CHAPTER 3

*Ex vivo* analysis of aberrant splicing induced by two donor site mutations in *PKLR* of a patient with severe pyruvate kinase deficiency

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Abstract

Two single-nucleotide substitutions in PKLR constituted the molecular basis underlying pyruvate kinase (PK) deficiency in a patient with severe haemolytic anaemia. One novel mutation, IVS5+1G>A, abolished the intron 5 donor splice site. The other mutation, c.1436G>A, altered the intron 10 donor splice site consensus sequence and, moreover, encoded an R479H substitution. We studied the effects on PKLR pre-mRNA processing, using ex vivo produced nucleated erythroid cells from the patient. Abolition of the intron 5 splice site initiated two events in the majority of transcripts: skipping of exon 5 or, surprisingly, simultaneous skipping of exon 5 and 6 (∆5,6). Subcellular localization of transcripts suggested that no functional protein was produced by the IVS5+1A allele. The unusual ∆5,6 transcript suggests that efficient inclusion of exon 6 in wild-type PKLR mRNA depends on the presence of splice enhancing elements in exon 5. The c.1436G>A mutation caused skipping of exon 10 but was mainly associated with a severe reduction in transcripts although these were, in general, normally processed. Accordingly, low amounts of PK were detected in nucleated erythroid cells of the patient, thus correlating with the patient’s PK-deficient phenotype. Finally, several low-abundant transcripts were detected that represent the first examples of ‘leaky-splicing’ in PKLR.

Introduction

The enucleated mature red blood cell depends solely on anaerobic glycolysis for the constant generation of ATP in order to maintain cellular integrity and function. Disturbances in glycolysis shorten its cellular survival and frequently result in nonspherocytic haemolytic anaemia of variable severity. The most common cause of chronic nonspherocytic haemolytic anaemia is pyruvate kinase (PK) deficiency (MIM 266200). PK catalyses the final step of glycolysis, converting phosphoenolpyruvate to pyruvate with the concomitant generation of ATP. PK deficiency is transmitted as an autosomal recessive disease, and molecular analysis has identified more than 130 mutations in the structural gene (PKLR) in association with PK deficiency. The majority of these mutations constitute single-base substitutions, predicting amino acid changes of conserved residues in structurally and functionally important domains of PK. In contrast, mutations that affect PKLR transcription or processing of its pre-mRNA are less frequently encountered.

Pre-mRNA processing is a critical aspect of gene expression and comprises the precise removal of introns in such a way that the exons are joined to form mature mRNAs with intact
translational reading frames. The splicing reaction is carried out by the spliceosome which consists of five small ribonucleoprotein complexes U1, U2, U4, U5, and U6 snRNPs and a large number of non-snRNP proteins. The spliceosome is assembled \textit{in vivo} onto the pre-mRNA as it is being synthesized, thus providing an intimate relationship between transcription and pre-mRNA processing. Changes in splice site choice, \textit{e.g.} as result of mutation, arise from changes in the assembly of the spliceosome and are thought to be regulated by altered binding of the initial factors to the pre-mRNA and the formation of early spliceosome complexes. Fifteen percent of point mutations in human genes, associated with disease, affect the conserved splice site signals. However, mutations can affect pre-mRNA processing in a multitude of ways. Therefore, in order to fully comprehend the contribution of any specific mutation on disease, it is important to consider mRNA biogenesis, quality control, and metabolic fate.

In this study we report on the identification of two single-base substitutions in \textit{PKLR} of a patient with severe PK deficiency. A novel G>A substitution at the invariant GT dinucleotide of intron 5 and a similar substitution concerning the last nucleotide of exon 10, both affected the 5’ splice site. Since pre-mRNA processing is a nuclear event and the reticulocyte is devoid of RNA synthesis, we studied the effects of these mutations on \textit{PKLR} pre-mRNA processing using \textit{ex vivo} produced nucleated erythroid cells from the patient. Multiple aberrant transcripts were identified and characterized. Subsequent subcellular localization of mRNAs and Western Blot analysis established the relevance of each transcript with regard to the PK-deficient phenotype of the patient. In addition, the characterization of one unusual transcript which lacked both exon 5 and 6 as a result of the intron 5 donor site mutation, suggests that efficient inclusion of exon 6 in wild-type \textit{PKLR} mRNA depends on the presence of splice enhancer elements in exon 5 in the primary transcript.

