Splicing of phenylalanine hydroxylase (PAH) exon 11 is vulnerable: Molecular pathology of mutations in PAH exon 11

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A B S T R A C T
In about 20–30% of phenylketonuria (PKU) patients, phenylalanine [Phe] levels can be controlled by cofactor 6R-tetrahydrobiopterin (BH4) administration. The phenylalanine hydroxylase (PAH) genotype has a predictive value concerning BH4-response and therefore a correct assessment of the mutation molecular pathology is important. Mutations that disturb the splicing of exons (e.g. interplay between splice site strength and regulatory sequences like exon splicing enhancers (ESEs)/exon splicing silencers (ESSs)) may cause different severity of PKU. In this study, we identified PAH exon 11 as a vulnerable exon and used patient derived lymphoblast cell lines and PAH minigenes to study the molecular defect that impacted pre-mRNA processing. We showed that the c.1144T>C and c.1066-3C>T mutations cause exon 11 skipping, while the c.1139C>T mutation is neutral or slightly beneficial. The c.1144T>C mutation resides in a putative splicing enhancer motif and binding by splicing factors SF2/ASF, SRp20 and SRp40 is disturbed. Additional mutations in potential splicing factor binding sites contributed to elucidate the pathogenesis of mutations in PAH exon 11. We suggest that PAH exon 11 is vulnerable due to a weak 3’ splice site and that this makes exon 11 inclusion dependent on an ESE spanning position c.1144. Importantly, this implies that other mutations in exon 11 may affect splicing, since splicing is often determined by a fine balance between several positive and negative splicing regulatory elements distributed throughout the exon. Finally, we identified a pseudoexon in intron 11, which would have pathogenic consequences if activated by mutations or improved splicing conditions. Exonic mutations that disrupt splicing are unlikely to facilitate response to BH4 and may lead to inconsistent genotype-phenotype correlations. Therefore, recognizing such mutations enhances our ability to predict the BH4-response.

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1. Introduction

Hyperphenylalaninemia (HPA) is the result of a defect in the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) [1]. The reaction is catalyzed by phenylalanine hydroxylase (PAH, EC 1.14.16.1) requiring the essential cofactor tetrahydrobiopterin (BH4) [2]. In the majority of cases hyperphenylalaninemia (HPA) is caused by mutations in the PAH gene, resulting in different phenotypes classified according to Phe levels in the blood ranging from mild HPA, mild PKU to classic PKU. PKU is a very heterogeneous disease and belongs to the most common inherited diseases in amino acid metabolism [3]. As elevated Phe levels cause severe brain damage, it is compulsory to start treatment as early as possible.

Over 500 mutations have been reported in the coding sequence as well as in the intervening sequence of the PAH gene (Online database, http://www.pahdb.mcgill.ca/) [4]. More than half of these are classified as missense changes. Several PKU mutations have been shown to affect protein folding, thereby causing accelerated degradation and/or aggregation [5]. The measurement of enzymatic activities in vitro of mutant proteins can generally be used in predicting HPA’s, but it has also been suggested that up to 50% of exonic mutations may perturb pre-mRNA splicing, thereby leading to more deleterious effects on protein function, irrespective of the predicted amino acid change [6].

In 20–30% of PKU patients (all phenotypes), Phe levels may be controlled through BH4 (sapropterin dihydrochloride [7]) therapy [8]. Only
the patient's full genotype determines BH4-responsiveness [9,10], but
genotype–phenotype correlations are not always reliable as discordant
results have been observed between patients with common genotypes
[11]. Exonic mutations that disrupt splicing are unlikely to facilitate
BH4-response and recognizing such mutations enhances our ability to
predict BH4-responsiveness. It is therefore important to correctly assess
the molecular pathology of PAH mutations.

Cis-acting elements such as exon splicing enhancers (ESE) or exon
splicing silencers (ESS) participate in exon recognition in a finely bal-
anced interplay with splice site strengths and this fine balance can be
disturbed through deleterious effects of mutations in these elements.
When bound to ESEs serine/arginine-rich proteins (SR proteins) pro-
mote exon definition by directly recruiting the splicing machinery
through their RS domain and/or by antagonizing the action of nearby
silencer elements [12].

