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Molecular basis for pseudo vitamin D-deficiency rickets in the Hannover pig

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Abstract

The molecular basis for pseudo vitamin D deficiency rickets (PDDR) in the Hannover pig model was determined in the current study. Consistent with the inability of Hannover PDDR pigs to maintain ambient levels of 1,25-dihydroxyvitamin D (i.e., 1,25D), the bioactivation enzyme cytochrome P450C1 (or CYP27B1) was determined to contain coding-region deletions that rendered the enzyme ineffective due to frame-shift mutations and expression of a premature termination codon. Expression levels of P450C1mRNA were up-regulated in response to the low-1,25D high-parathyroid hormone state of the PDDR animals. In a complementary manner, cytochrome P450C24 mRNA was not detectable in PDDR pigs. Two different deletions were detected within the Hannover pig strain in which the *P450C1* coding region contained either 173 bp or 329 bp deletions that resulted in the expression of non-sense products beginning within the I-helix region and extending through the truncated C-terminal domains. The boundaries for the deletion segments aligned with derived mRNA processing sites. This observation was consistent with an mRNA processing error as the causative factor for the coding-region deletions. Based upon the expression of a non-functional P450C1 enzyme, the Hannover pig model for PDDR was determined to be identical to the human disease in which enzyme-inhibitory mutations are the molecular basis for the calcium disorder.

Keywords: Pig; Vitamin D; Rickets; Mutation; RNA deletion

1. Introduction

A deficiency in vitamin D was determined by pioneering research in the early 1900's to be the basis for rickets, a calcium-phosphate disease of bone [1,2]. Subsequent studies revealed the presence of a genetic disorder in which rickets developed in the presence of sufficient circulating levels of vitamin D (i.e., target tissues displayed an apparent resistance to vitamin D) [3,4]. Discovery of the necessity to bioactivate vitamin D to the nutrient hormone 1,25-dihydroxyvitamn D_3 (1,25VD) [5-7] made it was possible to differentiate the vitamin D-resistance disorder into two distinct disease types. In type-I resistance, a lesion was demonstrated in the 1,25VD synthetic pathway [8] in which low 1,25VD circulating levels were observed in the presence of normal vitamin D status. This disease was subsequently named pseudo vitamin D-deficiency rickets (PDDR) [9]. In a contrasting manner, patients with Type-II resistance displayed elevated levels of 1,25VD, however, they expressed target-organ refractoriness to the molecular actions of 1,25VD. Based upon the target-organ resistance to 1,25VD, the disease was named hereditary vitamin D-resistant rickets (HVDRR) [10].

Recognizing the advantage of using animal models to study human disease, a pig model for PDDR was developed [11,12] shortly after the disorder was described in humans [3]. The pig inbreeding studies demonstrated the disorder to be transferred in an autosomal recessive manner and the phenotypic expression was identical to human PDDR with the same bone and serological parameters (i.e., rachitic bone lesions in the presence of low levels of serum calcium, phosphate and 1,25VD, and elevated serum parathyroid hormone). Extensive studies on the function of 1,25VD in the PDDR pig demonstrated normal actions for the secosteroid's target organ function, serum transport and metabolic turnover rates [13,14]. In a related manner, renal synthesis of 1,25VD was observed to be low or non detectable in PDDR pigs [12]. The collective metabolic results, therefore, implicated a dysfunctional 25-hydroxyvitamind D_3 1-hydroxylase enzyme (i.e., cytochrome P450C1 or CYP27B1) in the 1,25VD bioactivation pathway. Further support for

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this concept was obtained when the human *P450C1* gene and *PDDR* gene loci were found to be co-localized on chromosome 12 (12q13.2-13.4) [15]. Molecular studies in humans utilized sequence information for the cloned 1-hydroxylase enzyme to identify P450C1 mutants as the molecular basis for PDDR [16-18]. Reverse transcriptase-polymerase chain reaction (i.e., RT-PCR) cloning technology was used in the current study to clone pig renal P450C1 and determine the molecular basis of PDDR in the Hannover pig model. Deletion mutations were detected in the coding region for several C-terminal domains of cytochrome P450C1 that resulted in the synthesis of a dysfunctional 1-hydroxylase enzyme.