\textbf{Subjects and methods}

\textbf{Patient}

The patient is a 31 year old Dutch woman, diagnosed at birth with severe, transfusion-dependent haemolytic anaemia due to PK deficiency. Other causes of anaemia were ruled out. Splenectomy, performed at the age of four years, improved her condition only slightly. From age 15 years on, the transfusion dependency has gradually decreased and is, to date, limited to periods of infection or during pregnancy, in general when blood hemoglobin falls below 6.4 g/dL. Throughout her life she has displayed a remarkable heat-sensitivity which led to haemolytic crises not only during febrile episodes but also as a result of prolonged
Aberrant processing of PKLR pre-mRNA

exposure to sunlight. Her heterozygous parents were asymptomatic. Appropriate informed consent was obtained from the patient and the normal control individual, a healthy female with no haemolysis and normal glycolytic enzyme activities.

**Biochemical analysis**

PK and hexokinase (HK) enzyme activities were measured according to standard methods. Thermal stability of PK was determined by the PK heat stability test.

**Molecular analysis of PKLR**

Genomic DNA was isolated by standard methods. The erythroid-specific promoter and individual exons of PKLR, including flanking intronic regions, were amplified by PCR as described and sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA). The IVS5+1G>A and c.1436G>A mutations were confirmed by HphI and MscI (New England Biolabs, Beverly, MA) digestion of independently amplified PCR products.

**In vitro production of human nucleated erythroid cells**

Nucleated erythroid cells from the patient and a normal control individual were produced from light-density cells as described. Briefly, light-density cells were collected from 50 mL venous blood by Ficoll-Paque Plus (Amersham-Pharmacia Biotec, Uppsala, Sweden) density centrifugation. Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM), containing 20% fetal bovine serum and supplemented with human stem cell factor (10 ng/mL) (a gift from dr. H. Lokhorst), erythropoietin (1 U/mL) (Janssen Cilag, Tilburg, The Netherlands), interleukin-3 (1 ng/mL) (Biosource Europe, Bruxelles, Belgium), dexamethasone (10⁻⁶ M), and β-estradiol (10⁻⁶ M) (both from Sigma, St Louis, MI). Cells were grown at 37°C at 5% CO₂ and maintained at 1–2×10⁶ cells/mL. Proliferation and cellular morphology was monitored every other day by May-Grünwald-Giemsa stained cytocentrifuged smears. Cells were harvested after 10 days and at that moment the culture consisted of more than 95% (pro-)erythroblasts. Subsequently, RNA was isolated or cells were used for polysome profile analysis. The transcriptional and translational active state of the cells with regard to PK was verified by polysome analysis and Western Blot (see ‘Discussion’).

**RT-PCR and estimation of allelic differences in transcript levels**

Total RNA was isolated from nucleated erythroid cells using RNABee reagent (Campro Scientific, Veenendaal, The Netherlands), according to instructions of the manufacturer.
PCR was performed using the GeneAmp RNA PCR Core Kit (Roche, Branchburg, NJ). 1.0 µg total RNA was reverse transcribed using random hexamers as primers. Because aberrant splicing events may not be confined to the immediate proximity of the location of both mutations, an extensive region was amplified from \(PKLR\) cDNA. In case of the IVS5+1G>A mutation, a region encompassing exons 4 to 7 was amplified with 30 pmol of primers PK-C4F: 5’-ACATTGCGCGACTCAACTTCTCC-3’ (exon 4, nts 338–360) and PK-C7R: 5’-TGATCTTTGATGCCGTCCTTCC-3’ (exon 7, nts 931–909). The effects of the c.1436G>A mutation were studied by amplification of exons 9 to 12 with primers PK-C9F: 5’-GGGCAACTTCCCTGTGGGGC-3’ (exon 9, nts 1230–1250) and PK-C12R: 5’-GATGGGGTACAAGGGTAGGGCTTAC-3’ (exon 12, nts +44 to +22, relative to the termination codon). In both cases, the reverse primer was 6-FAM labeled. Samples were heated for 5 minutes at 95°C and subjected to 35 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 60 seconds), followed by an elongated extension step of 10 minutes after the last cycle. Controls without RNA as well as controls in which the reverse transcription step was omitted were included. Labeled fragments were separated on an ABI 310 Genetic Analyzer (Applied Biosystems). Sizing (bp) and peak areas of fragments were calculated using GeneScan Analysis Software (Applied Biosystems).

