Feline acute intermittent porphyria: a phenocopy masquerading as an erythropoietic porphyria due to dominant and recessive hydroxymethylbilane synthase mutations

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Human acute intermittent porphyria (AIP), the most common acute hepatic porphyria, is an autosomal dominant inborn error of heme biosynthesis due to the half-normal activity of hydroxymethylbilane synthase (HMB-synthase). Here, we describe the first naturally occurring animal model of AIP in four unrelated cat lines who presented phenotypically as congenital erythropoietic porphyria (CEP). Affected cats had erythrocytosis, brownish urine, fluorescent bones, and markedly elevated urinary uroporphyrin (URO) and coproporphyrin (COPRO) consistent with CEP. However, their uroporphyrinogen-III-synthase (URO-synthase) activities (deficient in CEP) were normal. Notably, affected cats had half-normal HMB-synthase activities and elevated urinary 5-aminolevulinic acid (ALA) and porphobilinogen (PBG), the deficient enzyme and accumulated metabolites in human AIP. Sequencing the feline HMB-synthase gene revealed different mutations in each line: a duplication (c.189dupT), an in-frame 3 bp deletion (c.842_844delGAG) identical to that causing human AIP and two missense mutations, c.250G>A (p.A84T) and c.445C>T (p.R149W). Prokaryotic expression of mutations c.842_844delGAG and c.445C>T resulted in mutant enzymes with <1% wild-type activity, whereas c.250G>A expressed a stable enzyme with ~35% of wild-type activity. The discolored teeth from the affected cats contained markedly elevated URO I and III, accounting for the CEP-like phenocopy. In three lines, the phenotype was an autosomal dominant trait, while affected cats with the c.250G>A (p.A84T) mutation were homozygous, a unique recessive form of AIP. These animal models may permit further investigation of the pathogenesis of the acute, life-threatening neurological attacks in human AIP and the evaluation of therapeutic strategies. GenBank Accession Numbers: GQ850461–GQ850464.

INTRODUCTION

The human porphyrias are inborn errors of metabolism each resulting from the decreased activity of a specific enzyme in the heme biosynthetic pathway. They are classified as ‘hepatic’ or ‘erythroid’ reflecting the predominant site of their respective accumulated porphyrins or porphyrin precursors. The acute hepatic porphyrias present with life-threatening neurological attacks, while the erythropoietic porphyrias have photosensitive cutaneous manifestations due to the photo-activation (HCP) of accumulated porphyrins in the skin and anemia is sometimes present. The three common acute hepatic porphyrias, acute intermittent porphyria (AIP), hereditary coproporphyria (HCP) and variegate porphyria (VP) are inherited as autosomal dominant traits, whereas the erythropoietic porphyrias, congenital erythropoietic porphyria (CEP) and erythropoietic protoporphyria (EPP) are autosomal recessive disorders. In each of the human
porphyrias, the specific enzymatic defects, underlying gene mutations, resultant biochemical abnormalities and clinical phenotypes have been characterized (reviewed in 1).

Although a variety of naturally occurring models have been identified in domestic animals for each of the erythropoietic porphyrias (2–9), including cats suspected to have CEP (10,11), there are no reported naturally occurring models for the hepatic porphyrias. Whereas the animal models for CEP were first suspected because of their reddish urine and/or their erythrodonitia (7,8,12,13), animals with a hepatic porphyria presumably would be difficult to diagnose since their acute attacks would be non-specific, possibly leading to their rapid neurological demise. Here, we report the biochemical and molecular genetic characterization of four unrelated lines of feline hepatic AIP. Therefore, the hepatic and splenic URO-synthase activities were determined in erythroid and/or non-erythroid tissues from affected cats and/or of healthy feline controls (n = 8). Similarly, their urinary COPRO I and III isomers were 7- to 17-fold and 6- to 9-fold greater, respectively, than the respective mean normal levels (Table 1). Of particular note, there was a 2- to 4-fold increase in the urinary heme precursor ALA in two lineages and a 3- to 13-fold increase in precursor PBG concentration in affected cats from all lineages.

Tissue porphyrins were also markedly elevated in the porphyric cats. In liver and spleen, URO I concentrations were 8- to 230-fold and 31- to 3500-fold increased, respectively, over wild-type, and URO III was 13- to 401-fold and 36- to 2460-fold greater, respectively, than wild-type levels (Table 2). COPRO I was 3- to 48-fold and 28- to 370-fold increased, respectively, than wild-type levels (Table 2). COPRO I was 3- to 48-fold and 28- to 370-fold elevated in liver and spleen, respectively, whereas COPRO III was 3- to 21-fold and 7- to 98-fold increased, respectively.

Erythrodonitia was observed in the affected cats of all four AIP lineages from tooth eruption which occurs typically at 3 weeks of age. It remained prominent in the teeth of older Saskatchewan and Massachusetts cats. The brown erythrodonitia fluoresced pink under Wood’s light (Fig. 1), similar to that seen in affected human CEP patients. In teeth and femurs, both URO I and III isomer levels were >100-fold greater than wild-type levels for the Saskatchewan and Massachusetts lineages, whereas only 1- to 6-fold elevated in teeth and femur, respectively, from an affected Missouri cat (Table 3). Approximately 10-fold lower accumulations of 7-COOH URO were observed and only traces of the further decarboxylated and less water-soluble porphyrins were detected.

The markedly elevated porphyrin levels in urine, tissue, teeth and bone were consistent with CEP, with the exception of the elevated porphyrin precursors, ALA and PBG, the elevated URO- and COPRO III isomers, and the dominant inheritance in three lineages that suggested AIP. Therefore, the URO-synthase and HMB-synthase activities were determined in erythroid and/or non-erythroid tissues from affected cats from each lineage and from healthy control cats (Table 4). The hepatic and splenic URO-synthase activities were normal or elevated (from 1- to 1.6-fold) compared with age- and sex-matched feline control activities in the dominantly inherited AIP lines, whereas the HMB-synthase activities

### RESULTS

#### Biochemical studies

UPLC analysis of the urinary porphyrin concentrations from affected cats revealed markedly elevated URO I and III isomer concentrations, 42- to 680-fold and 38- to 610-fold greater, respectively, than those of their wild-type littermates and/or of healthy feline controls (n = 8). Similarly, their urinary COPRO I and III isomers were 7- to 17-fold and 6- to 9-fold greater, respectively, than the respective mean normal levels (Table 1). Of particular note, there was a 2- to 4-fold increase in the urinary heme precursor ALA in two lineages and a 3- to 13-fold increase in precursor PBG concentration in affected cats from all lineages.
were about half-normal in affected cats from all four porphyric lines. Similarly, URO-synthase activities in erythrocytes were from 0.7- to 3.5-fold of mean wild-type activity, whereas HMB-synthase was from 20 to 50% of wild-type levels, supporting a diagnosis of AIP rather than CEP.