2. Materials and methods

2.1. Materials

PCR oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) or through the Center for Genetics in Medicine, University of New Mexico Health Sciences Center (Albuquerque, NM). AMV and M-MLV reverse transcriptase, Taq polymerase, deoxynucleotides, and T4 DNA ligase were obtained from Promega (Madison, WI). Restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, MA). RNase Inhibitor was obtained from Chimerx (Milwaukee, WI). The PCR-Script Amp Cloning Kit was purchased from Stratagene (LaJolla, CA). QIAquick Gel Extraction Kit was obtained from Qiagen, Inc. (Valencia, CA). TRIzol Reagent, Platinum Taq DNA Polymerase High Fidelity, DH5α-competent cells, and electrophoresis grade agarose were acquired from Life Technologies (Rockville, MD).

2.2. Pigs, treatment and RNA isolation

Two Hannover piglets that were homozygous for PDRR were derived by a described breeding scheme in which the genetic defect was transferred in an autosomal recessive manner [19]. Phenotypic expression was verified in each animal by changes in serological parameters (i.e., high serum PTH and alkaline phosphatase and low serum 1,25 VD and calcium) and animals were euthanized at six weeks of age. Wild-type weanling pigs were raised for six weeks on a diet deficient in vitamin D and treated intramuscularly with carrier ethanol (-D group) or vitamin D_3 (+D group-)(i.e., 125 mg of vitamin D_3 at days -9 and -2 before sacrifice)(special recognition to R. Horst, USDA, Ames, IA). Kidney cortex and bone tissue were rapidly collected from euthanized pigs, cut into $\sim$0.5 cm^3 pieces and frozen in liquid nitrogen. Total RNA was extracted using TRIzol Reagent and a tissumizer at 50% power (Tekmar Company, Cincinnati, OH). RNA samples were phenol/chloroform extracted and stored at –70°C in DEPC-treated sterile water prior to use in RT-PCR. The absence of DNA and quality of isolated RNA was confirmed by agarose-formaldehyde gel electrophoresis.

2.3. RT-PCR

Total RNA (5 μg) was denatured at 65°C (10 min) in the presence of oligo dT_{18} (100 pmols), rapidly chilled at 4°C, adjusted to contain manufacturer's buffer, 0.1 mM dNTPs, and 25 units of RNase Inhibitor (Chimerx). The mixture was reverse transcribed at 42°C for 2 hr using 5 Units of AMV-RT (Promega) in a total volume of 30 μl. A portion of the first-strand cDNA product (2 μl) was used as target DNA in subsequent PCR reactions containing 10 pmol of each primer, 2 mM $MgSO_4$, manufacturer's buffer, 2 mM dNTPs and 1 unit of Platinum Taq Polymerase High Fidelity (Life Technologies). The DNA primers were designed to amplify the entire coding sequence of pig P450C1 [20]. The primers were used as a hemi-nested set (i.e., a common 5′ amplimer with inner and outer 3′ amplimers). Sequence of the common 5′ amplimer was 5′-ATGACCCAAACCCTCAAGCT, the outer 3′ amplimer was 5′-ATCCCTTGGTCTTATGCCTA and the inner 3′ amplimer was 5′-GGTGATGATGACAGCCTCCT. PCR parameters for the sequential hemi-nested protocol were: denaturation (2 min, 94°C), followed by 25 cycles at 94°C for 30 s, 48°C for 30 s (first PCR using outer 3′-amplimer) or 50°C for 30 s (second PCR using inner 3′-amplimer), with 68°C extension for 1.5 min, and a final extension at 68°C for 5 min.