In order to estimate relative transcript levels from both alleles of the patient, RT-PCR product, encompassing exons 9 to 12, was digested by MscI and subjected to agarose gel electrophoresis. The c.1436G>A mutation in exon 10 creates a unique recognition site for this enzyme and RT-PCR product from the 1436A allele renders fragments of 204 and 336 bp after digestion. The product from the other allele remains uncut (540 bp). Equal allelic amounts of exon 10 as amplified from the patient’s genomic DNA served as a control, yielding digestion fragments of 212 and 50 bp in case of the 1436A allele whereas the DNA-PCR product from the other allele is uncut (262 bp).

**Subcloning of RT-PCR products**

RT-PCR products were cloned using the pGEM-T Easy Vector System Kit (Promega, Madison, WI). Plasmid DNA was isolated (Qiaprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and 50 to 65 inserts were sequenced with the vector-derived T7 primer.

**Polysome profile analysis and \(\Delta5\) transcript instability**

Ten minutes before harvesting the cultured nucleated erythroid cells, cycloheximide was added to a concentration of 100 µg/mL. Cells were washed three times with ice-cold PBS containing 1 µg of cycloheximide per mL and lysed in 200 µL of 20 mM Tris-HCl (pH 7.6),
Aberrant processing of PKLR pre-mRNA

15 mM KCl, 1.5 mM Mg-acetate, 0.1 mM EDTA, 0.1% Triton X-100, and 7 mM β-mercaptoethanol. Cells were kept on ice for 1 minute and Na-desoxycholate and Igepal (Sigma) were added to a final concentration of 0.5%. The suspension was incubated for 30 minutes on ice. Cell debris and nuclei were removed by a 5 minute centrifugation at 4°C at 10,000 rpm. The supernatant was immediately layered onto isokinetic 15-32% (w/w) sucrose gradients in 20 mM Tris-HCl pH 7.6, 100 mM K-acetate, 3 mM Mg-acetate, 0.1 mM EDTA, and 7 mM β-mercaptoethanol. Gradients were centrifuged for 45 minutes at 50,000 rpm in a SW55 Beckman rotor, and harvested by upward displacement through a spectrophotometer, set at 260 nm. Ten fractions of 0.25 mL were collected into tubes containing 0.125 mL phenol/chloroform. RNA was isolated and 0.15 μg RNA of each fraction was used for RT-PCR and subsequent fragment analysis. To study the ∆5 transcript (i.e. the transcript lacking exon 5) instability, cells were isolated, lysed, and kept on ice. Aliquots of lysates were taken at 0, 10, 30, and 60 minutes and RNA was isolated for RT-PCR (primers PK-C4F and PK-C7R) and subsequent fragment analysis.

SDS-PAGE and Western Blot analysis
10^7 cultured nucleated erythroid cells of the patient and control were lysed in 100 μL buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM β-mercaptoethanol, 10 mM ε-aminocaproic acid, 10 mM EDTA, 1 mM PMSF. Samples were adjusted for protein content, subjected to 10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked for 1 hour at room temperature in 5% BSA, 0.1% Tween-20 in phosphate buffered saline (PBST) and incubated overnight with a polyclonal anti-PK antibody in 1% BSA, PBST. The membrane was washed 3 times with PBST and incubated for 2 hours with 1:10,000 diluted anti-rabbit IgG-HRP-linked antibody (Cell Signaling Technology, Beverly, MA) in 1% BSA, PBST. Bands were visualized by Enhanced Chemiluminescence (PerkinElmer, Boston, MA), according to instructions of the manufacturer.

Results

Glycolytic enzyme activities
Typical haematological parameters of the patient are displayed in Table 1. Despite a reticulocytosis of 638‰, the patient was severely anaemic. The PK activity was in the normal range but was very low considering the massive reticulocytosis, illustrated by the strongly elevated HK activity. Furthermore, PK from the patient showed reduced thermal stability, indicated by the 90% decrease in PK activity in the heat stability test (Figure 1).
Table 1. Haematological parameters and glycolytic enzyme activities of the patient.