**Hematological studies**

Hematological studies were performed in wild-type and affected cats from the Massachusetts line and compared with reference feline values at the University of Pennsylvania School of Veterinary Medicine. The low erythrocytic hemoglobin, hematocrit and iron values, and increased reticulocyte counts were consistent with a mild compensated hemolytic anemia, whereas the low mean corpuscular volumes in the affected cats indicated a mild microcytic anemia (Supplementary Material, Table S1).

**Quantitative real-time PCR of feline ALAS1 mRNA**

Multiplex TaqMan® assays were employed to quantitate *ALAS1* mRNA concentrations relative to the expression of the endogenous housekeeping control *RPS11* gene, encoding the feline 40S ribosomal protein S11, in wild-type and mutant cat lines as described in the Materials and Methods section. The average *ALAS1* mRNA level in the Saskatchewan cat liver was elevated 3.1-fold over wild-type cat liver levels.

**Structure of the wild-type feline *HMBS* and *UROS* genes and transcripts**

To investigate the specific genetic defects responsible for the porphyric phenotype in these cats, it was necessary to determine the normal feline genomic organizations and cDNA sequences of *HMBS* and *UROS*, which were not completely annotated in the GenBank database. The exon boundaries for *HMBS* determined by genomic and RT–PCR cDNA sequencing are shown in Supplementary Material, Table S2. The coding sequence of the cDNA for the feline housekeeping *HMBS* was 1086 nt long, encoding a peptide of 361 amino acids and that for feline erythroid *HMBS* was 1035 nt long, encoding a protein of 344 residues. Both housekeeping and erythroid feline HMB-synthase had the same number of amino acid residues as their human counterparts with 95% amino acid identity.

Of note, RT–PCR of feline *HMBS* revealed alternative transcripts generated from both the erythroid and the housekeeping promoters (Fig. 2A and C). One housekeeping *HMBS* mRNA had the usual exon 1 to 3 splice pattern seen in other species, whereas the alternative form contained a 48 bp insertion consisting of the distal end of erythroid exon 2 (Fig. 2A). Semi-quantitative RT–PCR indicated that the relative abundance of the two housekeeping transcripts were about equal (Fig. 2B). The presence of an in-frame TGA stop codon in the alternatively spliced exon 2 predicted a non-functional housekeeping alternative HMB-synthase truncated polypeptide of 18 amino acids. The erythroid transcripts were

**Table 2. Tissue and erythrocyte porphyrins in affected and wild-type cats**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Porphyrin</th>
<th>Wild-type Mean pmol/g tissue (range) n = 2–3</th>
<th>Saskatchewan Mean fold over wild-type (range) n = 3; 1b</th>
<th>Massachusetts Mean fold over wild-type (range) n = 2–3; 1b</th>
<th>Missouri Mean fold over wild-type (range) n = 1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>URO I</td>
<td>2.27 (2.10–2.45)</td>
<td>233 (44.8–561)</td>
<td>85.3 (36.2–148)</td>
<td>8.04</td>
</tr>
<tr>
<td></td>
<td>URO III</td>
<td>0.47 (0.43–0.52)</td>
<td>401 (89.6–950)</td>
<td>167 (59.3–347)</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>7-COOH I + III</td>
<td>3.34 (3.21–3.48)</td>
<td>24.8 (10.2–53.4)</td>
<td>16.9 (5.82–29.5)</td>
<td>6.85</td>
</tr>
<tr>
<td></td>
<td>6-COOH I + III</td>
<td>1.10 (0.94–1.27)</td>
<td>12.4 (7.44–26.4)</td>
<td>9.02 (3.64–13.7)</td>
<td>6.01</td>
</tr>
<tr>
<td></td>
<td>5-COOH I + III</td>
<td>1.61 (0–3.22)</td>
<td>13.0 (3.26–28.5)</td>
<td>9.19 (0.45–13.5)</td>
<td>5.18</td>
</tr>
<tr>
<td></td>
<td>COPRO I</td>
<td>2.87 (2.75–2.98)</td>
<td>42.1 (13.5–92.5)</td>
<td>47.5 (28.1–75.1)</td>
<td>2.79</td>
</tr>
<tr>
<td></td>
<td>COPRO III</td>
<td>0.80 (0.58–1.03)</td>
<td>21.4 (4.98–52.4)</td>
<td>17.4 (8.45–22.1)</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>PROTO IX</td>
<td>22.89 (13.3–32.5)</td>
<td>1.18 (1.04–1.24)</td>
<td>0.92 (0.48–1.23)</td>
<td>1.13</td>
</tr>
<tr>
<td>Spleen</td>
<td>URO I</td>
<td>3.16 (2.78–3.59)</td>
<td>3500</td>
<td>699 (218–1271)</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>URO III</td>
<td>1.43 (0.93–1.30)</td>
<td>2465</td>
<td>510 (223–716)</td>
<td>35.6</td>
</tr>
<tr>
<td></td>
<td>7-COOH I + III</td>
<td>0.59 (0.40–0.86)</td>
<td>1066</td>
<td>161 (85.2–268)</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td>6-COOH I + III</td>
<td>0.98 (0.77–1.12)</td>
<td>106</td>
<td>18.6 (6.22–33.4)</td>
<td>5.34</td>
</tr>
<tr>
<td></td>
<td>5-COOH I + III</td>
<td>0.11 (0–0.32)</td>
<td>1616</td>
<td>165 (33.8–302)</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>COPRO I</td>
<td>0.90 (0.85–0.98)</td>
<td>372</td>
<td>62.2 (30.5–108)</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td>COPRO III</td>
<td>0.79 (0.70–0.89)</td>
<td>97.6</td>
<td>10.7 (1.99–15.1)</td>
<td>6.57</td>
</tr>
<tr>
<td></td>
<td>PROTO IX</td>
<td>11.7 (4.72–17.4)</td>
<td>15.4</td>
<td>29.1 (5.52–73.4)</td>
<td>3.03</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>URO I</td>
<td>0.61 (0.0–1.22)</td>
<td>398</td>
<td>19.4</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>URO III</td>
<td>0.1 (0.0–0.1)</td>
<td>3710</td>
<td>203</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>7-COOH I + III</td>
<td>0.28 (0.0–0.55)</td>
<td>643</td>
<td>40.4</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>6-COOH I + III</td>
<td>0.47 (0.1–0.86)</td>
<td>55.3</td>
<td>6.00</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5-COOH I + III</td>
<td>0.76 (0.1–1.52)</td>
<td>20.3</td>
<td>3.12</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>COPRO I</td>
<td>0.07 (0.0–0.14)</td>
<td>509</td>
<td>39</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>COPRO III</td>
<td>0.1 (0.0–0.1)</td>
<td>1210</td>
<td>871</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>PROTO IX</td>
<td>1.46 (0.57–2.35)</td>
<td>56.7</td>
<td>13.0</td>
<td>5.2</td>
</tr>
</tbody>
</table>