Relatively few studies have investigated mRNA processing defects
owing to exonic sequence variation in the PAH gene. The mutation
c.611A>G, putatively p.Y204C, was investigated for a role in mRNA pro-
cessing when in vitro assessment of the mutant enzyme did not demon-
strate significantly reduced activity and correspond to the phenotype in
the PKU patients [13]. Analysis of the PAH mRNA in a patient lympho-
blast cell line showed that the c.611A>G mutation masquerades as a
missense mutation, but actually creates a new 5′ splice site resulting in
a 96nt deletion at the 3′ end of PAH exon 6. This study, together, with
the finding that a synonymous mutation, c.1197A>T, causes exon 11
skipping instead of being neutral [14] and our recent study of a
c.30C>G synonymous mutation, which creates an ESS with unex-
skipping instead of being neutral [14] and our recent study of a
the phenotype and the possibility of BH4-response. Herein, we established
molecular pathology which ultimately may relate to both the patient's
pre-mRNA processing may be required to determine a mutation's
expected effects on mRNA splicing, shows that more detailed analysis of
PAH pre-mRNA processing may be required to determine a mutation's
molecular pathology which ultimately may relate to both the patient's
phenotype and the possibility of BH4-response. Herein, we established
and validated a PAH exon 11 minigene, which allows testing the impact
of PAH exon 11 missense and splice site mutations on mRNA splicing.
Both natural mutations and several artificial mutations were investigat-
ed to gain insight into the splicing mechanism of PAH exon 11. The
pathology of the two exonic mutations, c.1139C>T and c.1144T>C, was
analyzed by transfection of the minigene reporter, by RNA affinity puri-
ification and results were confirmed by analysis of patient cell lines.

2. Materials and methods

2.1. Patient specimens

Patient samples analyzed in this work were previously reported
among a large cohort of Turkish PKU patients [10]. Table 1 summa-
rizes the genotypes and phenotypes of the 4 patients analyzed in
this study. Patient with a mild HPA, have been identified as BH4-
responsive by the oral loading test.

2.2. Generation of patient cell lines and cell culture

Peripheral blood lymphocytes from PAH deficient patients were
transformed with Epstein–Barr virus [16] to generate lymphoblast
cell lines. Lymphoblast cell lines and Chang human liver epithelial
cells were cultured in RPMI 1640 (Sigma Aldrich, St. Louis, MO,
USA) and 5% fetal calf serum. COS-1 cells were cultured in Dulbecco's
Modified Eagle's Medium (DMEM, Sigma) with 10% fetal calf serum.

To perform nonsense-mediated mRNA decay analysis, lympho-
blast cells were cultured overnight in presence of 10 ng cyclohexi-
mide (Sigma) prior to mRNA extraction.

2.3. Minigene construction

Initially, a 1946-bp fragment of human PAH including exon 10, intron
10, exon 11, intron 11 reduced to 988 bp and exon 12 was synthesized by
GenScript (NJ, USA). A start codon was added to exon 10 and a KpnI site
was removed from intron 10 to facilitate cloning. In addition, intron 11
was extended by inserting at an EcoRI site a PCR amplified 963 bp frag-
ment amplified using AccuPrime Pfx SuperMix (Invitrogen). Five unique
minigene constructs were prepared: WT, c.1139C>T, c.1144T>C, c.1066-
3C>T and c.1197A>T. The minigenes were cloned into the polylinker of
cDNA3.1 + vector (Invitrogen) by using KpnI and XhoI restriction en-
zeymes. The correct insertion was verified by sequencing with BigDye
Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism
3100 Sequencer.

Other mutations used for the characterization of eventual splicing
regulatory elements caused by the mutations of interest were either
introduced by site-directed mutagenesis with QuikChange XL II kit
(Agilent Technologies, CA, USA) or ordered from GenScript. These
nucleotide changes include: c.1139C>A, c.1139C>G, c.1144T>A,
c.1144T>G, c.1169A>G, c. 1146C>A, c.1144C + 1146A, c.1139T>C
and 1144C, c.1144-1146delITC and the insertion of a known wild type
and mutant ESE sequence from the ACADM gene [17].