Semi-quantitative PCR utilized 0.2 μg of total RNA with 50 units of M-MLV reverse transciptase, 0.4 mM dNTPs, with supplied buffer to 25 μl volume. PCR was conducted on the reverse transcribed sample for 24 cycles (linear range) using Taq polymerase (1 unit), 0.4 mM dNTP, 0.1 pmol of the 5′ and 3′ amplimers and supplied buffer at 1.5 mM Mg. Cycling parameters were 94°C for 30 sec, 55°for 30 sec and an extension at 72°C for 2 min. The 5′ and 3′ primers were: GADPH, 5′-amplimer, 5′-TCCTGCACCACCAACTGCTTA and 3′-amplimer, 5′-ACCACCCTGTTGCTGTAGCCA; P450C1, 5′-amplimer, 5′-CGTGCTCCTGAGCTGGGTTCC and 3′-amplimer, 5′-CCAGCTGGGCATCGCCATAGTCAGGAGCGTGGACACAAAC. Statistical difference between treatment groups was determined using the unpaired t-test.

Cloning of P450C1-PCR products were size-verified by gel electrophoresis on 1% TBE agarose gels and purified using the Qiagen QIAquick Gel Extraction Kit. The PCR product was cloned into pPCR-Script Amp SK (+) vector using the PCR-Script Amp cloning kit (Stratagene) and positive colonies identified using X-Gal color selection. Clones containing the P450C1 insert were PCR amplified and sequenced. The sequence data was analyzed by the GCG software suite [21] in order to compare DNA and derived amino acid sequences for the two mutant pigs against wild-type sequences from the present and prior study [20].

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wt  atgacccaaa ccctcaagct cgcttccaga gtgttccatc gcgtctgccg tgctcctgag 60
M1  atgacccaaa ccctcaagct cgcttccaga gtgttccatc gcgtctgccg tgctcctgag
M2  atgacccaaa ccctcaagct cgcttccaga gtgttccatc gcgtctgccg tgctcctgag
                                    Exon 1
wt  ctgggttcca gaggctccga ctcagcgcct cggggattgg ccgacctccc aggcccctcc  120
M1  ctgggttcca gaggctccga ctcagcgcct cggggattgg ccgacctccc aggcccctcc
M2  ctgggttcca gaggctccga ctcagcgcct cggggattgg ccgacctccc aggcccctcc

wt  acgcctggtt tccttgccga acttttctgc aaggggggtc tgtcacggct gcacgagcta  180
M1  acgcctggtt tccttgccga acttttctgc aaggggggtc tgtcacggct gcacgagcta
M2  acgcctggtt tccttgccga acttttctgc aaggggggtc tgtcacggct gcacgagcta
      ▼
wt  caggtgcagg gtgccgcgcg ctttggccca gtgtggttgg ccagtttcgg gaaggtgcgc  240
M1  caggtgcagg gCgccgcgcg ctttggccca gtgtggttgg ccagtttcgg gaaggtgcgc
M2  caggtgcagg gtgccgcgcg ctttggccca gtgtggttgg ccagtttcgg gaaggtgcgc
                 Exon 2
wt  acggtgtacg tggcggcccc tacgctcgtc gagcagctgc tacgacagga gggacccttg  300
M1  acggtgtacg tggcggcccc tacgctcgtc gagcagctgc tacgacagga gggacccttg
M2  acggtgtacg tggcggcccc tacgctcgtc gagcagctgc tacgacagga gggacccttg

wt  cccgagcgct gcagcttctc accctggacg gagcaccgtc gccgacgcca gcggtcttgc  360
M1  cccgagcgct gcagcttctc accctggacg gagcaccgtc gccgacgcca gcggtcttgc
M2  cccgagcgct gcagcttctc accctggacg gagcaccgtc gccgacgcca gcggtcttgc
                  ▼
wt  ggactgctca ccgcggaagg tgaagaatgg cagaggctcc gcagtctcct ggccccgttg  420
M1  ggactgctca ccgcggaagg tgaagaatgg cagaggctcc gcagtctcct ggccccgttg
M2  ggactgctca ccgcggaagg tgaagaatgg cagaggctcc gcagtctcct ggccccgttg
                                          Exon 3
wt  ctcctccggc ctcaagcggc agcccgctat gccgggaccc tgcatgacgt ggtccaggac  480
M1  ctcctccggc ctcaagcggc agcccgctat gccgggaccc tgcatgacgt ggtccaggac
M2  ctcctccggc ctcaagcggc agcccgctat gccgggaccc tgcatgacgt ggtccaggac

