confirm this result, and to show that this detected difference was not an mRNA truncation or a deletion, probe E was prepared. This probe covered the same cDNA region but was end-labeled on the opposite end of the probe strand as probe B. Probe E was also cleaved internally (Fig. 2 *Lower, E*, arrowhead). Furthermore, the sites of cleavage of probes B and E, deduced from the sizes of the cleavage products, mapped to the same site (Fig. 2 *Upper*, ×). These results were consistent with an RNA insertion, a very small

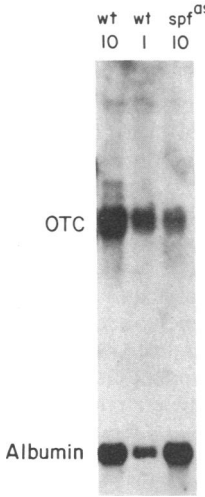

FIG. 1. RNA gel blot of wild-type (wt) and *spf*^{ash} male mouse liver mRNA. Ten micrograms and 1 μg of wild-type mouse poly(A)$^+$ mRNA were compared to 10 μg of *spf*^{ash} mouse mRNA. The blot was probed with a labeled rat OTCase cDNA and then reprobed with a mouse serum albumin cDNA.

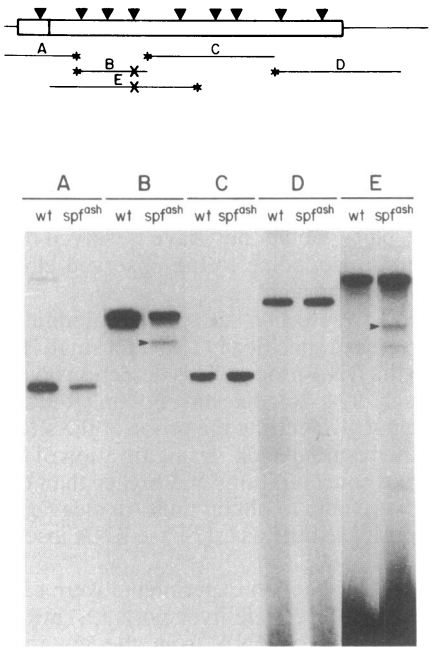

FIG. 2. S1 nuclease protection assay of wild-type (wt) and *spf*^{ash} mouse liver mRNA. A diagram of the mouse OTCase cDNA spMO-3 and the probes (A–E) used in the S1 nuclease assays is shown (*Upper*). The open bar represents the protein coding region, the shorter bar at the left represents the leader peptide encoding region, and the horizontal lines to the left and right represent the 5′ and 3′ untranslated regions, respectively. Triangles above the diagram depict the position of introns interrupting the genomic sequence (2). The end-labeled double-stranded DNA probes, A–E, are represented as lines coinciding with their positions in the cDNA, with the asterisk depicting the ^{32}P-labeled nucleotide of the antisense (probe) strand. The Xs in probes B and E represent the sites of S1 nuclease cleavage demonstrated below. (*Lower*) Results of S1 nuclease protection assays with probes A–E. Probes were hybridized with wild-type (wt) or *spf*^{ash} mouse liver poly(A)$^+$ mRNA and then digested with S1 nuclease. Internal S1 nuclease cleavage sites are detected in the *spf*^{ash} OTCase mRNA with probes B and E (arrowheads). The location of these cleavages is shown (×) in the diagram above.

(<10 bases) deletion, or a detectable point mutation, in some but not all of the *spf*^{ash} OTCase mRNA. This region of interest maps to the site of splicing of exon 4 to exon 5 (2).

PCR Amplification and cDNA Cloning. To characterize further the splice junction between exons 4 and 5 in the *spf*^{ash} cDNA, we amplified this region by the PCR (19). Using oligonucleotide primers complementary to sequences in exons 3 and 5, cDNA from normal or *spf*^{ash} mRNA was subjected to 40 cycles of amplification. Aliquots of the products were separated by denaturing polyacrylamide gels, transferred to a nylon membrane, and hybridized to a radiolabeled normal mouse OTCase cDNA fragment. As shown in Fig. 3, normal mouse cDNA produced a single prominent amplification product of the expected size (228 bp). Two major amplification products were produced from *spf*^{ash} mouse cDNA, however: one of normal size and one elongated. The nucleotide sequence of clones derived from the amplified normal mouse cDNA agreed exactly with previously isolated cDNAs (Fig. 4).