2.4. Transient transfection experiments

Transient transfection experiments were conducted with FuGENE 6
Transfection reagent (Roche Applied Biosciences) as described [17].
Chang or COS-1 cells were seeded at 2×10^5 (resp. 3×10^5) cells per
35 mm well and transfected with 0.8 µg of minigene construct DNA.
Co-transfections with the vectors for SF2/ASF, SRp40 (generous gifts
from Adrian Krainer, Cold Spring Harbor, NY), hnRNP H (generous gift
from Mark McNally, University of Wisconsin) and hnRNPA1 (generous gift
from Benoit Chabot, University of Sherbrooke, Canada) were per-
formed as described [17]. After 48 h, cells were harvested in 300 µl
RLT buffer and stored at −80 °C for either later processing or RNA ex-
traction was continued according to the manufacturer's protocol of
Qiagen RNA blood mini kit.

2.5. Analysis of RNA processing

Analyses of illegitimate PAH transcripts from patient lymphoblasts
were performed according to previously described methods [18].
After harvesting, total RNA was extracted from patient lymphoblast
cell lines using Qiagen RNA blood mini kit. One microgram of isolated
RNA was reverse-transcribed with iScript™ cDNA Synthesis kit
(BioRad, CA, USA) containing a mix of Oligo (dT) and random
hexamer primers. The cDNA from patient lymphoblasts covering
exons 9–13 was PCR-amplified using primers PAHX9fwd (5′-TG-
GCTTGCCTCTCTGGGTGC-3′) and PAHDrev (5′-GACCATATTCTGGC-
ATGGCTTAA-3′). Amplification from PAH was performed with a
minigene-specific primer pair to exclude detection of endogenous PAH:
forward primer 11s2 (5′-GGTAACGAGCGCCAACTATGTCTTCG-3′) and
reverse 11as (5′-AGACTCGAGGTAGTCTTATATTT-3′). The amplifi-
cation products were analyzed by 1% agarose gel electrophoresis. PCR
products were gel extracted, purified and sequenced using the BigDye
Terminator Cycle sequencing v1.1 (Applied Biosystems) on an ABI Prism
3130xl Sequencer.

Table 1

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<tr>
<th>Genotype</th>
<th>Variation</th>
<th>Phenotype</th>
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<tr>
<td>1. c.1066-3C&gt;T/c.1066-3C&gt;T</td>
<td>c.1139C&gt;T/c.1144T&gt;C</td>
<td>Mild HPA</td>
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<td>2. c.1106-3C&gt;T/c.1208C&gt;T</td>
<td>IVS10-3C&gt;T</td>
<td>Mild HPA</td>
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<tr>
<td>3. c.1144T&gt;C/c.1144T&gt;C</td>
<td>p.F382L/p.F382L</td>
<td>Mild HPA</td>
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2.6. RNA oligonucleotide affinity purification

The affinity purification of RNA binding proteins utilized 3'-biotin-labeled RNA oligonucleotides as described (DNA Technology Denmark) [15]. For each purification 100 pmol of RNA oligonucleotide was coupled to 100 μl of streptavidin-coupled magnetic beads (Invitrogen) and incubated with Hela nuclear extract (Cilibioch S.A., Belgium) [17]. After washing, bound proteins were investigated by western blotting using a monoclonal mouse antibody SF2/ASF (AK96 from Zymed Laboratories (Invitrogen)), SRp40, SRp20, hnRNPH or hnRNPA1 (sc-33418, sc-13510, sc-10042 and sc-10029 — Santa Cruz Biotechnology, Santa Cruz, CA).

2.7. Expression of PAH proteins and activity assay

PAH activities were determined using a novel mass spectrometry method for quantification of Phe and Tyr in cell lysates [19]. Mutations in the human PAH cDNA sequence in pcMV-FLAG-PAH were introduced by site-directed mutagenesis using QuikChange XL II kit from Agilent Technologies (Santa Clara, CA, USA) and confirmed by DNA sequence analysis. Expression plasmids were transfected into COS-1 cells using FuGENE 6 (Roche Applied Biosciences) and harvested after 48 h.

Cell lysates were prepared and enzyme activity was determined using previously described methods. The amount of Tyr produced was determined by LC ESI-MSMS.

Protein concentrations of all sample types were determined using Pyrogallol Red protein dye binding assay [20]. Specific PAH activities are expressed in μM/mg total protein, with μM equal to nmol Tyr produced per min.