wt  cttgtgcggc gactgcggag ccagcgggga ctgggcgctg ggcctcccgc cctggttcgg  540
M1  cttgtgcggc gactgcggag ccagcgggga ctgggcgctg ggcctcccgc cctggttcgg
M2  cttgtgcggc gactgcggag ccagcgggga ctgggcgctg ggcctcccgc cctggttcgg
                                          ▼
wt  gacgtggcag gagagtttta taagtttgga ctagaaggca ttgcggcggt gctgttgggt  600
M1  gacgtggcag gagagtttta taagtttgga ctagaaggca ttgcggcggt gctgttgggt
M2  gacgtggcag gagagtttta taagtttgga ctagaaggca ttgcggcggt gctgttgggt

wt  tcccgcctgg gctgcctgga acccgaagtg ccgccagaca cagagacctt catccgcgcg  660
M1  tcccgcctgg gctgcctgga acccgaagtg ccgccagaca cagagacctt catccgcgcg
M2  tcccgcctgg gctgcctgga acccgaagtg ccgccagaca cagagacctt catccgcgcg
                       Exon 4
wt  gtgggatcgg tgtttgtgtc cacgctcctg accatggcga tgcccagctg gctgcaccgc  720
M1  gtgggatcgg tgtttgtgtc cacgctcctg accatggcga tgcccagctg gctgcaccgc
M2  gtgggatcgg Cgtttgtgtc cacgctcctg accatggcga tgcccagctg gctgcaccgc
                                                            ▼
wt  ctcgtgccgg gaccctgggc ccgcctctgc cgcgactggg accagatgtt tgcatttgcc  780
M1  ctcgtgccgg gaccctgggc ccgcctctgc cgcgactggg accagatgtt tgcatttgcc
M2  ctcgtgccgg gaccctgggc ccgcctctgc cgcgactggg accagatgtt tgcatttgcc

wt  caggagcacg tggagcggcg agaggccgag gctgccatga agagccaggg aaagcctgag  840
M1  caggagcacg tggagcggcg agaggccgag gctgccatga agagccaggg aaagcctgag
M2  caggagcacg tggagcggcg agaggccgag gctgccatga agagccaggg aaagcctgag
                                    Exon 5
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Fig. 1. Pig P450C1 Coding Sequence. Proposed exon organization is identified and noted (▼) and a putative intra-exon cryptic RNA processing sequence underlined.