<table>
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<td>11.8–14.4</td>
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<td>1.94</td>
<td>3.7–5.0</td>
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<td>MCV (fL)</td>
<td>118</td>
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<td>PK (U/gHb)</td>
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<td>6.9–14.5</td>
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<tr>
<td>HK (U/gHb)</td>
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**PKLR sequence analysis**

DNA sequence analysis of the patient’s *PKLR* gene revealed compound heterozygosity for two single base substitutions. The first mutation, on the paternal allele, concerned a guanine to adenine change at nt +1 of intron 5: IVS5+1G>A. This novel mutation abolishes the invariant GT dinucleotide of the intron 5 donor splice site. On the maternal allele we detected a previously reported guanine to adenine change at the last nt of exon 10 (c.1436G>A).5,28 Apart from encoding an arginine to histidine change at residue 479, this mutation disrupts the consensus sequence of the intron 10 donor splice site. No other mutations were detected.

![Figure 1. Thermal stability of PK](image-url)
Aberrant processing of PKLR pre-mRNA

A. Transcript no. bp WT P

1. 588 (584) 98 13
2. 411 (407) 1 2
3. 493 (495) 1 2
4. 461 (453) ND 49
5. 273 (268) ND 32
6. 635 (636) ND 1

B. Transcript no. bp WT P

7. 537 (540) 92 70
8. 368 (358) 2 2
9. 473 (474) 6 11
10. 382 (373) ND 1
11. 186 (191) ND 2
12. 313 (306) ND 1
13. 537 (540) ND 13
in coding region, flanking intronic sequences or the erythroid-specific promoter. Because both mutations were likely to affect correct PKLR pre-mRNA processing, we studied the effects of these mutations using erythroid cells, actively involved in RNA synthesis. For this, we cultured nucleated erythroid cells from the light-density fraction of venous blood of the patient and a control subject. After 10 days of culture, the initial 10^8 light-density cells of the patient rendered a culture consisting of 9.5 × 10^7 cells. More than 95% of these cells were morphologically identified as (pro-)erythroblasts. Total RNA was isolated from these cells.

The IVS5+1G>A mutation leads to skipping of exon 5 or simultaneous skipping of exon 5 and 6

For comprehensive analysis of aberrant processing induced by the IVS5+1G>A mutation, fluorescent RT-PCR products encompassing PKLR cDNA exons 4 to 7 were separated by capillary electrophoresis, cloned and evaluated. DNA sequence analysis of the cloned RT-PCR product of the control confirmed normal processing of wild-type PKLR transcripts as reflected by the majority of inserts (Figure 2A, transcript 1).

In agreement with this, one major 586 bp fragment was detected by fragment analysis (Figure 3A). In addition, clonal analysis revealed two rare inserts that reflected low level, alternative processing of wild-type PKLR pre-mRNA. One of these transcripts lacked exon 6 (∆6) whereas the other transcript lacked 95 bp of the 3’-end of exon 6 due to processing at a cryptic donor site at nt 600 in exon 6. (Figure 2A, transcripts 2 and 3). These transcripts were, likely, represented by the 411 and 493 bp fragments, as detected by fragment analysis (Figure 3A).

DNA sequence analysis of cloned RT-PCR product of the patient revealed six distinct inserts. Three of these reflected the same transcripts as detected in the control, i.e. normally processed with regard to exon 5 (Figure 2A, transcripts 1 to 3; Figure 3B, 411, 493, and 586 bp fragments). We postulate that these transcripts were not associated with the IVS5+1G>A mutation because donor site mutations abolish normal splicing completely20 and, moreover,
Aberrant processing of PKLR pre-mRNA

The transcripts were detected in both the patient and the control. Consequently, they were derived from the other allele of the patient. The three other inserts encountered in the cloned RT-PCR product of the patient reflected transcripts aberrantly processed with regard to exon 5. They were not detected in the control (see above) and, thus, likely associated with the IVS5+1G>A mutation. Of these three transcripts, two were abundant. They lacked either exon 5 (Δ5) or both exon 5 and 6 (Δ5,6) (Figure 2A, transcripts 4 and 5), and corresponded to fragments of 461 and 273 bp, as detected by fragment analysis (Figure 3B). The third,

Figure 3. Detection of aberrant transcripts associated with the IVS5+1G>A mutation. Fluorescent-dye labeled RT-PCR products of total RNA from nucleated erythroid cells of a control subject (panel A) and the patient (panel B) were separated by capillary electrophoresis. Primers amplified a region of PKLR cDNA encompassing exons 4 to 7 and RT-PCR products are indicated by arrows. Peak heights reflect the fluorescence signal of the individual fragments.
low-abundant, transcript contained the first 51 nt of intron 5 due to the use of a cryptic donor site at nt +52 in intron 5 (Figure 2A, transcript 6) and, likely, corresponded to the 635 bp fragment, as detected by fragment analysis (Figure 3B). No clones were encountered that could match the minor 374 bp fragment, detected by fragment analysis (Figure 3B). When reviewing the different transcripts identified in the patient and control, we speculate that the corresponding transcript lacks exon 5 and the 95 bp from the 3’-end of exon 6.