3. Results

3.1. PAH exon 11 is flanked by a weak 3′ splice site

We initially assessed the strength of all splice sites in the PAH gene to identify exons that are weakly defined and thus vulnerable to mutations affecting splicing regulatory elements [12]. Table 2A displays all splice sites of the 13 exons of the PAH gene and the calculated strengths using the maximum entropy model (MaxEntScan http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) which showed that exon 11 is vulnerable with a weak 3′ splice site. The c.1066-3C>T mutation, which is located in the 3′ splice site, was previously been demonstrated to result in exon 11 skipping [18], although this change only modestly weakens the score (3.16 to 2.11, Table 2B) and the mutant splice site retains within the splice site consensus. Moreover, c.1197A>T, an exonic mutation that only decreases the MaxEnt splice site ME score 3′ from 9.16 to 7.65 has also been reported to cause exon 11 skipping [14]. Together these data indicated that exon 11 is weakly defined and is likely to be dependent on exonic splicing enhancer sequences. We therefore hypothesized that mutations in exon 11 might disrupt the fine balance between exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) and thus result in exon 11 skipping. We decided to investigate some of the missense mutations reported to be BH4 responsive.

3.2. Aberrant PAH splicing in patient lymphoblast cell lines

PAH pre-mRNA processing was investigated using patient derived lymphoblast cell lines. RT-PCR was used to amplify a fragment from PAH exon 9 to the end of the coding region. In patients 1, 2 and 3 the expected 463 bp fragment was observed along with a 329 bp fragment (Fig. 1A). This alternative product is most pronounced in patient 1 who is homozygous for the c.1066-3C>T mutation. DNA sequence analysis of the purified 329 bp band showed that exon 11 was missing. This analysis indicates that the c.1066-3C>T and c.1144T>C mutant alleles cause exon 11 skipping in patient cells.

Skipping of exon 11 results in deletion of 134 bp from the PAH mRNA leading to a shifted reading frame and replacement of the 97 C-terminal codons with 21 missense codons followed by three in frame premature stop codons in exon 12, which is the penultimate exon of the PAH gene. The first premature stop codon in exon 11 skipped PAH mRNA is located 53 nucleotides upstream of the last exon–exon junction. This corresponds to the required minimal distance (50–55 nt) upstream of the last exon, which typically triggers degradation of the premature stop codon containing mRNA by the Nonsense Mediated Decay (NMD) system [21]. There are, however, examples where NMD is triggered by premature stop codons located even closer to the last exon–exon junction [21]. To determine whether NMD is degrading the aberrantly spliced PAH mRNA, we treated patient cells overnight with cycloheximide (CHX), to block NMD. Assessment of PAH mRNA following CHX treatment showed a dramatic increase in the 329 bp cDNA product lacking exon 11 which demonstrates that the aberrantly spliced PAH mRNA is degraded by NMD (Fig. 1B). The presence of the full length product confirms that the c.1139C>T mutant allele does not lead to exon 11 skipping.

The minor DNA species migrating close to the wild type transcript is a heteroduplex formed between cDNA strands of the wild type and exon 11 deleted mutant. This was confirmed by sequencing.

3.3. Minigene analysis confirms results from patients’ lymphoblasts

To further elucidate the molecular mechanism of aberrant PAH exon 11 splicing and to enable testing of exon 11 mutations where cell lines are not available, a PAH minigene was constructed. The minigene harbors exons 10, 11 and 12, with intron 10 and a shortened intron 11 (Fig. 2A). We tested the exon 11 minigens c.1066-3C>T, c.1139C>T, c.1144T>C, in addition to c.1197A>T that was previously reported to cause exon 11 skipping [14,22] and c.1169A>G which is a prevalent BH4 responsive allele in the Turkish population [10]. Analysis of COS-1 cells transfected with the minigenes shows that the mutations c.1197A>T, c.1144T>C and c.1066-3C>T lead to exon 11 skipping, whereas the c.1169A>G mutation is neutral and the c.1139C>T mutation seems to have a slightly positive effect on exon 11 inclusion (Fig. 2B). These results are consistent with our analysis of the patient cells and underline that the splicing of PAH

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<th>Table 2A PAH splice site strength.</th>
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5′ splice site 9mer, 3′ splice site 23mer, maximum entropy scores from http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html.

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<th>Table 2B Maximum entropy (ME) score variation for 3′ splice site of PAH exon 11 upon c.1066-3C&gt;T mutation.</th>
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transcripts from our minigene mimics that of the endogenous PAH gene. In addition, the minigene analysis confirms that the c.1197A>T, c.1144T>C and c.1066-3C>T mutations compromise splicing and that the observed mis-splicing in patient cells does not result from a linked mutation located outside the sequenced region of the gene or from the other mutant allele present in the compound heterozygous patients.

