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Abstract

3-Methylcrotonyl-CoA carboxylase (MCC) deficiency is an autosomal recessive disorder of leucine catabolism. MCC is a heteromeric mitochondrial enzyme composed of biotin-containing alpha (MCCA) and smaller beta (MCCB) subunits encoded by MCCA and MCCB, respectively. We report studies of the c.1054G-->A mutation in exon 11 of MCCB detected in the homozygous state in a patient with MCC deficiency. Sequence analysis of MCCB cDNA revealed two overlapping transcripts, one containing the normal 73 bp of exon 11 including the missense mutation c.1054G-->A (p.G352R), the other with exon 11 replaced by a 64-bp sequence from intron 10 (cryptic exon 10a) that maintains the reading frame and is flanked by acceptable splice consensus sites. In expression studies, we show that both transcripts lack detectable MCC activity. Western blot analysis showed slightly reduced levels of MCCB using the transcript containing the missense mutation, whereas no MCCB was detected with the transcript containing the cryptic exon 10a. Analysis of the region harboring the mutation revealed that the c.1054G-->A mutation is located in an exon splice enhancer sequence. Using MCCB minigene constructs to transfect MCCB-deficient fibroblasts, we demonstrate that the reduction in utilization of exon 11 associated with the c.1054G-->A mutation is due to alteration of this exon splice enhancer. Further, we show that optimization of the weak splice donor site of exon 11 corrects the splicing defect. To our knowledge, this is the first demonstration of a point mutation disrupting an exon splice enhancer that causes exon skipping along with utilization of a cryptic exon.
CRYPTIC EXON ACTIVATION BY DISRUPTION OF AN EXON SPLICE ENHANCER: A NOVEL MECHANISM CAUSING 3-METHYLCROTONYL-CoA CARBOXYLASE DEFICIENCY*
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3-Methylcrotonyl-CoA carboxylase (MCC) deficiency is an autosomal recessive disorder of leucine catabolism. MCC is a heteromeric mitochondrial enzyme composed of biotin-containing α (MCCA) and smaller β (MCCB) subunits encoded by MCCA and MCCB, respectively. We report studies of the c.1054G>A mutation in exon 11 of MCCB detected in the homozygous state in a patient with MCC deficiency. Sequence analysis of MCCB cDNA revealed two overlapping transcripts, one containing the normal 73 bp of exon 11 including the missense mutation c.1054G>A (p.G352R), the other with exon 11 replaced by a 64 bp sequence from intron 10 (cryptic exon 10a) that maintains the reading frame and is flanked by acceptable splice consensus sites. In expression studies we show that both transcripts lack detectable MCC activity. Western blot analysis showed slightly reduced levels of MCCB using the transcript containing the missense mutation while no MCCB was detected with the transcript containing the cryptic exon 10a. Analysis of the region harboring the mutation revealed that the c.1054G>A mutation is located in an exon splice enhancer sequence. Using MCCB-minigene constructs to transfect MCCB-deficient fibroblasts we demonstrate that the reduction in utilization of exon 11 associated with the c.1054G>A mutation is due to alteration of this exon splice enhancer. Further, we show that optimization of the weak splice donor site of exon 11 corrects the splicing defect. To our knowledge this is the first demonstration of a point mutation disrupting an exon splice enhancer that causes exon skipping along with utilization of a cryptic exon.

Accurate and efficient removal of introns from pre-mRNA is essential for gene expression. The information present in the consensus splice site signals – the 5’ splice site, branch site and 3’ splice site – is necessary but not always sufficient to define exon-intron boundaries (1,2). On average, these signals appear to provide about half of the information required for exon and intron recognition in human transcripts (3). Sequences flanked by consensus splice site sequences but not known to be retained in mature transcripts are common in introns; some may be “cryptic exons” that for unknown reasons are not normally included in mature mRNAs; others may be “true” exons that have not been recognized because they are alternatively spliced in some developmental or tissue-specific fashion (1,4,5).