Nucleotide sequencing of the normal length product from *spf*^{ash} cDNA showed an unexpected nucleotide change. The last nucleotide of exon 4 was A instead of G (Fig. 4). This change was seen in each of six independent clones from the *spf*^{ash} cDNA, produced from two independent PCR amplifications, as well as in the direct sequencing of the gel-purified normal length amplified cDNA. This nucleotide transition predicts that the normal-sized pOTCase protein in *spf*^{ash} mice contains an amino acid substitution of histidine for arginine at amino acid 129.

The nucleotide sequence of the longer cDNA from the *spf*^{ash} mouse was even more interesting (Fig. 4). This cDNA contained the G to A transition discussed above, followed immediately by a 48-bp insertion. The sequence of this insertion agreed completely with the published sequence of the 5′ end of intron 4 (2). The following six nucleotides in the genomic sequence (2) are a good match to the consensus sequence (27, 28) for a splice donor site (Fig. 4 *Lower*, cryptic site). Thus, a cryptic splice donor site 48 bases into intron 4 is used to create the elongated mRNA and from it, the elongated pOTCase.

Genomic DNA Clones. We generated and analyzed genomic DNA clones from wild-type and *spf*^{ash} mouse OTCase genes to show that the point mutation was specific for the *spf*^{ash} mutation and to exclude the possibility that two nucleotide differences were present to produce the *spf*^{ash} phenotype—e.g., that the G to A transition decreased use of the normal splice site and that a second mutation within the intron activated the cryptic site. Oligonucleotide primers for PCR were prepared based on the published sequence of introns 3 and 4 (2). These primers flank exon 4 such that a 250-bp DNA fragment would result from amplification. Products of PCR amplification of genomic DNA from an *spf*^{ash} male and a wild-type male littermate were cloned and sequenced. The sequences of these DNAs agreed with those published (2), with the exception of the same G to A transition in the genomic DNA of the *spf*^{ash} mouse. Specifically, no other mutations were found surrounding the activated cryptic site, which was identical in normal and *spf*^{ash} DNAs.

Biogenesis of OTCase *in Vitro*. We next proved that the two types of *spf*^{ash} mRNA were sufficient to account for the OTCase proteins translated by mutant liver mRNA. We substituted the PCR-amplified cDNAs, as restriction fragments, for the analogous region of the normal mouse cDNA in a transcription vector. These cDNAs were transcribed *in vitro*, and the mRNA generated was translated *in vitro* as a reticulocyte lysate. As shown in Fig. 5, insertion of the point mutation into the pOTCase coding region did not affect the electrophoretic mobility of the translated pOTCase. On the other hand, the elongated cDNA programmed the synthesis of an elongated pOTCase protein, identical in mobility to the elongated pOTCase translated from *spf*^{ash} male liver mRNA.

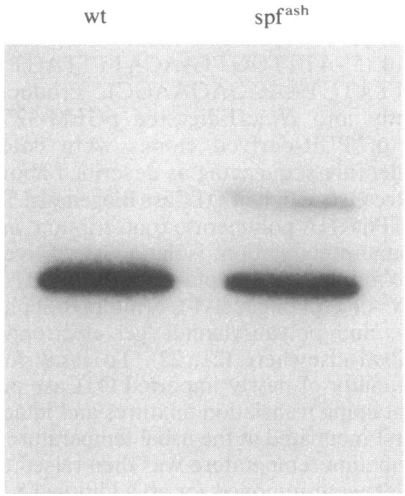

FIG. 3. PCR products of wild-type (wt) and *spf*^{ash} mouse liver cDNA. Amplification products were resolved in a denaturing urea/polyacrylamide gel, electrophoretically transferred to a nylon membrane, and probed by hybridization to a labeled mouse OTCase cDNA fragment. Note the two products in the *spf*^{ash} sample compared to the single wild-type one.