The minigene analysis also indicates that splicing of wild type PAH exon 11 results in small amounts of exon 11 skipping, consistent with the fact that it is weakly defined and dependent on ESE's [12].

As PAH is primarily expressed in the liver, we also transfected Chang cells with PAH minigene constructs. A higher degree of mis-splicing of the c.1144T>C minigene (Fig. 2C) was observed, but the

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### Fig. 1. PAH pre-mRNA splicing analysis in patient lymphoblast cells.


The cells were passaged the day before harvesting and supplemented with CHX.

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### Fig. 2. PAH pre-mRNA splicing in COS-1 and Chang cells transfected with PAH minigenes.

(A) Schematic description of the PAH minigene harboring exons 10 to 12. Start and stop codons were added to complete the reading frame. (B) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1139C>T, c.1144T>C, c.1066-3C>T, c.1169A>G and c.1197A>T mutations. (C) Analysis of transfection in Chang liver cells with minigenes harboring WT, c.1139C>T, c.1144T>C and c.1066-3C>T mutations. (D) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1144T>C and c.1066-3C>T mutations with and without an optimized 3' splice site. Amplification of PAH exons 10–12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.
degree of mis-splicing was inconsistent between separate transfection experiments. As such, COS-1 cells were used in subsequent experiments. The results from Chang cells indicated that the c.1144T>C mutation may result in different degrees of mis-splicing varying from complete skipping to the same degree of skipping as observed in the COS-1 cells. This also illustrates that the degree of mis-splicing may vary depending on the cell type.

Heteroduplex formation of wild type and exon 11 deleted mutant cDNAs was confirmed by sequencing in both cell lines.

3.4. Correction of the 3′ splice site of exon 11 by mutagenesis

To investigate the contribution of the weak 3′ splice site to exon skipping in PAH exon 11, the 3′ splice site was optimized. The four guanosine nucleotides (c.1066-8 to c.1066-11) that interrupt the polypyrimidine tract were replaced with thymidines. This substitution increased the maximum entropy score from a weak 3.16 to a robust 9.58. In the wild type minigene construct, improved exon 11 inclusion was observed (Fig. 2D). Moreover, when the optimized polypyrimidine tract is included in the c.1066-3C>T and c.1144T>C constructs, aberrant splicing is no longer observed. At position −3 upstream of the 3′ splice site, a thymidine base is considered to match the consensus motif. PAH exon 11 skipping owing to the c.1066-3C>T mutation is an indication to the weakness of the splice site owing to guanosine bases at −8 to −11. Amelioration of mis-splicing owing to c.1144T>C with the corrected polypyrimidine tract suggests that the c.1144T>C mutation disrupts the function of an ESE, which is required for recognition of the weak 3′ splice site.

3.5. The c.1144T>C mutation disrupts an ESE, which is required for inclusion of PAH exon 11

The c.1144T>C mutation causes exon 11 skipping. To elucidate whether the mechanism underlying pre-mRNA mis-splicing is owing to the disruption of an ESE or creation of an ESS in the minigene constructs, various proportions of the sequence surrounding position c.1144 were deleted (Fig. 3A). Minigenes with a 9 bp deletion (c.1141 to c.1149) showed complete exon 11 skipping, whereas the 6 bp deletion (c.1144 to c.1149) has a slightly less dramatic effect, and the 3 bp deletion (c.1144 to 1146) has a minor deleterious effect on exon 11 inclusion (Fig. 3B). This shows that an ESE element is located in this region of exon 11, but it may also suggest that this ESE is complex and may bind more than one splicing factor, since 6 bp and 9 bp had to be deleted in order to completely abolish splicing. Alternatively, the 3 bp deletion recreates an ESE sequence (see below). To further characterize this putative ESE, we analyzed the wild type and mutant sequences with the Human Splicing Finder (HSF) program (http://www.umd.be/HSF/HSF.html) and made site specific mutations in the region. The HSF program suggested that the c.1144T>C mutation disrupts a TTCCAG(C) ESE, which could be a binding site for the splicing stimulatory factor SRp40 (SRSF5). Moreover, the HSF program also suggested that a TTCCAG motif, which is a putative ESE [23] is disrupted both by the c.1144T>C mutation and the c.1146C>A mutation, but that the c.1144T>C/c.1146C>A double mutation creates a new ESE AGCT. These predictions were consistent with our minigene analysis (Fig. 3B) and suggest that the TTCCAG sequence functions as an ESE, perhaps by binding of SRp40 (SRSF5) and/or other factors. The HSF analysis also suggested that although the 3 bp deletion removes part of