3. Results

Wild-type pig P450C1 cloned in the current study from renal tissue was 1512 nucleotides in length (Fig. 1) and coded for a 504 amino acid protein (mw 52 kDa). The coding sequence was identical to the previously cloned pig enzyme from LLC-PK1 kidney cells [20]. Intron locations derived from the coding sequence had identical alignments to the processing sites for both the human and mouse P450C1 gene (i.e., eight introns) [22,23]. RT-PCR products

```
wt  gaggacttgg gatctggggc gcacctgacc tacttcctct tccgggaaga gctgccagcc  900
M1  gaggacttgg gatctggggc gcacctgacc tacttcctct tccgggaaga gctgccagcc
M2  gaggacttgg gatctggggc gcacctgacc tacttcctct tccgggaaga gctgccagcc

wt  ccgtccatcc tggggaatgt gacagagttg ctactggctg gagtggacac ggtgtccaac  960
M1  ccgtccatcc tggggaatgt gacagagttg ctactggctg gagtggacac g.........
M2  ccgtccatcc tggggaatgt gacagagttg ctactggctg gagtggacac g.........

wt  acactctcct gggctctcta tgaactctct cggcaccctg aagtccagat ggcactccat  1020
M1  .......... .......... .......... .......... .......... ..........
M2  .......... .......... .......... .......... .......... ..........
                                  Exon 6
wt  tctgagatca aaactgcttt gggccccagc tccagtgccc acccatcagc cactgttcta  1080
M1  .......... .......... .......... .......... .......... ..........
M2  .......... .......... .......... .......... .......... ..........

wt  tcccagctgc ccctgcttaa ggcagtggtc aaggaagtgc taagactgta ccctgtggta  1140
M1  .......... .......... .......... .......... ....actgta ccctgtggta
M2  .......... .......... .......... .......... .......... ..........
                                Exon 7
wt  cctggaaact cccgtgtgcc agacaaagac atttgtgtgg gtgactacat tatccccaaa  1200
M1  cctggaaact cccgtgtgcc agacaaagac atttgtgtgg gtgactacat tatccccaaa
M2  .......... .......... .......... .......... .......... ..........

wt  aatacactgg tcactctgtg tcactatgcc acttcaaggg accctgccca gttcccagag  1260
M1  aatacactgg tcactctgtg tcactatgcc acttcaaggg accctgccca gttcccagag
M2  .......... .......... .......... .......... .......... ..........
                                                Exon 8
wt  ccaaattctt ttcgtccAGc tcgctggctg ggggaatgtc cagcccccca cccatttgca  1320
M1  ccaaattctt ttcgtccagc tcgctggctg ggggaatgtc cagcccccca cccatttgca
M2  .......... .........c tcgctggctg ggggaatgtc cagcccccca cccatttgca

wt  tctctccccc ttggctttgg caagcgcagc tgcatgggga gacgcctggc agagcttgag  1380
M1  tctctccccc ttggctttgg caagcgcagc tgcatgggga gacgcctggc agagcttgag
M2  tctctccccc ttggctttgg caagcgcagc tgcatgggga gacgcctggc agagcttgag

wt  ctgcaaatgg ctttggccca gatcttgatc cactttgagg tgcagcctga gccaggttct  1440
M1  ctgcaaatgg ctttggccca gatcttgatc cactttgagg tgcagcctga gccaggttct
M2  ctgcaaatgg ctttggccca gatctTGAtc cactttgagg tgcagcctga gccaggttct
                                Exon 9
wt  gccccaatca gacccatgac ccggactgtt ctggtacctg agagaagcat caatctacag  1500
M1  gccccaatca gacccatgac ccggactgtt ctggtacctg agagaagcat caatctacag
M2  gccccaatca gacccatgac ccggactgtt ctggtacctg agagaagcat caatctacag

wt  tttgtggaca gatag  1515
M1  tttgtggaca gatag
M2  tttgtggaca gatag
```

for the kidney *P450C1* coding region from two different Hannover-PDDR pig mRNA samples gave shorter products than was observed for wild-type mRNA (Fig. 2). The smaller PCR products were due to deletions of 173 and 329 bp as determined by DNA sequence analysis of the cloned products. The 173-nucleotide deletion in mutant pig-1 (M-1) occurred between nucleotides 950 and 1123. The 5′cut site for the deletion in mutant pig-2 (M-2) was the same as in pig M-1, however, the 3′-cut site occurred 156 bp further downstream at nucleotide 1279. Both deletions caused a downstream frame-shift that resulted in the expression of premature stop codons and derived mRNA lengths of 1342 nucleotides for M-1 and a shorter transcription product of 1186 nucleotides for M-2. In both instances, the

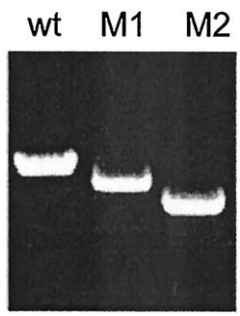

Fig. 2. Kidney RT-PCR Products. Size of coding sequence for wild-type (wt)(1552 bp), mutant-1 (M-1)(1379 bp) and mutant-2 (M-2)(1223 bp) derived PCR products using the primer set defined in Methods and Materials.