The c.1436G>A mutation causes skipping of exon 10 and is associated with strongly reduced transcript levels

Using primers spanning exons 9 to 12, we studied the effect of the c.1436G>A mutation on pre-mRNA processing. Fragment analysis (data not shown), and DNA sequence analysis of the cloned RT-PCR product of the control confirmed normal pre-mRNA processing in the majority of transcripts (Figure 2B, transcript 7). In addition, two minor transcripts were detected that lacked either exon 11 (Δ11) or 67 bp of the 5’-end of exon 11 due to alternative processing at a cryptic splice acceptor site at nt 1502 in exon 11 (Figure 2B, transcripts 8 and 9).

The cloned RT-PCR product of the patient revealed four distinct transcripts associated with the c.1436G>A mutation. Three of these were low-abundant transcripts that had the absence of exon 10 in common. They lacked exon 10 itself (Δ10), exon 10 and 11 together (Δ10,11), or exon 10 and 67 bp of the 5’-end of exon 11. (Figure 2B, transcripts 10 to 12). The majority of c.1436G>A associated transcripts was normally processed and, consequently, contained an adenine at nt 1436 (Figure 2B, transcript 13). Three transcripts were not associated with the c.1436G>A mutation and, thus, originated from the IVS5+1A allele. As in the control, the majority of these transcripts was normally processed whereas a minority resulted from alternative processing with regard to exon 11 (Figure 2B, transcripts 8 and 9).

Remarkably, when comparing the composition of clones from the patient, only 12 of the 63 clones represented transcripts derived from the 1436A allele. A similar asymmetrical clonal distribution was observed in the before-mentioned experiments regarding the IVS5+1G>A mutation: of the 56 selected clones, only six clones represented transcripts from the 1436A allele. This prompted us to estimate the relative amounts of transcripts from each allele of the patient. Therefore, we compared MscI digestion fragments of exon 10-containing RT-PCR and DNA-PCR products. The c.1436G>A mutation in this exon introduces a unique recognition sequence for MscI. Agarose gel electrophoresis of digested DNA-PCR product showed that half the amount of this 262 bp PCR product was cut into 212 and 50 bp fragments (Figure 4, the 50 bp fragment is not shown). In contrast, the two RT-PCR product digestion fragments (336 and 204 bp), representing the 1436A allele, were considerably less in amount than the undigested product (540 bp) which represented the 1436G allele (Figure 4).
Aberrant processing of PKLR pre-mRNA

The Δ5,6 nonsense transcript is, in part, associated with polysomes whereas the Δ5 transcript is unstable

To examine whether the identified transcripts were translationally active, cell-extracts were fractionated on sucrose gradients. Absorbance profiles of samples of the control and patient indicated the presence of polysomes in fractions 4 to 10 (Figure 5A and 5B). RNA obtained from each fraction was amplified by RT-PCR using primers PK-C4F and PK-C7R, and PCR products were analyzed by fragment analysis. As depicted in Figure 5C, the majority of wild-type mRNA was associated with polysomes. Low levels of the two other transcripts were present in all fractions, although we note that the mRNA alternatively spliced at the cryptic donor site in exon 6, appeared to accumulate in the lighter fractions, containing inactive ribosomes.