Fig. 3. PAH pre-mRNA splicing in COS-1 cells transfected with PAH minigenes. (A) Sequences showing the precise location of the deletions and the inserted MCAD ESE. (B) Analysis of COS-1 cells transfected with minigenes harboring WT, 3 bp, 6 bp and 9 bp deletions and analysis of COS-1 cells transfected with minigenes harboring WT, c.1144T>C, c.1146C>A and c.1144T>C+c.1146C>A mutations. (C) Analysis of COS-1 cells transfected with minigenes harboring WT and all possible nucleotide variations at positions c.1139 and c.1144. (D) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1139C>T, c.1144T>C, c.1139C>G and substitutions with wild type and mutant MCAD ESE sequences. Amplification of PAH exons 10–12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.
this TTCCAG motif it also recreates several potential ESE sequences, explaining the lack of a drastic effect from this mutation. Consistent with our transfection results HSF analysis suggested a deleterious impact of both the 6 bp and the 9 bp deletions. Moreover we also mutated positions c.1139 and c.1144 to all possible substitutions and also created a double mutation. Interestingly, this showed that c.1139C>G and c.1144T>G both cause skipping and that introduction of the c.1139C>T mutation together with the c.1144T>C mutation compensates for the splicing defect (Figs. 3C, D). These data were not consistent with the HSF analysis predictions and may contradict that the ESE functions by binding of SRp40 and they suggest that the c.1139C>T mutation has a positive effect on splicing.

Finally, we substituted the PAH ESE region with a functional ESE from the ACADM gene [17], which has been demonstrated to function in several other genes [17,24]. Surprisingly, the wild type MCAD ESE could only partly substitute the PAH ESE sequence. As expected the mutated MCAD ESE was also non-functional in the PAH context (Figs. 3A, D).

Taken together our data are consistent with a model where the c.1144T>C mutation causes exon 11 skipping by disrupting the function of an ESE, which is required for recognition of the weak 3′ splice site.

3.6. Analysis of the PAH exon 11 ESE by RNA affinity purification

To identify proteins that bind the PAH exon 11 ESE, RNA affinity purification was performed. RNA oligonucleotides containing c.1139C>T and c.1144T>C mutant sequences were incubated in HeLa cell nuclear extracts (Fig. 4). Western blot analysis showed strong binding of SF2/ASF (SRSF1) to the WT sequence, which was abolished by both the c.1139C>T and c.1144T>C mutant sequences. While factor binding was less robust for SRp20 (SRSF2) and SRp40 (SRSF5) the same pattern was observed. No difference was observed for binding of hnRNPA1. This may indicate that c.1144T>C causes exon 11 skipping by disrupting binding of an SR protein to an ESE motif that includes c.1144. However, it is not clear, if all three SR proteins bind an identical sequence motif or if they bind overlapping motifs, which are all disrupted by the c.1144T>C mutation. Alternatively one SR protein may bind the RNA while the others are associated via protein–protein interaction through their RS-domains. Moreover, it is unclear why binding of SR proteins is disrupted by c.1139C>T, as this mutation is shown to improve splicing in our minigene studies (see Figs. 2 and 3). This could, however, be due to steric hindrance by binding of another protein to the new ESE created by the c.1139C>T mutation. In support for this notion, we observed increased binding of hnRNPH to the c.1139C>T sequence and this would block binding of the SR proteins to the flanking ESE harboring position c.1144. The hnRNPH protein is, however, typically a negative regulator of splicing [15].

Further analysis is required in order to identify conclusively the involved splicing regulatory proteins that bind the exon 11 ESE.