Additional cis-acting sequence elements that function as splicing enhancers and silencers exist in the genome (6). Exon splice enhancers (ESEs)1 are short sequences within exons that augment exon recognition and the control of alternative splicing (1). ESEs bind SR proteins, a family of essential splicing proteins which activate splicing by defining exons and recruiting the splicing machinery to the adjacent intronic splice consensus sequence (6-8). ESEs appear to be common and are present in most, if not all exons including constitutive exons (6,9). Unlike transcriptional enhancers, ESEs function in a strongly position-dependent manner, enhancing splicing when present downstream of a 3’ splice site or upstream of a 5’ splice site. Other cis-acting sequences, often in introns, repress exon recognition (6,10). In some instances ESEs appear to compensate for “weak” 5’ or 3’ splice signals in introns. Strengthening of the splice consensus sites of an enhancer-dependent exon by site directed

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mutagenesis may eliminate dependence on the enhancer (8,10).

Single nucleotide substitutions in the coding regions of genes are the most commonly recognized type of mutation underlying inherited human diseases (11). The molecular pathophysiology of nonsense mutations results from a combination of premature termination of translation and nonsense-mediated mRNA decay (NMD) (12,13). The NMD pathway is activated by nonsense mutations (or frame shifting deletions or insertions) that occur 5' of the last exon-intron junction and minimizes the potential for problems caused by truncated proteins by reducing the abundance of the abnormal transcript (12,13). The deleterious effects of missense mutations are usually attributed to their effects on protein stability, folding or function (14) while synonymous mutations are often assumed to have no pathophysiologic consequences. Recently, our understanding of the potential pathological effects of single nucleotide substitutions has been expanded to include alterations that inactivate ESEs causing exon skipping (10,15-17). The importance of considering the possibility of RNA-processing phenotypes in the analysis of the consequences of mutations is emphasized by the growing list of exonic variations that alter RNA-processing and thereby cause or modify disease (see list of 34 “missense” and 26 “silent” point mutations causing exon skipping reviewed in (6)).

3-methylcrotonyl-CoA carboxylase (MCC, EC 6.4.1.4) is a heteromeric mitochondrial enzyme composed of biotin-containing α subunits and smaller β subunits, encoded by MCCα (MCCC1; MIM 609010) and MCCβ (MCCC2; MIM 609014), respectively (18). Mutations in these genes can cause isolated MCC deficiency (MIM 210200 and 210210), a disorder of leucine catabolism inherited as an autosomal recessive trait with a variable phenotype that ranges from neonatal onset with severe neurological involvement to asymptomatic adults (11,18,19). Introduction of tandem mass spectrometry to newborn screening has resulted in a large increase in the number of inborn errors that can be detected, including several amino acidemias and organic acidurias. This technique has shown MCC deficiency to be one of the more frequently detected organic acidurias with an overall frequency of approximately 1 in 50,000 and a mainly mild phenotype (19,20).

Here we report detailed studies of a missense mutation (c.1054G>A, p.G352R) in exon 11 of MCCB that disrupts an ESE and causes MCC deficiency by utilization of a cryptic exon instead of the normal MCCB exon 11. The patient in which this mutation was detected (MCC019) is the child of a consanguineous union and presented at 7 months with failure to thrive (19).

**EXPERIMENTAL PROCEDURES**

*Cell cultures and carboxylase assays* - Skin fibroblasts were cultured in Dulbecco’s (transfected cells) or Earl’s minimal essential medium supplemented with 10% fetal calf serum, 2mM L-glutamine and antibiotics. To be able to sequence unstable transcripts nonsense mediated mRNA decay (NMD) was inhibited by adding emetine (100 µg/ml) in the culture medium 10 hours before harvesting the cells (21).

Activities of MCC and propionyl-CoA carboxylase (PCC) were assayed in fibroblast homogenates by measuring the incorporation of 14C-bicarbonate into acid-non-volatile products with established methods (22).

*Mutation analysis by RT-PCR and genomic PCR* - We extracted RNA and genomic DNA from cultured fibroblasts using RNA and DNA isolation kits from Qiagen and performed RT-PCR using 2-5 µg total cellular RNA with the cDNA cycle kit (Invitrogen) following the manufacturers’ instructions. All PCR reactions (50 µl) contained primers (100 ng each), standard PCR buffer (Gibco-BRL), dNTPs (200 µM) and Taq polymerase (2.5 U; Gibco-BRL). The sequences of all primers are listed in supplemental table 1.

Construction of wild type and mutant MCCB expression vectors - To introduce the c.1054G>A mutation and the transcript containing the cryptic exon (exon 10a) instead of exon 11, we amplified cDNA of patient MCC019 with primers DV4498 and DV4503 and subcloned the PCR products into the pTracer-MCCB-wild type construct (18) by using the BstEI and SfiI sites.