With regard to the patient, a substantial amount of Δ5,6 transcripts accumulated in the lighter fractions. Only part of this mRNA was polysome-associated (Figure 5D). The other transcripts were present in low amounts. Although there appeared to be a tendency for the normally processed 1436A mRNA to associate with polysomes (Figure 5D), levels were too low for accurate interpretation. The finding that the Δ5 mRNA was also present in low amounts was surprising because, thus far, this transcript had been the most abundant one. A possible explanation can be that the transcript was degraded during sample handling time (approximately one hour), prior to performing polysome analysis. Therefore, we performed a time-stability assay (see ‘Methods’). The results confirmed that once the cells were lysed, the Δ5 transcript was rapidly degraded (Figure 6).
Figure 5. Subcellular localization of \textit{PKLR} transcripts by RT-PCR analysis of polysomal fractions. (A, B) Polysome profile analysis of nucleated erythroid cellular extracts of the control and the patient. Polysomes were in gradient fractions 4 to 10. 40S, 60S, and 80S (monosomes) peaks are indicated by arrows. (C, D) Distribution of mRNAs from the control and the patient as determined by RT-PCR and subsequent fragment analysis. Primers amplified exons 4 to 7 from \textit{PKLR} cDNA. For each transcript, the amount of RT-PCR product is plotted against the fraction numbers. Transcript composition is indicated as follows: n, normally processed; \(\Delta 6\), deletion of exon 6; cryp6, spliced at the cryptic donor site in exon 6; \(\Delta 5,6\), deletion of exon 5 and 6; \(\Delta 5\), deletion of exon 5.

Figure 6. Instability of the exon 5 deleted transcript. Nucleated erythroid cell lysate from the patient was placed on ice and RNA was isolated at the indicated time intervals. RNA stability was analyzed by RT-PCR with primers spanning exons 4 to 7, and agarose gel electrophoresis. M: molecular mass marker.
**Aberrant processing of PKLR pre-mRNA**

**Low amounts of PK monomers are synthesized by the cultured nucleated erythroid cells of the patient**

Normally processed PKLR 1436A missense mRNA, albeit present at low levels, might direct active translation of R479H PK monomers. Using a polyclonal anti-PK antibody, a distinct band of the expected 63 kDa was detected by Western Blot in the nucleated erythroid cells of the normal control, corresponding to (erythroid-specific) PK monomers (Figure 7). The smaller, faint band most likely corresponded to the partially proteolysed form of 57–58 kDa. The same amount of total protein from nucleated erythroid cells of the patient displayed one faint, but distinct band of 63 kDa (Figure 7), indicative of low amounts of PK monomers.

![Image](figure7.png)

**Figure 7.** Detection of PK monomers in nucleated erythroid cells. Extracts of nucleated erythroid cells of the control and the patient were adjusted for protein content and separated by SDS-PAGE. Proteins were transferred to blot and PK monomers (63 kDa) were visualized by Western Blot, using a polyclonal anti-PK antibody.

**Discussion**

Severe pyruvate kinase deficiency in a Dutch patient with haemolytic anaemia was associated with two single nucleotide substitutions in the PKLR gene. One novel mutation, IVS5+1G>A, abolished the invariant dinucleotide at the donor splice site of intron 5. On the other allele, the previously reported c.1436G>A (R479H) missense mutation was detected which, additionally, altered the exonic consensus sequence of the intron 10 donor splice site. Both mutations were expected to exert their principal effect at the RNA level by interfering with correct processing of PKLR pre-mRNA. To study these effects, we used *ex vivo* produced nucleated erythroid cells from the patient. Both mutations induced aberrant processing of PKLR transcripts, and multiple mRNAs were identified. Subcellular localization of transcripts and Western Blot analysis established the relevance of each
transcript with regard to the PK-deficient phenotype of the patient. The transcript lacking exon 5 and 6 in particular was unusual which suggests the presence of a splice enhancer element in \textit{PKLR} exon 5.

The recognition of the exon is, in general, invoked by the pairing between the 3’ and 5’ splice site across an exon in a process called exon definition\cite{31}. Inactivation of the 5’ splice site involves disruption of U1 snRNP binding to the donor site during early spliceosome assembly\cite{13}. Consequently, the predominant effect of 5’ splice site mutations is skipping of the upstream exon or the use of cryptic splice sites, when present in the vicinity of the wild-type site\cite{20,32}. Indeed, the two major transcripts derived from the allele with the IVS5+1G>A mutation lacked exon 5: \(\Delta5\) and \(\Delta5,6\). In addition, we detected small amounts of a transcript with an in-frame insertion of 51 nt, resulting from processing at the cryptic donor site at nt +52 in intron 5. The fact that this cryptic site was used at such low frequency is likely the reflection of its markedly low 5’ splice site score\cite{33} of 63.0. The 132-bp deleted \(\Delta5\) transcript maintains the open reading frame, encoding a PK monomer (estimated mass 58 kDa) that lacks amino acids 126 to 169. In contrast, the 319 bp deleted \(\Delta5,6\) transcript contains a premature stop codon at nt 710 in exon 7. Thus, if synthesized, this monomer (estimated mass 14 kDa) would be severely truncated, lacking approximately 75% of its peptide chain.