3.7. Co-transfection with splicing factors leads to change in splicing pattern

Because the RNA affinity studies had suggested a possible role for SF2/ASF (SRSF1) and SRp40 (SRSF5) in binding to the ESE, we performed co-transfection of the PAH minigenes with expression plasmids for SF2/ASF (SRSF1) or SRp40 (SRSF5) human proteins to see if the mutant ESE could be compensated by increasing the amounts of these SR proteins. However, instead of correcting splicing, over-expression of SF2/ASF (SRSF1) resulted in strong activation of a previously unknown pseudoexon (exon 11a) comprising 286 bp of intron 11 and severely reduced inclusion of exon 11 (Figs. 5A, B and Supplementary Figs. S1, S2). The c.1139C>T mutant minigene had slightly less pseudoexon inclusion, consistent with the fact that this mutation results in improved splicing, possibly because it creates a new ESE, which results in stronger definition of exon 11. In line with this, pseudoexon inclusion in response to SF2/ASF (SRSF1) overexpression was nearly abolished when the weak 3′ splice site is improved, showing that this pseudoexon activation is only possible because exon 11 is weakly defined (Fig. 5C).

Similar results as above were obtained from co-transfecting the different minigenes with SRp40 (SRSF5) (Supplementary Fig. S1). Co-transfection of hnRNPA1 and hnRNPH, two negative regulators of splicing, did not result in changes in splicing pattern or splicing efficiencies (data not shown).

When patient cells are treated with cycloheximide, the new exon 11a can be amplified from the patient’s cDNA using an exon 11a specific primer (Supplementary Fig. 2). This shows that the pseudoexon inclusion is not merely an artifact produced only from the minigenes, but that some level of pseudoexon inclusion is possible from the endogenous PAH gene, although the relevance of this is unclear. The pseudoexon could have deleterious effects if it is activated by mutations or by improved splicing conditions.

3.8. Enzyme activities of c.1139C>T and c.1144T>C are reduced compared to wild type PAH

The expression of c.1139C>T (p.T380M) and c.1144T>C (p.F382L) mutant proteins showed reduced activities compared to wild type PAH protein (Supplementary Fig. S3). Residual activity for mutant p.T380M was found to be 38% of wild type activity, whereas p.F382L activity was lower with 18%. Patients with these mutations exhibit a mild phenotype, which is in accordance with residual PAH activity of 25%.

The p.F382L mutant has previously been expressed in a eukaryotic expression system with a residual activity of 56%. But in this case, the amino acid change was caused by c.1146G [25].

4. Discussion

Correlation between genotype and disease phenotype is fundamental to inform about treatment in inherited diseases. The utility of PAH gene...
exon 11 skipping if NMD is not blocked prior to analysis. In order to cell lines. Similar analyses of patient cells may therefore easily overlook information of the degree of exon 11 skipping when analyzing patient derived exons. Moreover, we demonstrate that NMD may lead to an underestimation of splice site strength may help to identify vulnerable our hypothesis that exon 11 is vulnerable and exempli

Fig. 5. SF2/ASF overexpression in COS-1 cells transfected with PAH minigenes leads to pseudoexon activation. (A) Schematic overview of PAH splicing showing inclusion of a pseudoexon sequence from intron 11. (B) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1139C>T, c.1144T>C, c.1066-3C>T and c.1169A>G mutations with and without co-transfection of a vector overexpressing SF2/ASF. (C) Analysis of COS-1 cells transfected with minigenes harboring WT, c.1144T>C and c.1066-3C>T mutations with and without and optimized 3’ splice site and co-transfection of a vector overexpressing SF2/ASF. Amplification of PAH exons 10–12 was done with minigene-specific primers. Transfection experiments were performed as at least two independent transfections.

genotyping is increasingly relevant as efficient newborn screening is a facilitator of patients having their first clinic visit often within the first week of life. As Phe levels in early identified patients will not have reached peak concentrations, discerning disease phenotype often relies on the PAH genotype. The PAH genotype may also inform on the utility of BH4 therapy. Furthermore, in vitro biochemical characterization of missense mutations may provide clues as to the efficacy of BH4. However, in recent years it has become increasingly clear that a so called “splicing code” is also in operation and mutations in the coding sequence may affect pre-mRNA processing and thus overrule what can be predicted based on assumed amino acid substitutions. The splicing code is poorly defined, but it is clear that not all exons are equally subject to aberrant splicing by mutations affecting cis-acting splicing regulatory elements. The so-called “weak exons” may often be on the verge of not being recognized, whereas the splicing machinery easily recognizes other well-defined exons. Since the primary determinants for exon definition is the strength of their flanking splice sites, we first evaluated the splice sites of all exons of the PAH gene. From this analysis exon 11 clearly stood out by having the weakest 3’ splice site of all exons of the PAH gene, and we therefore hypothesized that exon 11 could be a vulnerable exon to aberrant mRNA processing. This is further corroborated by the fact that mutations that only very modestly affected splice site strength had been reported to cause aberrant splicing of exon 11 [13,18]. Consequently, we investigated in more detail how exonic mutations may affect PAH exon 11 splicing.