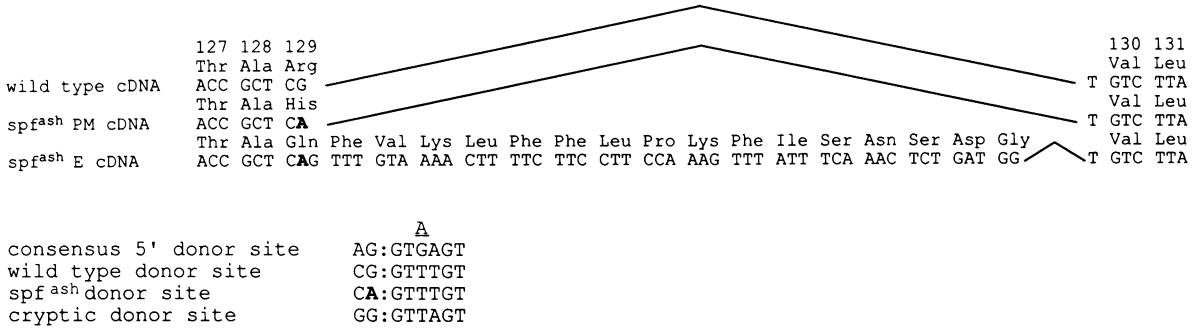

FIG. 4. Comparison of relevant cDNA and genomic sequences from wild-type and *spf*^{ash} mice. (*Upper*) The sequence of wild-type cDNA from codon 127 to codon 131 is aligned with the sequences of the *spf*^{ash} mouse cDNAs containing the point mutation (PM) and the elongation (E). The lines connecting exons 4 and 5 indicate the splicing products. The A created by the *spf*^{ash} transition is shown in boldface type in both the PM and E cDNAs. The putative translation products are given above the corresponding cDNAs. (*Lower*) The consensus 5′ splice donor site (27) is compared to the wild-type OTCase exon 4 donor site, the mutated donor site, and the cryptic site within intron 4, which is used to create the elongated cDNA. The A resulting from the *spf*^{ash} mutation is shown in boldface type.

Mixing of the translation products of the point mutation mRNA and the elongated mRNA reproduced the pattern of pOTCase translation from *spf*^{ash} liver mRNA.

Furthermore, in experiments not shown, when the *spf*^{ash} OTCase precursors were incubated with isolated rat liver mitochondria, both pOTCase proteins were imported and cleaved to mature subunits. After the mitochondria had been solubilized, the newly formed OTCase subunits were assayed for assembly. A portion of the wild-type OTCase and the missense substituted OTCase bound to a δ-*N*-phosphonoacetyl-L-ornithine affinity column, indicating that the newly formed mature subunits had assembled into an active trimer. None of the elongated subunits bound to the affinity ligand, however, confirming the observations of Rosenberg *et al.* (12). To determine more specifically the fate of the elongated OTCase subunits, mitochondria were incubated for additional times at 37°C. The newly cleaved, elongated OTCase subunits were unstable under these conditions, with an apparent half-life of <2 hr, while both the wild-type OTCase and the missense substituted protein were stable.

DISCUSSION

We show here that the OTCase gene in the *spf*^{ash} mouse contains a point mutation, changing the last nucleotide of the fourth exon from G to A. This mutation produces two dramatically different effects on OTCase biogenesis. First, this exon mutation has an unexpectedly drastic effect on the use of the adjacent splice donor site. The splice site is used to produce only 5% of wild-type levels of properly spliced mRNA. An additional mRNA, using a cryptic splice donor within intron 4, is also produced at 5% of wild-type levels. Apparently, 90% of the pre-mRNA transcripts are degraded, either after unproductive mis-splicing or without removal of intron 4. Second, translation of the mRNAs produces two pOTCase proteins. One contains a missense mutation, changing arginine 129 to histidine. This substitution, however, does not inhibit mitochondrial import or assembly of the precursor, nor does it have any detectable effect on the enzyme's activity. The second precursor contains an insertion of 16 amino acids encoded by 48 bases of intron 4. The internal insertion does not affect mitochondrial import but apparently prevents proper folding or subunit trimerization so that the unassembled elongated subunits are degraded rapidly within mitochondria.