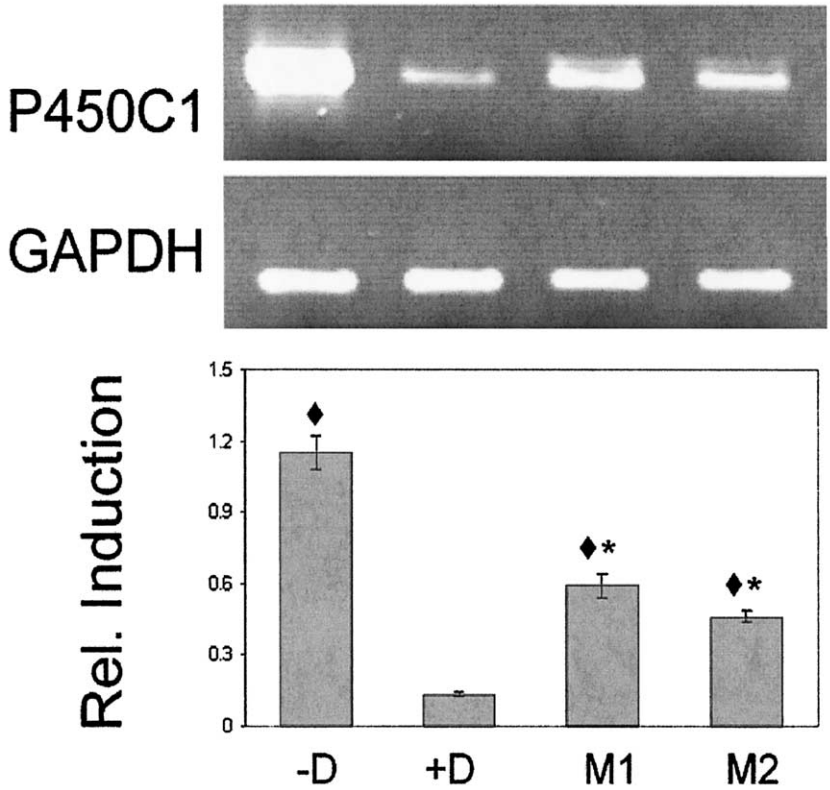

Fig. 3. Expression Levels for Kidney P450C1. Message levels for kidney P450C1 was estimated using quantitative PCR conditions for induced (-D) and suppressed (+D) wild-type (wt) and mutants M-1 and M-2 animals. P450C1 expression levels are gel-visualized and four independent measurements were standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with results shown in bar graphs as mean +/- SD (N = 4). ♦, Significantly different from +D group ($p < 0.001$); *, Significantly different from -D group ($p < 0.01$).

deletion-mutations coded for a truncated enzyme (336 and 359 amino acids for M-1 and M-2, respectively, compared to 504 for the wild-type enzyme) that lacked functional domains beginning within the I-helix and continuing through the C-terminal beta-sheet region [24]. Such conformational changes predictably rendered the enzymes catalytically ineffective due to lack of a heme-binding region and domains associated with active-site functionality and electron transfer from the mitochondrial ferredoxin iron-sulfur molecule. The extra-renal site for P450C1 expression in bone was also analyzed by RT-PCR and was observed to express the same wild-type sequence and PDDR deletion aberrations (Choe, S, Serda, R and Omdahl, J, data not shown).

Relative expression levels for renal P450C1 and P450C24 mRNA were estimated for wild-type and PDDR mutant animals using semi-quantitative PCR in which results were normalized to gyceraldehyde-3-phosphate dehydrogenase (GAPDH) message levels. *P450C1* mRNA levels for mutant samples were compared to wild-type RNA obtained from animals that were induced (-D) or suppressed (+D) for P450C1 enzyme expression (Fig. 3). Wild-type and Hannover-mutant kidney mRNA samples had a higher *P450C1* message level than observed in mRNA from enzyme suppressed (+D) animals ($p < 0.001$) (Fig. 3). However, the *P450C1* mRNA level for both mutant kidney samples was estimated to be about 2-fold lower than the enzyme message level in wild-type vitamin D-deficient (-D) P450C1 induced animals ($p < 0.01$) (Fig. 3). Whether this observed difference in mRNA levels was due to accelerated mRNA turnover or lower transcription regulation in the PDDR animals was not determined in the current study.