aThe porphyrins in each tissue were determined at least twice. The mean (and range) of these determinations are indicated.
bNumber of affected cats studied. Note that only one Missouri cat was available; no Oregon cats were available.
cThe limit of detection of porphyrins is 0.1 pmol/g tissue.
sequenced from RT–PCR products amplified with an erythroid-specific 5'-primer and an exon 4 primer common to both the erythroid and housekeeping transcripts. The only difference between the two erythroid transcripts was the absence of the terminal four nucleotides of exon 2 in a form that was estimated to be a minor species (\(\sim 10\% - 20\%\)) based on its relative abundance in the sequencing reaction. The shorter transcript was spliced at a non-canonical (GC) 5'-splice donor site (Supplementary Material, Table S2). In this case, the absence of the four nucleotides at the end of erythroid exon 2 (the first exon in \(HMBS\) erythroid transcripts) made no difference in the erythroid coding sequence as the erythroid start codon is in exon 3.

For feline \(UROS\), analysis of genomic organization and RT–PCR sequences identified the housekeeping and erythroid promoter regions, the 3' boundary of exon 1, and confirmed that these and all remaining intron/exon boundaries were identical to those in the human gene and transcripts (data not shown). The cDNA sequence for the feline housekeeping and erythroid \(UROS\) were both 795 nt long, and encoded a single protein of 264 amino acids.

**Identification of \(HMBS\) mutations causing feline AIP**

Sequencing of the \(UROS\) gene of affected cats from all four lineages did not identify a putative disease-causing exonic or

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**Table 3. Bone and tooth porphyrins in affected cats**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Porphyrin</th>
<th>Wild-type Mean nmol/g dry weight (range)</th>
<th>Saskatchewan Mean fold over wild-type (range)</th>
<th>Massachusetts Mean fold over wild-type (range)</th>
<th>Missouri Mean fold over wild-type (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(n = 3^b)</td>
<td>(n = 1^b)</td>
<td>(n = 3^b)</td>
<td>(n = 1^b)</td>
</tr>
<tr>
<td>Tooth</td>
<td>URO I</td>
<td>0.09 (0.03–0.13)</td>
<td>104</td>
<td>132 (24.7–330)</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>URO III</td>
<td>0.06 (0.01–0.09)</td>
<td>80.8</td>
<td>122 (21.7–312)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>7-COOH I</td>
<td>0.03 (0–0.05)</td>
<td>8.79</td>
<td>14.6 (1.69–31.7)</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>COPRO I</td>
<td>0.0 (0–0.01)</td>
<td>ND</td>
<td>7.45 (0–22.4)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>COPRO III</td>
<td>0.01 (0–0.02)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Femur</td>
<td>URO I</td>
<td>0.12 (0.06–0.17)</td>
<td>265</td>
<td>101 (40.4–183)</td>
<td>4.62</td>
</tr>
<tr>
<td></td>
<td>URO III</td>
<td>0.07 (0.04–0.10)</td>
<td>255</td>
<td>97.4 (41.8–166)</td>
<td>5.82</td>
</tr>
<tr>
<td></td>
<td>7-COOH I</td>
<td>0.04 (0.02–0.05)</td>
<td>35.8</td>
<td>9.98 (5.45–17.4)</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>COPRO I + III</td>
<td>ND</td>
<td>traces</td>
<td>traces</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detectable.

*The porphyrins in each cat’s tissue were determined at least twice and averaged. The mean (and range) of these averages are indicated.

*Number of affected cats studied. Note that only one Saskatchewan and one Missouri cat were available; no Oregon cats were available.

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**Figure 1.** Feline AIP facial phenotype masquerading as CEP. (A and B) Photographs of an affected cat from the Saskatchewan line. (C and D) Wild-type and affected cats, respectively, from the Massachusetts line. (A, C and D) Photographed under white light and (B) under ultraviolet light from a Wood’s lamp.
HMB-synthase isozymes were similar to those of the human housekeeping and erythroid feline HMB-synthase. Prokaryotic expression of housekeeping and erythroid HMB-synthase cDNAs were purified to homogeneity. The expressed erythroid and housekeeping isozymes had molecular weights as determined by SDS–PAGE of ~43.0 kDa, respectively, similar to those of the respective human enzymes (17). The enzymatic activities (~700–1200 U/mg protein) of the feline HMB-synthase isozymes were similar to those of the human recombinant HMB-synthase isozymes (18). Prokaryotic expression of the three feline missense AIP mutations c.842_844delGAG (p.delG281), c.445C>T (p.R149W) and c.250G>A (p.A84T) resulted in mutant enzyme proteins with ~35% of mean wild-type expressed feline HMB-synthase activity, respectively (Table 5). Thermostability studies of the expressed housekeeping A84T protein at pH 8.0 and 50°C revealed a prolonged half-life of 6.9 h relative to the half-life of 1.9 h for the wild-type enzyme. Although the activity of the pure enzyme was only ~35% of wild-type, it is likely that its greater thermostability results in a somewhat higher *in vivo* activity. Indeed, in erythrocytes from an affected OregonAR cat, the activity was 47% of wild-type (Table 4).

### Table 4. HMB-synthase and URO-synthase activities in tissues of affected and wild-type cats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme</th>
<th>Wild-type Mean U/mg protein (range)a</th>
<th>Saskatchewan Fold change of mean wild-type specific activity (range)a</th>
<th>Massachusetts Mean U/mg protein (range)a</th>
<th>Missouri Mean U/mg protein (range)a</th>
<th>OregonAR Mean U/mg protein (range)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>HMB-synthase</td>
<td>0.030 (0.026–0.033)</td>
<td>0.46 (0.26–0.58)</td>
<td>0.49 (0.38–0.67)</td>
<td>0.46</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>URO-synthase</td>
<td>3.28 (2.22–4.75)</td>
<td>1.64 (1.63–1.66)</td>
<td>1.01 (0.86–1.15)</td>
<td>1.25</td>
<td>NA</td>
</tr>
<tr>
<td>Spleen</td>
<td>HMB-synthase</td>
<td>0.064 (0.029–0.101)</td>
<td>NA</td>
<td>0.45 (0.27–0.55)</td>
<td>0.30</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>URO-synthase</td>
<td>9.83 (5.83–14.1)</td>
<td>1.57</td>
<td>1.31 (0.49–2.71)</td>
<td>0.92</td>
<td>NA</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>HMB-synthase</td>
<td>0.017 (0.009–0.023)</td>
<td>0.23</td>
<td>0.18</td>
<td>0.30</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>URO-synthase</td>
<td>0.5 (0.42–0.59)</td>
<td>2.76 (1.63–1.66)</td>
<td>3.51 (0.86–1.15)</td>
<td>0.97</td>
<td>0.73</td>
</tr>
</tbody>
</table>

NA, not available.

aNumber of affected cats studied. Note that only one Missouri cat was available; no Oregon cat liver or spleen autopsies were available.