*MCCB minigene constructs* - To examine splicing between exons 9 and 12 of the MCCB gene, we constructed a MCCB minigene by modifying a vector (pBK-RSV-OAT) which had previously been shown to function as an *in vivo*
splicing template (12). First a multiple cloning site was introduced between the \textit{XbaI} and the \textit{PstI} sites of the vector. Then an 8.9 kb fragment containing the genomic DNA sequence between the 3’ end of exon 9 (DV5050) and the 5’ end of exon 12 (DV5049) of MCCB was amplified from genomic DNA of patient MCC019. This sequence was now introduced into the multiple cloning site at the \textit{SpeI} and \textit{SacI} sites (Fig. 3a).

To optimize the donor splice site of MCCB intron 11 (CAGgtataa), we used site directed mutagenesis to change it into the consensus donor sequence (CAGgta) (Fig. 3a). To do this, overlapping forward (DV5111) and reverse (DV5112) primers containing both the c.1054G>A mutation and an optimized intron 11 donor splice site (c.1072+4 taa>agt) were designed. Primer DV5111 was used with a reverse primer in intron 11 (DV5094) producing a 1952 bp genomic fragment containing a \textit{XmaI} site. Primer DV5112 was used with a forward primer in intron 11 (DV5094) producing a 1381 bp genomic fragment containing a \textit{XcaI} site. These two PCRs were combined and amplified by using the outer primers DV5110 and DV5094, producing a 3233 bp genomic fragment containing both the c.1054G>A mutation and an optimized donor splice site of intron 11. This fragment was subloned into pBK-MCCB-G352R using the \textit{XcaI} and \textit{XmaI} sites to obtain the pBK-MCCB-G352R Δss construct.

Similarly, to introduce the wild type sequence, we amplified a 3233 bp genomic fragment from control DNA and subcloned it into the pBK-MCCB-G352R construct.

We verified that all the inserts and constructs had the indicated changes and stayed in frame by sequencing all the exons and flanking intronic sequences.

**Transfections** - For expression studies, the constructs were transiently transfected into an immortalized MCCB-deficient reference cell line by electroporation as described (19). The reference cell line originates from skin fibroblasts of a patient homozygous for MCCB Q43X, shows no detectable MCC activity and does not express detectable MCCB protein. We harvested the cells 48 hrs after transfection and assayed for MCC and PCC activities.

**Protein extraction and Western blot** – Cell lysates were prepared by harvesting confluent fibroblasts from a 75 cm² flask by trypsinization. The cells were washed with 5 ml PBS and centrifuged for 5 minutes at 200x g. The pellet was resuspended in 150 µl protein extraction reagent (M-Per, Piercenet) and homogenized in a mortar. The homogenate was centrifuged for 5 minutes at 14’000x g. The supernatant was transferred into a new tube and the protein concentration was measured using the A280 method (Nanodrop; Witec).

Proteins were separated by SDS-PAGE (50 µg per lane) and detected by immunoblotting. Anti-MCCB (Abnova) was used at a dilution of 1:1000. Signals were detected using the ECL detection kit (Amersham Biosciences). 4 µg of protein was processed similarly as loading control and probed with a monoclonal Anti-β-Actin antibody (Sigma).

**RESULTS**

Patient MCC019 presented with severe isolated MCC deficiency shown by deficient MCC activity (2.2% of median control value) together with normal PCC activity in homogenates of primary fibroblasts (Fig. 1). Virtually no MCCB protein was detected in fibroblasts by Western blotting (Fig. 1).

Sequence analysis of MCCB RT-PCR cDNA of this proband revealed two overlapping transcripts (19): one contained the normal 73 bp of exon 11 with a missense mutation c.1054G>A (p.G352R); in the other, exon 11 was replaced by a sequence of 64 bp from intron 10 (MCCB-exon10a). This cryptic exon maintains the reading frame and is flanked by acceptable splice consensus sites [ctttagAAA…..ATGgtaagt; average score of 86, (23)].