Considering the abundance of \(\Delta5\) and \(\Delta5,6\) transcripts and their putative involvement in protein synthesis, we determined the subcellular localization of the respective mRNAs by polysome analysis. First, we established the transcriptional and translational activity of the cultured nucleated erythroid cells with regard to PK, using blood from a normal control individual. Wild-type \textit{PKLR} mRNA was associated with polysomes, and PK monomers were detected by Western Blot, confirming the translationally active state of the cultured cells. With regard to the \(\Delta5,6\) transcript, a substantial amount accumulated in the lighter fractions and only part of the mRNA was polysome-associated. Thus, translation of \(\Delta5,6\) mRNA was inefficient. Moreover, it is most likely that such a severely truncated protein, if stable, would be non-functional.

The \(\Delta5\) transcript was unstable during polysome analysis. Western Blot analysis failed to identify any PK of the predicted size (Figure 7) which may be directly related to this instability or, alternatively, the mutant protein encoded by the \(\Delta5\) mRNA may be unstable. If translated, a nonfunctional protein is to be expected since the deletion of residues 126 to 169 includes a number of amino acids essential for the enzyme’s function\cite{28,34-37}.

For two reasons, the detection of the \(\Delta5,6\) mRNA was noteworthy. Firstly, this mRNA examplifies another nonsense transcript insensitive to nonsense mediated mRNA decay (NMD)\cite{38-40}. This surveillance mechanism rapidly degrades mRNAs with premature translation termination codons, thereby preventing the potentially deleterious effects of
Aberrant processing of PKLR pre-mRNA

truncated proteins. The relative abundance of Δ5,6 mRNA suggests that this nonsense transcript bypasses NMD, similar to recently reported fibrinogen Aα and β-globin nonsense mRNAs. Secondly, simultaneous skipping of exon 5 and 6 is unusual by itself. Splice site mutations are rarely associated with multiple exon skipping and only a few examples are available. Our results suggest that the IVS5+1G>A mutation-induced skipping of exon 5 enhanced skipping of exon 6. Additional downstream (exon 7) or upstream (exon 4) skipping events were excluded (data not shown). We hypothesize that, in wild-type PKLR pre-mRNA, efficient inclusion of exon 6 depends on the presence in the primary transcript of, yet undefined, splice enhancing elements in exon 5. Likely candidates are exonic splice enhancers (ESEs). ESEs are cis-acting exonic sequence motifs that promote exon definition by serving as RNA binding sites for specific serine/arginine-rich (SR) proteins. The role of ESEs in constitutive splicing is now appreciated and binding of SR proteins to constitutive exons plays an important role in the splicing reaction. ESEs are very common in human genes and might be present in most, if not all, human exons. Interference with the function of exonic cis-elements has been shown to be a common mechanism for inappropriate exon skipping. The effects of ESE-disrupting mutations have, to our knowledge, thus far been limited to the ESE-containing exon. Recently, however, ESEs were found to be capable of activating not only weak splice sites within the same exon, but also weak splice sites of flanking adjacent exons, across a 430 bp intron. These in vitro results, combined with the small size of PKLR intron 5 (134 bp), the weak splice sites flanking exon 6 (3' splice site score intron 5, 82.1; 5' splice site score intron 6, 72.4), and the fact that exon 6 is aberrantly processed in wild-type pre-mRNA (Figure 2A), strengthen our hypothesis regarding the requirement of upstream exonic splice enhancing elements for efficient inclusion of PKLR exon 6. Such a regulatory mechanism may also explain why point mutations in an upstream exon can cause skipping of only the downstream exon. It is noteworthy to realize that the observation of splice site mutations leading to multiple exon skipping can easily been obscured by the frequently applied investigative approaches to study such mutations that involve the use of primers located only in the immediate upstream and downstream exons. Consequently, multiple exon skipping events will go by undetected.