### DISCUSSION

Naturally occurring animal models of human inborn errors of metabolism provide the unique opportunity to investigate the

### Molecular modeling

Molecular models of wild-type and mutant feline HMB-synthase enzymes were generated using SWISS-MODEL and the crystal structures of *Escherichia coli* (19) and of human HMB-synthase (20) as templates. The p.delG281 model predicted an alteration of the β2, beta structure in the third domain of the enzyme; the deletion of one of the two glycines twisted the polypeptide chain such that the residue 281–288 sequence, VWSLDGSD, was rotated ~180° (Fig. 3A). This was predicted to result in a disruption of the surface-located third of the beta sheet structure composed of beta strands β13, β23 and β33 (20). The p.R149W model predicted the mutant protein to have a normal quaternary structure. By alignment, R149 was equivalent to residue R131 in *E. coli*, which lies in the catalytic site and interacts with the cofactor. Modeling of the dipyrrole cofactor into the active site of the wild-type feline HMB-synthase predicted that the substitution of arginine 149 by a bulky tryptophan would result in a clash between the cofactor and this mutated residue (Fig. 3B). The p.A84T model also predicted a normal quaternary structure compared with wild-type with only minor shifts in residues to accommodate the slightly larger threonine residue (Fig. 3C).

### Prokaryotic expression of housekeeping and erythroid HMB-synthase

The housekeeping and erythroid feline *HMBS* cDNAs were prokaryotically expressed and their respective enzymes were purified to homogeneity. The expressed erythroid and housekeeping isozymes had molecular weights as determined by SDS–PAGE of ~40.5 and ~43.0 kDa, respectively, similar to those of the respective human enzymes (17). The enzymatic activities (~700–1200 U/mg protein) of the feline HMB-synthase isozymes were similar to those of the human...
molecular pathology and disease pathogenesis relative to their human counterparts. In addition, these animal analogues permit the evaluation of new or improved therapeutic strategies, particularly for neurological disorders, that are not possible in human studies due to the limitations of human experimentation (21–23). Although knock-in mice have been generated for AIP and VP (24,25) to date, no naturally occurring models of the acute porphyrias have been identified.

The four unrelated lines of AIP cats described here were initially diagnosed as having CEP. These cats had prominent erythrodontia, brownish urine and fluorescent teeth under UV light, all of which mimicked CEP. Subsequent porphyrin analyses demonstrated markedly elevated urinary URO I and COPRO I concentrations, further suggesting the diagnosis of CEP. These findings signaled an erythropoietic porphyria to the referring veterinarians. However, the absence of cutaneous photosensitivity, the presence of ~3- to 13-fold elevated
Table 5. Identification and in vitro expression of feline HMB-synthase mutations causing AIP

<table>
<thead>
<tr>
<th>AIP line</th>
<th>Exon</th>
<th>cDNA mutation</th>
<th>Predicted protein alteration</th>
<th>Specific Activity U/mg (n)</th>
<th>Percent of expressed WT activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
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<td>None</td>
<td>925 (10)</td>
<td>100</td>
</tr>
<tr>
<td>Saskatchewan</td>
<td>5</td>
<td>c.189dupT</td>
<td>p.Leu64SerfsX65</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Massachusetts</td>
<td>14</td>
<td>c.842_844delGAG</td>
<td>p.delGly281</td>
<td>2.20 (1)</td>
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</tr>
<tr>
<td>Missouri</td>
<td>9</td>
<td>c.445C &gt; T</td>
<td>p.Arg149Trp</td>
<td>0.22 (3)</td>
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<tr>
<td>OregonAR</td>
<td>6</td>
<td>c.250G &gt; A</td>
<td>p.Ala84Thr</td>
<td>318 (2)</td>
<td>34.4</td>
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</table>

*The c.189dupT mutation predicts a frameshift that results in a substitution of Ser for Leu in codon 64 followed by a premature TGA stop in codon 65 of the normal 361-residue enzyme.

The c.189dupT mutation predicts a frameshift that results in a substitution of Ser for Leu in codon 64 followed by a premature TGA stop in codon 65 of the normal 361-residue enzyme.

urinary PBG and the autosomal dominant inheritance in three of the lineages were not consistent with the features of human CEP. For two lines of previously described cats considered to have a CEP (10,11,14,15), it was suggested that these differences with human CEP were either species-related or due to a different enzymatic defect (15).

Enzymatic studies revealed that the porphyric cats from all four lines had normal to slightly increased tissue and erythrocytic URO-synthase activities, the enzyme deficient in human CEP, whereas erythrocytic HMB-synthase activities were approximately half-normal, indicating a diagnosis of AIP. The finding of remarkably elevated URO and COPRO isomers in the urine, tissues, teeth and bones of the AIP cats (Tables 1–3) explained their erythroodontia and misdiagnosis of CEP.

That the porphyric cats masqueraded as CEP can be explained by the decreased HMB-synthase activity which resulted in constitutively induced hepatic ALAS1 and the resultantly increased heme biosynthesis as indicated by the increased levels of urinary ALA, PBG and tissue porphyrins (Tables 1 and 2). The urinary profile of these porphyric precursors and porphyrins is remarkably similar to that of the human AIP patients during acute attacks (26,27), including the markedly elevated levels of both URO I and II, which in the affected cats were increased over 100-fold in the urine. In human CEP, the major accumulated porphyrins, URO I and COPRO I, are primarily responsible for the phenotypic manifestations including the brownish-appearing teeth and reddish urine, and the cutaneous lesions due to the photoactivation of the URO and COPRO isomers deposited in the skin. The erythroodontia led previous investigators to identify the porphyric cats as an animal model of CEP.