In contrast to P450C1 mRNA expression, the P450C24 mRNA levels were observed only in kidney from wild-type animals treated with vitamin D (+D) and suppressed for P450C1 expression. In contrast, kidney RNA from the (-D) wild-type and PDDR animals deficient in 1,25D (the major up-regulator of P450C1) did not display enough P450C24 mRNA to be detected using the employed RT-PCR method. Although the hallmark for 1,25D treatment is the up-regulation of P450C24 expression, PDDR Hannover pigs have been reported [25] to not induce P450C24 expression in response to 1,25D treatment. Such results have been attributed to a genetic defect in *P450C24* gene expression [25] although this possibility was not evaluated in the current study.

4. Discussion

The molecular hallmark of PDDR in humans is a functional mutation in cytochrome P450C1 (CYP27B1), which

Fig. 4. Intron-splicing Scheme and Protein-expression Products. (A) Derived intron-exon boundaries are shown for wild-type (wt) and two RNA-splicing mutants in which exon 6 was deleted in mutant M-1 and exons 6, 7 and part of 8 were deleted in mutant M-2. (B) Schematic protein sequences for regions downstream of RNA deletion sites (i.e., C-terminal side of deletions) for mutants M-1 and M-2 are compared to sequence for wild-type (wt) enzyme.

is the rate-limiting enzyme in the vitamin D bioactivation pathway. Mutations that result in an inactive enzyme have been documented in different functionally important domains throughout the human enzyme [26]. To date, the availability of large-animal models to study the nutrient-genetic PDDR disorder is limited to the Hannover pig. Studies in the current paper were designed to obtain a molecular understanding of the enzyme lesion in the Hannover pig PDDR model.

The genetic analysis of numerous PDDR patients have resulted in the characterization of distinct single-point mutations within the coding region of the *P450C1* gene [15,16,18] that render the enzyme catalytically inactive. In addition, a seven base insertion has also been observed in exon 8 of the human *P450C1* gene that caused a frame shift and premature termination with loss of enzyme function [27]. The current study documents for the first time deletions in the *P450C1* gene that involved the natural ablation of entire exons, which resulted in attendant frame shifts and the expression of premature termination codons. In the current study, two pig PDDR deletion mutants were documented. Based upon exon-intron boundaries that are identical to those for the mouse and human P450C1 gene, one PDDR mutant (M-1) was determined by sequence-alignment analysis to involve the deletion of exon-6 (173 bp). The sequence within exon 6 codes for the conserved arginine residue in the I-helix region that is required for oxidation of the heme-iron during the catalytic cycle of P450C1. Exon 6 also codes for the J helix and the surface K-helix domain that binds the ferredoxin electron-transfer molecule, which provides electrons for reduction of molecular oxygen during the mixed-function oxidase catalytic cycle for P450C1. The other PDDR mutant (M-2) had a more extensive deletion (329 bp) that involved the loss of both exons 6 and 7 and approximately 1/3 of exon 8 in which the deleted exon 7 and 8 regions coded for substrate-binding and conformational domains that extend through the meander region. Nonsense and premature termination-codon expression in the post-deletion sequence resulted in the loss of general conformational integrity, the critical heme-binding domain (i.e., cysteine pocket), and substrate-binding residues located in the C-terminal β-sheet regions. Recognizing that cytochromes P450 are highly sequence and conformationally-dependent hemoproteins (i.e., loss of function in human PDDR with single amino acid mutation in a conformational domain) [16–18,26] it can be predicted that the truncated and missense P450C1 enzymes from the Hannover pigs are not catalytically functional.