Although not invariant, the guanine at the –1 nt of the 5' splice site is very common in primates (78%). The predominant feature of mutating the –1 nt is skipping of the upstream exon. Normal processing of pre-mRNA may be observed, albeit usually at low levels. Previous studies regarding the c.1436G>A mutation were unable to demonstrate aberrant splicing induced by this mutation. Instead, low levels of normally processed transcript were detected. Our results confirmed the association of the c.1436G>A mutation with a
strong reduction in transcript levels. Furthermore, we demonstrate here that this mutation does effect pre-mRNA processing by the characterization of three aberrant transcripts that lacked exon 10. The majority of 1436A transcripts, however, was normally processed which may be a reflection of the modest decrease in 5’ splice site score of the mutated donor site (71.5), compared to the wild type donor site (83.9). Of interest was the presence of the Δ10,11 transcript. The equal amounts of transcripts lacking exon 11 detected in control and patient’s RNA suggests, however, that simultaneous skipping of exon 10 and 11 in this case more likely reflects ‘leaky splicing’ (see below) that led to skipping of exon 11 on transcripts that had already skipped exon 10 due to the c.1436G>A mutation.

During this study we identified low-abundant alternative transcripts, not associated with mutation. They likely resulted from inefficient processing or ‘leaky splicing’ events as has been reported for other genes.56,57 We report here for the first time alternative processing of wild-type PKLR pre-mRNA. Correct processing of exon 6 and 11 appeared to be inefficient and in both cases the exons were either skipped or alternatively processed at exonic cryptic splice sites. Although such low-abundant transcripts are not likely to result in clinical expression, their characterization may render employable knowledge. For example, the identification of a PKLR splice site mutation (IVS6–2A>T),10 prompted the investigators to analyze the patient’s cDNA. This revealed no abnormalities other than a 67-bp deletion of the 5’ end of exon 11 which, in turn, led the authors to conclude that this aberrant transcript was related to the intron 6 acceptor site mutation.10 Figure 2B clearly shows that the 67-bp deletion from exon 11 was a result of inefficient processing of this exon in wild-type PKLR pre-mRNA. We propose that the actual effect of the IVS6–2A>T mutation has escaped detection and remains to be established.

No functional PK monomers were produced from the patient’s IVS5+1A allele. However, we detected low amounts of PK monomers in nucleated erythroid cells of the patient which is in agreement with the low levels of normally processed 1436A missense mRNA. Hence, the patient can be regarded as pseudo-homozygous for R479H PK. Recently, Valentini et al. characterized recombinant wild type and R479H mutant PK, expressed in Escherichia coli.58 Structural data concerning this mutant showed that the His479 side chain was fully solvent-exposed and that the crystal structure of R479H PK was identical to that of the wild-type (recombinant) protein.58 Thus, structural perturbations induced by the R479H substitution are likely to be limited. Furthermore, kinetic parameters of the catalytically active tetrameric mutant enzyme appeared to be essentially unaffected.58 Altogether, we postulate that the PK-deficient phenotype of the patient is directly related to strongly reduced amounts of R479H PK tetramers. The only discrepancy comes from the patient’s heat-sensitivity (see ‘Subjects’) which suggests decreased stability of the R479H PK tetramer during febrile episodes or even
Aberrant processing of PKLR pre-mRNA prolonged exposure to sunlight. This is in agreement with reduced thermal stability of the patient’s PK \textit{in vitro} (Figure 1) but contrasts with the nearly unaffected thermal stability of recombinant R479H PK,\textsuperscript{58} thereby underscoring the differences in fate and function of mutant enzymes \textit{in vivo} and \textit{in vitro}.\textsuperscript{59} Insight into the basic mechanisms of PKLR pre-mRNA processing and the factors that influence splice site selection may eventually result in therapeutic possibilities designed to target the splicing pathway. Such approaches have been shown to be feasible by the successful treatment of nucleated erythroid cells from thalassemic patients using antisense oligonucleotides that restored correct splicing\textsuperscript{60,61} and may well be applied to other human diseases like PK deficiency.

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\section*{References}


20. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair
34. Cohen-Solal M, Préhu C, Wajcman H, et al. A new sickle cell disease phenotype associating Hb S trait, severe pyruvate kinase deficiency (PK Conakry), and an α2


Aberrant processing of PKLR pre-mRNA


54. Lam BJ, Hertel KJ. A general role for splicing enhancers in exon definition. RNA. 2002;8:1233-1241.