To identify the mutations causing the enzyme deficiency in these cats, it was necessary to establish the genomic organization and intron/exon boundaries of the feline HMBs and UROS genes. The housekeeping HMB-synthase enzyme was remarkably conserved among mammals: the feline amino acid sequence had 96% identity with canine, 95% with human and porcine, 92% with bovine and 91% with murine proteins. Sequence analysis of the feline HMBs gene in the affected cats revealed a unique HMB-synthase disease-causing mutation in each feline porphyric line. The Massachusetts three-base, in-frame deletion (p.delG281) has been reported to cause AIP in humans (28) and the Missouri missense mutation (c.445C>T; p.R149W) was previously described in a human patient with AIP (16). Of note, HMB-synthase residues A84 and R149, mutated in the Missouri and OregonAR feline lineages, respectively, were conserved in 12 available mammalian sequences, suggesting their importance for enzyme function (Supplementary Material, Fig. S2).

Of the four lineages, affected cats from the Saskatchewan line appeared to have the most severe clinical manifestations and biochemical abnormalities. The c.189dupT single base duplication caused a frameshift mutation that resulted in a truncated protein of 64 residues. This truncated enzyme protein could not have activity, because the dipyromethene cofactor in the active site is covalently linked to cysteine 261 of the 361 residue HMB-synthase housekeeping polypeptide. Furthermore, reverse transcription of total RNA isolated from the liver of an affected cat heterozygous for this mutation, and PCR amplification of the resultant cDNAs, did not detect the mutation-containing amplicon, suggesting that the mRNA was subject to nonsense-mediated decay (29).

The Massachusetts lineage AIP cats had the next most severe AIP phenotype, based on their elevated urinary and tissue porphyrin levels. The p.delG281 enzyme’s deleted glycine (L262 in the E. coli HMB-synthase) was predicted to cause a major disruption of the beta sheet structure of the third domain of the enzyme (Fig. 3A) and may alter its known hydrophobic interactions with domains 1 and 2 that surround the active site (19). Indeed, little activity (0.24% of wild-type) was found for the purified recombinant protein. This is similar to the 0.8% residual activity reported for the identical mutation in a human AIP patient (28). The c.842_844delGAG mutation that gives rise to the glycine deletion designated to codon 281 could be designated as any of six equivalent three base deletions at the feline and human HMBs sequence CAGGAGGAGT. Although it was previously described as a (841–843delGGA) deletion at codon 281 in humans (28), now that the official nomenclature body has specified that it should be designated as the most 3’ possible deletion, the c.842_844delGAG designation is the accepted one. This also means that one could just as well say it was the invariant glycine at codon 280 that was deleted (Supplementary Material, Fig. S2), as the deletion of either gives rise to the glycine deletion designated to codon 281 could be designated as any of six equivalent three base deletions at the feline and human HMBs sequence CAGGAGGAGT. Although it was previously described as a (841–843delGGA) deletion at codon 281 in humans (28), now that the official nomenclature body has specified that it should be designated as the most 3’ possible deletion, the c.842_844delGAG designation is the accepted one. This also means that one could just as well say it was the invariant glycine at codon 280 that was deleted (Supplementary Material, Fig. S2), as the deletion of either gives the identical mutated product. Thus, for purposes of nomenclature, we use the accepted designation, but for purposes of function, show invariant glycine codon 280 as the deletion in Supplementary Material, Fig. S2 that shows all four feline mutations affect invariant nucleotides among 14 vertebrate species.
incorporation (19). Modeling of the feline R149W HMB-synthase with the crystal structure of the human protein indicated possible interference with the cofactor’s normal position. In the human crystal structure, R149 participates in a critical hydrogen bond with the dipyromethane cofactor (20). Although an R149Q mutation in human AIP was reported to be CRM negative (31) and the purified feline mutant enzyme had essentially no activity, the phenotypic heterogeneity of AIP mutations was underscored by the relatively mild phenotype of the feline R149W mutation.

Notably, affected cats from the Saskatchewan, Massachusettts and Missouri lines were heterozygous for their respective mutations, consistent with the autosomal dominant inheritance of the phenotype, their half-normal HMB-synthase activities and their essentially undetectable activities when their mutations were expressed in vitro (Table 5). In contrast, the OregonAR line was unique. The two affected cats were homozygous for the c.250G > A mutation which expressed ~35% of wild-type activity in vitro (Table 5) and 47% in vivo (Table 4). Thus, affected OregonAR cats have autosomal recessive AIP and provide an example of a homozygous mutation that reduces the enzymatic activity to a level similar to that in the autosomal dominant forms. Modeling of the p.A84T mutation predicted little change in the quaternary structure of the enzyme (Fig. 3C), consistent with its higher expressed and in vivo enzymatic activity. Homozygous AIP in humans is extremely rare and has been reported in four patients in three families (32–35), all characterized by prominent neurological manifestations including disartria, cerebral atrophy and psychomotor retardation. Of note, two had erythrodoanitia (32,35) and one had a urinary porphyrin profile similar to that of the OregonAR cats (35).

Unlike most human AIP patients, the AIP cats had constitutionally elevated levels of PBG and porphyrins similar to the levels in human AIP patients having an acute attack (26,27). Sequence analysis of alternative housekeeping HMBS transcripts provided a possible mechanism for this acute porphyric state. The ATG start codon of the housekeeping HMB-synthase isozyme begins in exon 1 which is normally spliced in-frame to coding sequences in exon 3. However, the alternative splice site in the feline wild-type housekeeping transcript spliced exon 1 to the distal end of erythroid exon 2 that contains an in-frame stop codon and generates a presumably inactive 18-residue HMB-synthase peptide. Semi- quantitative RT–PCR (Fig. 2B) indicated that the alternatively spliced housekeeping transcript accounted for about half of the total HMBS RNA. Therefore, in wild-type cats, the HMB-synthase activity already is markedly reduced, and introduction of an HMBS mutation would further lower the HMB-synthase activity. The result would be highly deficient heme biosynthesis, which would negatively feedback and induce the ALAS1 activity, thereby causing the chronically increased porphyrins in the AIP cats. In support of this hypothesis, the hepatic ALAS1 mRNA level was 3.1-fold elevated over wild-type in the most severely affected Saskatchewan AIP cat line. The feline alternative splicing would only reduce the activity of the housekeeping isozyme, as the observed alternative splicing of the erythroid transcript decreased the length of the 5'-untranslated end of the transcript, but did not alter the erythroid enzyme protein.