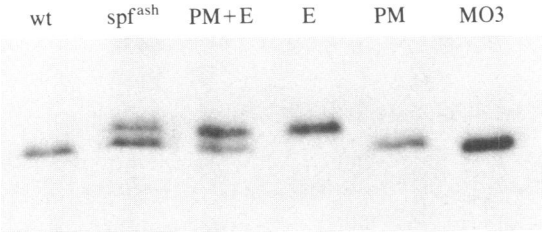

FIG. 5. *In vitro* translation of pOTCase proteins encoded by liver mRNAs and by *in vitro* transcribed mRNA. mRNA was synthesized *in vitro* by SP6 RNA polymerase transcription of cDNAs encoding wild-type mouse OTCase (lane MO3), *spf*^{ash} mouse OTCase containing the point mutation (lane PM), or *spf*^{ash} mouse OTCase containing the elongation (lane E). The mRNA samples were translated in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate, and the proteins were resolved by SDS/PAGE, fluorography, and autoradiography. Lane PM+E contains a mixture of the translation products from the point-mutated and the elongated *spf*^{ash} OTCase transcripts. Similarly, total liver poly(A)$^+$ mRNA from wild-type (wt) or *spf*^{ash} mice was translated, then immune precipitated with anti-OTCase antiserum, and resolved on the same gel.

Effects of an Exon Nucleotide on an Adjacent Splice Site. The *spf*^{ash} mutation shows unusually drastic effects of a nucleotide change within an exon on the use of an adjacent splice donor site. It has been recognized that the conserved sequences of the consensus splice sites extend to the adjacent exon nucleotides (27, 28). Nevertheless, there are few examples of naturally occurring mutations within the exon portions of splice sites that affect mRNA splicing. The only natural precedent for the *spf*^{ash} mutation is the *ry*^{5208} mutation at the *rosy* (xanthine dehydrogenase) locus of *Drosophila melanogaster*, which changes the last nucleotide of the first exon from G to A (29). Although enzyme production from this allele is negligible, the effect of the mutation on pre-mRNA splicing has not been reported. A mutation identical to the *spf*^{ash} mutation has been created in rabbit β-globin exon 2 by DNA manipulation. This G to A change had no effect on mRNA splicing *in vivo* (30). In an *in vitro* splicing extract, the mutation had a modest effect, still allowing correct splicing at >50% of wild-type efficiency (31).

The most obvious resolution of the dichotomy between the drastic effects of the *spf*^{ash} mutation and the innocuous effects of the same mutation at the globin locus is that the *spf*^{ash} mutation occurs adjacent to a 5′ splice site, which already is only a poor match to the consensus sequence (Fig. 4). In fact, the wild-type donor site matches the 5′ splice site consensus only as well as the worst four matches reported by Mount (27). None of the naturally occurring donor sites reported matched as poorly as the *spf*^{ash} site. We have also detected, at low levels, mis-splicing of the normal OTCase gene, so as to join exon 3 to exon 5 (unpublished data). This may be a consequence of the inefficient use of the wild-type splice donor site of exon 4. The *spf*^{ash} mutation apparently converts a poor but functional site into a barely usable site.

Translation of Two pOTCase Proteins. The aberrant splicing of the *spf*^{ash} allele produces two translatable mRNAs, each in diminished amount. Both mRNAs contain mutations with markedly different effects on the biogenesis of their respective OTCase proteins. The missense substitution of histidine for arginine-129 has no apparent effect on OTCase biogenesis or enzyme function, despite considerable likelihood that the region surrounding residue 129 participates in necessary interactions between adjacent subunits in the trimeric enzyme (32). In contrast, it is easy to imagine how the insertion of the 16-amino acid peptide encoded by the intron sequence might prevent the assembly of OTCase, especially because the inserted peptide is rich in bulky and hydrophobic amino acids. Its effect in this regard has been demonstrated by the *in vitro* reconstruction of OTCase biogenesis. The elongated pOTCase is imported into mitochondria normally, and its mitochondrial leader peptide is accurately removed by proteolytic cleavage. The elongated OTCase subunit, however, does not adopt the trimeric conformation necessary to bind to a substrate affinity column. Furthermore, this elongated OTCase is selectively degraded.

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