Analysis of patient cells showed that both c.1066-3C>T and a missense mutation, c.1144T>C cause exon 11 skipping. This corroborates our hypothesis that exon 11 is vulnerable and exemplifies that simple comparison of splice site strength may help to identify vulnerable exons. Moreover, we demonstrate that NMD may lead to an underestimation of the degree of exon 11 skipping when analyzing patient derived cell lines. Similar analyses of patient cells may therefore easily overlook exon 11 skipping if NMD is not blocked prior to analysis. In order to enable investigation of patient mutations without the need of obtaining cells and to enable analysis of exon 11 splicing in more detail we established a minigene, which closely mimics the endogenous PAH gene. Minigenes confirmed that the c.1144T>C mutation affects splicing; moreover our testing of other artificial mutations (like c.1139C>G, c.1144T>G and c.1146C>A) shows that other exonic variants lead to exon 11 skipping. Minigene assessment demonstrated that the patient mutations c.1139C>T and c.1169A>G do not affect splicing. Our mutagenesis of the minigene showed very clearly, that it is the weak 3’ splice site that is responsible for the vulnerability of exon 11, since increasing the splice site strength by optimization of the polypyrimidine tract neutralized the effect of all the splicing mutations.

The c.1066-3C>T mutation has been first reported by Abadie et al. [18]. The PAH amplification by RT-PCR from lymphoblast cells of their patient revealed two different transcripts, corresponding to normal spliced PAH and exon 11 skipped PAH transcripts. However, the patient was compound heterozygous for p.R261Q/c.1066-3C>T and the normal PAH transcript may exclusively result from p.R261Q allele. A BH4-responsive, homozygous c.1066-3C>T patient has been reported by Desviat et al. [26]. We were not able to detect normal spliced PAH transcript in our homozygous patient for c.1066-3C>T. In addition, our patient was not responding to BH4 in an 8 h test. To our knowledge, PAH gene transcript amplification from a homozygous patient was not reported before. Lymphoblasts are not the primary tissue for PAH protein expression, but we speculate that very small amounts of normal spliced PAH transcript could be present in the liver. Faint bands observed in our transfected COS-1 and Chang cells indicate that low amounts of normally spliced PAH can be produced from the c.1066-3C>T. Even low amounts of PAH transcript would be stabilized by BH4 and result in lowered Phe levels upon BH4 treatment.

Because testing mutations using minigenes and/or patient cells is cumbersome there is a growing need for computer-based predictions of possible deleterious effects on splicing. The Human Splicing Finder
Homology: TTCCAG ESE motif is conserved in all species.

Consensus ESE
Human: ACCTGCACAGATTTCCACCC
Dog: NC_006597:
Mouse: NC_000076:
Boar Taurus (NW_001103925.1):
Pan troglodytes (NW_003457733.1):
Ret: NW_007742.3,
Locowood Africana (XM_004046331.1):
Human CYP2B6:

TTCCAG

Fig. 6. Alignment of PAH exon 11 sequences from different species. Alignment of sequences from different species shows that the TTCCAG (c.1144–c.1148) sequence is conserved in different species and that mutation (underlined G) in this element causes aberrant splicing in the human CYP2B6 gene.

program simultaneously analyzes wild type and mutant sequences for changes in splicing regulatory sequences and theoretical binding motifs for splicing factors. Although, such programs may provide useful hints to potential regulatory sequence motifs, which are changed by a mutation, the present study demonstrates that predictions cannot be used un-critically and that the different algorithms may produce contradictory predictions. In the present study the c.1139C>T mutation is predicted to be deleterious, instead it seems to improve splicing, which demonstrates why functional testing is still warranted. Alternatively, HSF predictions concerning the c.1144T>C mutation and in particular identifying TTCCAG (c.1144–c.1148) as an ESE may be correct. The importance of this sequence is further demonstrated by the fact that it is conserved in species ranging from elephants to mice (Fig 6). Moreover, comparison with our database of other exonic splicing mutations (unpublished observation)

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