Figure 3. Molecular modeling of feline HMB-synthase mutations. Modeling of feline HMB-synthase mutant structures against published crystal structures is described in Materials and Methods. The stereograms of regions of the HMB-synthase affected by AIP mutations are oriented for cross-eyed viewing. (A) Surface loop region of the wild-type and mutant p.delG281 proteins with the wild-type backbone carbons in gray and the mutant backbone carbons in green and the methionine 290 and 291 sulfurs colored in gold. Deletion of glycine 281 in the wild-type structure (see white arrow) results in a slight movement of the methyl group on methionine 56 as indicated by the white arrows. (B) The mutant backbone carbons are colored in gold. For the p.R149W mutation (B), the benzene ring of tryptophan residue 149 is predicted to be close to an acetic side-chain of the dipyromethane (DPM) cofactor as indicated by the white arrow. (C) The p.A84T mutation with addition of the threonine 84 hydroxy-methyl group predicted a slight movement of the methyl group on methionine 56 as indicated by the white arrows.

Modeling of the p.R149W missense mutation in the phenotypically milder Missouri line did not predict a structural effect, but predicted a potential alteration of cofactor dipyromethane binding (Fig. 3B) (30). R149 in the feline enzyme corresponds to R131 in the E. coli HMB-synthase model and substitution of this arginine blocks dipyromethane
The feline alternatively spliced form does not appear to be abundant in humans, as only one similarly alternatively spliced brain cDNA (GB:CR611708), which predicted an inactive truncated protein, was present out of 25 available, and only one such expressed sequence tag (EST) variant (GB:BF52797) was found in 189 human ESTs available in GenBank (Release February 2009). Note that both the alternatively spliced human cDNA and the EST were isolated from the same neuroblastoma clone. Although 10–50% alternative splicing deleting human HMBS exons 3 and 12 were reported to occur by unknown causes in peripheral leucocytes from normal individuals (36), these forms were not seen in mRNA isolated from transformed leucocytes from 57 unrel-ated AIP patients (37), confirming the absence of significant alternative splicing in humans.

It was notable that the erythrocyte porphyrins were markedly increased in affected cats from all four lines, particularly the URO and COPRO isomers. These porphyrins were presumably elevated in utero since the erythrodontia was observed when the teeth erupted at 3 weeks of age. This finding may partially explain the CEP-like phenotype, because the increased erythrocytic URO and COPRO isomers can enter the circulation where they gain access to tissues and bone. The mechanism responsible for this ‘erythroid component’ in the AIP cats may be, in part, due to erythrocyte uptake (i.e. by erythroblasts, reticulocytes and erythrocytes) of the elevated levels of plasma ALA and/or PBG synthesized in the liver due to the constitutively induced ALAS1 activity. The ALA and/or PBG would be converted to porphyrins by the normally high level of heme biosynthesis in the feline erythroid cells. In vitro studies indicate that ALA can diffuse across erythrocyte membranes where they are metabolized to porphyrins and then excreted into the plasma (38) and Clavero et al. (unpublished data). In support of this mechanism, high levels of ALA and PBG were responsible for the elevated URO and COPRO isomers in human homozygous AIP patients, resulting in the CEP-like manifestations of erythrodontia and splenomegaly (32,35).

In summary, these studies identified and characterized the first naturally occurring animal model of human AIP. It was notable that four lines of cats were identified clinically by their unique erythrodontia and that they, and presumably the previously reported feline porphyric lines (11,15), had masqueraded phenotypically and biochemically as CEP. In addition, the finding of the homozygous recessive AIP cats for the hypomorphic c.250G>A allele is a model for recessive human forms of this typically dominant hepatic porphyria. These animal analogues of human AIP should permit studies of the disease pathogenesis in a moderately large, easy to breed animal model, because such studies in genetically engineered mice have been limited to date (24). Characterization of the disease pathogenesis, including the acute central and peripheral nervous system pathology in a neurologically well-characterized animal may provide further understanding of the acute attack pathogenesis. Moreover, the effects of glucose and hematin treatments during an acute attack can be systematically determined clinically, pathologically and biochemically. Moreover, the AIP cats should permit the evaluation of novel methods to treat the acute attacks includ-ing pharmacological chaperone, gene replacement, partial hepatic transplantation or hepatic stem cell therapies.

MATERIALS AND METHODS

Porphyric cats

Affected porphyric cats were referred to the National Referral Center for Animals with Genetic Diseases at the University of Pennsylvania. Probands 1–4 (two males and two females) were affected Siamese cats, offspring of a sentinel-affected cat, who was referred in 1983 from the Toxicology Research Center at the University of Saskatchewan, Saskatoon, Canada (designated the Saskatchewan line). This line of cats was maintained until 1997, when six embryos from in vitro fertilization of affected ova and unaffected sperm were frozen in collaboration with Dr William Swanson of the Cincinnati Zoo. Also, 140 pellets of semen from affected males were collected and frozen. Proband 4 was from the Saskatchewan line re-established from embryos in 2008. Probands 5–15 (seven males and four females) were offspring of a second Siamese porphyric cat referred in 2002 from Westboro, MA (designated the Massachusetts line). Probands 16 and 17 (siblings, one male and one female) were short-haired domestic cats referred in 2008 from Liberty, Missouri (designated the Missouri line) and Probands 18 and 19 were short-hair females referred from Portland, Oregon (designated the Oregon line). The parents and a littermate of Proband 18 appeared phenotypically normal.

The affected cats from all lines, which are not known to be related to those reported previously (10,11,14,15), presented with brownish discolored teeth and brownish urine, both fluorescent under UV light, and no signs of skin hyperphotosensitivity (Fig. 1). Some appeared to have an intractable behavior when manipulated, but impairments in motor function affecting gait, coordination or muscle strength were not observed in any of the affected cat lines. Colonies were established for the Saskatchewan and Massachusetts line and several generations of each line confirmed the autosomal dominant inheritance of the porphyric phenotype. Colonies of the Missouri and Oregon lines could not be developed, because these cats had been neutered or were privately owned.

Peripheral Na2EDTA blood and serum samples for routine hematological studies and serum chemistries, and plasma and urine samples for analysis of porphyrin precursors and porphyrins were collected from the affected probands, their wild-type siblings when available, and unrelated healthy control cats.

Animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Affected and wild-type control cats from each line were sacrificed by intravenous overdose of sodium pentobarbital and blood, urine, various tissues, teeth and bones were obtained for the determination of the heme biosynthetic enzymatic activities and metabolites. Genomic DNA was isolated from peripheral blood and/or liver of all 19 probands, except for Probands 13 and 15 of the Massachusetts line, for sequencing of the HMB-synthase (HMBS) and/or URO-synthase (UROS) genes as described below.
URO-synthase and HMB-synthase enzymatic assays

Packed red blood cells from wild-type and porphyric cats were washed twice in phosphate buffered saline and lysed with three volumes of buffer containing 0.1 M KHEPES, pH 7.5, 0.1% Triton X-100, 1 mM DTT and 0.02% sodium azide. Liver and spleen were diced and homogenized in five volumes of buffer/g tissue in a Potter-Elvehjem mortar with a motorized pestle in buffer containing 50 mM KHEPES, pH 7.5, 150 mM KCl, 1 mM Na₂EDTA, 1 mM DTT and 0.1 mM PMSF. HMB-synthase and URO-synthase activities were determined as previously described (39–41). Briefly, for HMB-synthase, URO fluorescence was measured in a Ratio-2 System Filter Fluorometer (Farrand Optical Components & Instruments, Valhalla, NY, USA), using 405 nm interference (excitation) and 600 nm cutoff (emission) filters. For URO-synthase, URO I and URO III were separated and quantitated in an ACQUITY UPLC® (Ultra Pressure Liquid Chromatography) system (Waters Corporation, Milford, MA, USA) according to the following modifications of the method by Lim et al. (42). The column used for separation and quantitation was a 2.1 x 100 mm UPLC® BEH C18 with 1.7 μm particles (Waters Corporation). The porphyrin isomers were resolved using a 2 min concave (Waters #8) gradient from 10 to 30% acetonitrile in 1 M ammonium acetate, 0.02% sodium azide followed by 0.5 min at 30% acetonitrile, and a 1.5 min re-equilibration at 10% acetonitrile at a flow rate of 0.8 ml/min. Porphyrins were detected using an ACQUITY UPLC® Fluorescence (FLR) Detector (Waters Corporation) with excitation at 405 nm, emission at 619 nm, and a gain of 100. One unit of enzymatic activity was defined as that amount of enzyme that produced 1 nmol of product per hour at 37°C.

Porphyrin precursor and porphyrin isomer extraction and quantitation

All samples were immediately frozen after collection and kept frozen and in the dark until analyses. The porphyrin precursors, ALA and PBG, were detected and quantified in urine using the ALA/PBG Column Test kit from Bio-Rad Laboratories (Hercules, CA, USA) and the ClinRep® system, from Recipe Chemicals & Instruments, GmbH (Munich, Germany) following the respective manufacturer’s instructions. For the analysis of porphyrins, urine and plasma from wild-type and porphyric cats were deproteinized by adding 10 to 70% acetonitrile in 1 M ammonium acetate, 0.02% sodium azide followed by 0.5 min at 70% acetonitrile and a 1.5 min re-equilibration at 10% acetonitrile. For the analysis of porphyrins in bones and teeth, the osseous tissues were crushed to powder under liquid nitrogen with a mortar and pestle, vacuum dried and then weighed. The dried bone or tooth powder was dissolved in 500 μl of 6 N HCl overnight to extract the porphyrins, centrifuged at 7500g for 30 min and the porphyrins in the supernatant analyzed by UPLC as described above.

DNA and RNA extractions

Genomic DNA from both wild-type and porphyric cats was extracted from whole blood collected in EDTA and/or liver using the Puregene® Genomic DNA Purification Kit from Gentra Systems (Minneapolis, MN, USA) according to the manufacturer’s instructions. Total RNA was isolated from frozen liver and spleen, previously treated in RNAlater®-ICE (Ambion, Austin, TX, USA). The tissue samples were homogenized and total RNA was isolated with TRI Reagent® (Ambion).

Quantitative real-time PCR analysis of ALAS1 mRNA

Freshly excised feline liver was flash-frozen on foil-covered dry ice, transported at dry ice temperature and stored at −86°C. The frozen samples were treated with RNAlater®-ICE (Ambion, Austin, TX, USA) and then extracted with TRI Reagent® (Ambion) within 2 weeks of treatment. Total RNA was retrotranscribed with random decamers using the RETROscript® kit (Ambion) at 42°C for 1 h and 92°C for 10 min, according to the manufacturer's instructions. A total of 30–100 ng of cDNA was amplified by multiplexed real-time PCR in the Model 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the TaqMan® Gene Expression Master Mix (Applied Biosystems) in a reaction volume of 20 μl, containing 0.4 μM of the endogenous primers, 0.5 μM of ALAS1 primers and 2.5 μM of each probe. Cycling conditions were: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers and probes are listed in Supplementary Material, Table S3. The endogenous control was the housekeeping gene RPS11 encoding the feline 40S ribosomal protein S11. The endogenous and target TaqMan® assays were carried out in the same tube using 5'-labeled TET™ and 6FAM™ probes, respectively, both with a 3' TAMRA™ quencher dye. The results were analyzed with the SDS 7900HT Enterprise Database Software v2.2.2 from Applied Biosystems using the delta–delta Ct relative quantitation method. The assays were done in duplicate and the average of three separate wild-type feline ALAS1 transcript levels were compared with the transcript levels in an affected Saskatchewan cat liver in two separate assays.

Determination of the feline HMBS and UROS cDNA sequences and genomic structures

The initial genomic sequence for the feline HMBS and UROS genes, including their respective housekeeping and erythroid promoters, was obtained from the Ensembl Genome Browser (www.ensembl.org/index.html) using the low-coverage release (2×) assembly of the domestic cat (Felis catus), Genebuild: Ensembl, March 2008; database version 49 (mar2008.archive.ensembl.org/Felis_catus/index.html). Since the genomic sequencing of these genes has not yet been completed by the feline genome project (Ensembl), regions of unannotated sequence in each of these genes were amplified using primers designed to include the most conserved regions of the flanking intronic sequences. Primers used for amplification of feline HMBS are listed in Supplementary Material, Table S4.
Material, Table S3. These primers were designed to sequence ~150 and 100 bp of the 5’ and 3’ intronic regions, respectively, of each HMBS or UROS exon. PCR amplifications were performed using the HotStarTaq Master Mix kit (Qiagen Inc., Valencia, CA, USA), with the following specific conditions: 95°C for 15 min, 30 cycles of 95°C for 30 s, 50–65°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 10 min.