Upon closer analysis, it appears that each deletion occurred at a normal or pseudo (cryptic) mRNA splice sites. For example, the deletion of exon 6 in mutant M-1 is consistent with an error at the 3′-intron cut site that resulted in excision of the intervening intron 5-exon 6–intron 6 cassette (Fig. 4A). The coupling of exon 5 to exon 7 resulted in expression of a premature stop codon that resulted in a shortened message containing a 3′-missense region (Figs. 1 and 4B). Generation of the larger deletion mutant in M-2 can be explained through coupling of the 5′-splice donor with a pseudo 3′-splice acceptor site within exon 8 (Fig. 4A). The targeted 3′-cut site within exon 8 contained a polypyrimidine-cAG sequence (i.e., ccaaattcttttcgtccAG) (Fig. 1) that is a characteristic signature for intron 3′-cut sites [28] and appeared to function as a cryptic (pseudo) splice site during abnormal mRNA splicing events. Similar to the M-1 deletion mutation, deletion of the intervening cassette in mutant M-2 would result in a premature termination of the mRNA coding region that contains a 3′-missense region (Fig. 4B).

Tissue-specific splice variants have been documented for P450 enzymes that resullt in a number of aberrant mRNAs [29-31]. Recently, splice variants were identified and characterized for P450C1 in a human glioblastoma cell line [32]. The abnormal mRNAs were derived from a combination of complete exon deletions (exons 4 and 5), partial exon deletion (exon 8) and insertions of partial- and full-lengths of intron 2 between exons 2 and 3. Partial-length intron 2 insertions between exons 2 and 3 have also been noted in skin, prostate and colon but not in kidney, which is reported to express only full-length P450C1 mRNA [33]. The lack of P450C1 splice variants in kidney was also observed in the current study for both the induced and repressed regulatory states. The deletions could be explained on the basis of a gene recombination or transposable-like events. However, the coincidence of RNA excisions at predicted intron processing sites within the primary mRNA-transcript supports an RNA-processing error as the basis for the deletion mutations. DNA-level mechanistic studies into aberrant mRNA-splicing processes in the Hannover pig PDDR model are in progress.

Previous studies using the pig PDDR model have observed low to non-detectable levels of circulating 1,25VD [25]. Consistent with this observation, no 1-hydroxylase activity could be detected in crude renal-cortex preparations from PDDR pigs [12]). In contrast, a more recent study using partially purified P450 preparations from PDDR animals reported an increase in the 1-hydroxylation of 25OHD for both renal and hepatic mitochondrial enzyme preparations [34]. Therefore, the authors suggested that the suppressed circulating 1,25VD levels in PDDR pigs was not the result of an inactive P450C1 but rather could be related to a decrease in microsomal 1-hydroxylase activity [34]. Based upon these findings, the authors proposed that the pig PDDR was distinct from the human counterpart in which a defective mitochondrial P450C1 has been documented clearly [16-18]. Based upon recent developments, it is now possible to discuss the enzymatic mechanism for pig PDDR with greater clarity.

P450C1 of the vitamin D pathway is a highly labile enzyme that displays sensitivity to the detergents used in purification procedures (Bobrinokova, K and Omdahl, JL, personal observation). It is of interest to note, therefore, that the enzyme-purification procedure of Axen el.al. [35] used a detergent (i.e., emulgen) that is known to inhibit P450C1 activity. Consequently, activities measured in that study for partially purified P450s would predictably be low or devoid of P450C1 activity and would most reasonably represent a low level of non-specific 1-hydroxylase activity that that has been documented for hepatic mitochondrial CYP27A1 [36]. The low level of 1,25VD that can be produced from such an extra renal P450C1 source would appear from CYP27A1 knockout studies to be insignificant [37]. This point is further emphasized from recent P450C1 knockout studies in mice [38,39] in which serum 1,25VD levels decreased to undetectable levels and animals developed symptoms characteristic of PDDR. Therefore, it is apparent from the P450C1 gene-knockout studies and coding-sequence-deletion results in the current investigation that mitochondrial P450C1 is the enzyme in human and lower-vertebrates that is responsible for the development of PDDR.

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