To confirm the exonic sequences of the feline HMBS and UROS genes predicted by homology to the human sequences, RT–PCR of RNA from liver or spleen was performed and the cDNAs were sequenced. The SuperScript™ III One-Step RT–PCR System with Platinum® Taq DNA polymerase (Invitrogen Corp, Carlsbad, CA, USA) was used, with the following conditions: one cycle at 45–55°C, 94°C for 2 min, 30 cycles of 94°C for 15 s, 51–61.8°C for 30 s and 68°C for 1 min, with a final extension at 68°C for 5 min. In other cases, the first strand of the cDNA was obtained by retrotranscription with random decamers using the RETROscript® kit (Ambion, Austin, TX, USA) with incubation at 42°C for 1 h and then for 10 min at 92°C. PCR was performed with HotStarTaq Master Mix from Qiagen (Valencia, CA, USA) with the conditions: 95°C for 15 min, 30 cycles of 95°C for 30 s, 57–60°C for 30 s and 72°C for 30 s with a final extension at 72°C for 10 min. In some cases, ‘touch-down’ PCR was applied; after the reverse transcription step at 55°C for 30 min, with one step at 94°C for 15 s, a series of cycles with decreasing hybridization temperatures (by 1°C per three cycles) from 65 to 58°C, and an extension at 68°C for 1 min was performed for a total of 42 cycles. Primers sets for the amplification of HMBS cDNAs and corresponding genomic regions are listed in Supplementary Material, Table S3. Sequence analyses of the amplified fragments were performed on an Applied Biosystems Model 3730xl Genetic Analyzer and sequence comparisons were made using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA), CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA) and MacVector (MacVector, Inc., Cary, NC, USA). The four alternatively spliced feline housekeeping (1a and 1b) and erythroid-specific (2a and 2b) HMBS cDNA full-length coding sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide; HMBS1a: GQ850461; HMBS1b: GQ850463; HMBS2a: GQ850462; HMBS2b: GQ850464).

**Mutation analyses**

The promoters, all exons and the intron/exon boundaries of the HMBS and UROS genes were sequenced in both orientations in genomic DNAs isolated in 3% SeaKem® agarose (Lonza, Allendale, NJ, USA) gels from blood and/or liver of affected cats from each line. The identified mutations were confirmed by restriction analysis of genomic DNAs isolated from the porphyric (and non-porphyric) relatives in each line (primers listed in Supplementary Material, Table S3). In addition, at least 100 feline alleles were analyzed for these mutations in genomic DNA samples from wild-type domestic shorthair, domestic longhair, Somali, Abyssinian, Oicat, Persian, Bengal, Savannah, Himalayan mixed-breed, Siamese, Siamese mixed-breed, Ragdoll and Burmese cats. For the Saskatchewan mutation, a 91 bp region in exon 5 was amplified with primers FmH-insT-F and FmH-insT-R, with the following conditions: 95°C for 15 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 1.0°C/s to 72°C and 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were digested with HinfI (New England Biolabs, Ipswich, MA, USA) and visualized in a 4% Metaphor agarose gel (Lonza, Rockland, ME, USA). The c.189dupT duplication abolished the restriction site for HinfI in position 29 of the 91 bp amplified fragment. In wild-type cats, two bands in agarose gels of 29 and 62 bp were detected, while in porphyric cats, there were three bands of 92, 29 and 62 bp. For the Massachusetts mutation (c.842_844delGAG), a 144 bp region in exon 14 was amplified with primers FmH-del3-F and FmH-del3-R (Supplementary Material, Table S3) with the same conditions described above. The PCR products were digested with BseRI. The mutation abolished the BseRI restriction site located in position 59. In wild-type cats, two bands of 59 and 85 bp were detected, whereas in porphyric cats there were three bands of 141, 85 and 59 bp.

For the nucleotide change c.445C>T (p.R149W) in the porphyric cats from Missouri, primers were designed to modify the sequence by Amplification Created Restriction Site (ACRS) PCR (43) to generate an FspI restriction site in the wild-type sequence where the mutation was located. A region of 92 bp was amplified with primers FmH-R149W-F and FmH-R149W-R as described above, and afterwards digested with the enzyme FspI (New England Biolabs, Ipswich, MA, USA) and the products visualized as above. The mutation abolished the FspI restriction site created at position 70 by ACRS. In wild-type cats, two bands of 70 and 22 bp were detected, whereas in porphyric cats there were three bands of 92, 70 and 22 bp. For the Oregon mutation (c.250G>A, p.A84T), a region of 129 bp was amplified with primers FmH-A84T-F and FmH-A84T-R, with the same PCR conditions described above, and subsequently digested with the HhaI restriction enzyme. The mutation abolished an HhaI restriction site at position 71. In wild-type cats, two bands of 71 and 58 bp were detected, whereas in porphyric cats there was one band of 129 bp and in asymptomatic carriers there were three bands of 129, 71 and 58 bp.

**Prokaryotic expression of feline HMBS housekeeping and erythroid-specific cDNAs**

The feline HMBS cDNAs encoding the housekeeping and the erythroid isozymes were obtained by RT–PCR amplification of wild-type feline hepatic and/or splenic total RNA. The primers for each amplification contained BamHI and SalI restriction sites (Supplementary Material, Table S3). Using the megaprimer method (44), both cDNAs were amplified in two fragments, and reamplified by PCR using the more exterior primers to obtain the full-length cDNAs encoding each isozyme, which then were purified by electrophoresis and gel extraction (Qiagen Gel Extraction Kit, Qiagen, Valencia, CA, USA), digested with BamHI and SalI (New England Biolabs) and again gel purified. Site-directed mutagenesis using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) converted a specific BamHI/BglII site in the pSU vector (18) into a BamHI (see primers in Supplementary Material, Table S3. The vector
was then digested with BamHI and SalI, purified by gel extraction, and the feline BamHI/SalI-cut HMBS cDNAs ligated into the vector. Following transformation and colony purification, the constructs generated were confirmed by sequencing and then transformed into E. coli strain BL21+ for growth and purification of the feline HMB-synthase isozymes as previously described (18). The HMB-synthase activity in the bacterial lysates was determined as described above. Molecular sizes were approximated by electrophoresis in 12% SDS–PAGE using the EZ-Run Prestained Rec Protein Ladder (Fisher Scientific, Pittsburgh, PA, USA) as molecular weight standards.

Thermostability of recombinant feline HMB-synthase

The feline HMBS missense mutations were prokaryotically expressed in E. coli, and the cells were harvested and resuspended as previously described (18) in 50 mM HEPES pH 7.8, 0.5 M NaCl, 5 mM DTT, 330 μg/ml lysozyme and 5 μg/ml of each DNase and RNase. No protease inhibitors were added. The suspension was freeze-thawed three times in a dry-ice/ethanol bath and centrifuged at 10 000g for 30 min. The crude extracts were collected and adjusted to pH 7.4 and 8.0 with NaOH, then incubated at 37 and 50 °C for 1 h. Aliquots were collected every 15 min and placed into an ice bath for subsequent assay.

Molecular modeling

The molecular model for the feline HMB-synthase housekeeping or erythroid-specific isozymes was based on the crystal structure of E. coli HMB-synthase (19) (RCSB Protein Data Bank entry 1PDA) and the crystal structure of human HMB-synthase (20), (RCSB Protein Data Bank entry 3ECR). The feline models were obtained using the SWISS-MODEL Protein Modeling (swissmodel.expasy.org). The predicted protein models were visualized with the PyMOL molecular graphics program (www.pymol.org).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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REFERENCES