Amplification and sequencing of exon 11 from genomic DNA showed that the patient was homozygous for c.1054G>A (Fig. 2a). However, we considered the possibility that the patient had a deletion of one MCCB allele being hemizygous for MCCB-p.G352R but rejected this possibility because the patient is the product of a consanguineous union and because Southern blot analysis of genomic DNA after digestion with several restriction enzymes showed normal amounts of MCCB and no fragments of abnormal size (not shown). These results indicated that the patient indeed carries the missense mutation on both alleles.
To determine the functional consequences of the mutation, we subcloned both transcripts into pTracer vector and expressed them in an immortalized MCCB-deficient reference cell line (Fig. 1). Transfection of the MCCB wild type construct restored MCC activity from less than 1% of the median control value to 16%. Constructs with MCCB-G352R and MCCB-exon10a showed no rescue of activity, confirming the functional significance of these transcripts.

Western blot of lysates of cells transfected with the MCCB-G352R construct revealed slightly reduced levels of MCCB protein compared to those transfected with the wild type construct. No protein was detectable after transfection with the MCCB-exon10a construct (Fig. 1). This finding, together with Western blot data of untransfected patient fibroblasts, indicates that both variants lead to protein that is less stable than the wild type protein and in the case of MCCB-exon10a is rapidly degraded causing absence of MCC activity.

To visualize and assess the relative abundance of the two transcripts, we used primers in exon 10 (DV5020) and 12 (DV5019) to amplify either a 114 bp fragment (containing exon 11) or a 105 bp fragment (lacking exon 11 but containing the cryptic exon 10a) from cDNA and separated the products on a 12% acrylamide gel (Fig. 2b). From patient RNA the 114 bp and 105 bp fragments were produced in the ratio of 3 to 2, respectively, whereas only the 114 bp fragment was produced from control RNA. Both fragments with similar relative levels were also obtained using RNA extracted from patients’ lymphoblasts, indicating that this result is not specific for fibroblasts (not shown).

The effect of NMD on these steady-state levels of MCCB transcripts was investigated by repeating RT-PCR amplification in emetine treated cells. The most abundant MCCB transcript in RNA from emetine-treated patient cells was one lacking both exon 11 and exon 10a (Fig. 2c). This 41 bp transcript (MCCB-Δ10a,11) results in a frameshift followed by a stop codon. It was detected only after amplification of patient’s RNA after emetine treatment indicating that it is rapidly degraded by NMD under normal circumstances. To determine if the presence of a stronger donor site would reduce dependence on the ESE, we expressed a minigene construct containing the mutated ESE motif in combination with an optimized donor splice site (G352RΔss, Fig. 3a). Optimization of the donor splice site indeed corrected the splicing defect and led to amplification of a normal sized fragment (Fig. 3b).

These results confirm our hypothesis that MCCB c.1054G>A partially disrupts an ESE and that a functional ESE is no longer required when the splice donor site matches the consensus sequence.

**DISCUSSION**

About half of the disease causing nucleotide substitutions have been estimated to lead to aberrant splicing (6,16,27). Typically, the abnormal splicing results from inactivation of a splice site or creation of a new splice site, or interference with regulatory cis-elements, such as splicing enhancers or silencers.
Our study of the c.1054G>A (p.G352R) mutation, detected in the homozygous state in the MCCB gene of patient MCC019, illustrates a mechanism by which a missense mutation partially disrupts an ESE causing utilization of a cryptic exon (exon 10a) in place of exon 11. The c.1054G>A mutation is located in the center of a putative ESE motif responsive to the human SR protein SRp55 (24,25) and affects a highly conserved amino acid. Previous work has shown that ESEs can compensate for “weak” 5’ or 3’ splice signals in exons and that strengthening of the splice consensus sites of an enhancer-dependent exon generally eliminates enhancer dependence (10).

The inclusion of exon 10a in place of exon 11 does not change the reading frame and results in the replacement of the normal 24 amino acid sequence encoded by exon 11 by a novel sequence of 21 amino acid residues. Our expression studies clearly show that MCCB-exon10a produces no detectable MCCB, while some albeit slightly reduced levels of MCCB is produced by MCCB-G352R suggesting that the MCCB-exon10a and to a lesser extent the MCCB-G352R lead to an unstable protein product. This is supported by less MCCB protein in patient fibroblasts compared to control fibroblasts (Fig. 1). The discrepancy between the amount of MCCB protein seen in the fibroblasts transfected with wild type MCCB and the level of MCC activity could probably be explained by the fact that transfection efficiency was quite low (9%, data not shown) while at the same time the few transfected fibroblasts produced massive amounts of MCCB protein due to the CMV promoter present in the construct. The active MCC complex is a (αβ)6 dodecamer and overproduction of one of the subunits could lead to incomplete and therefore inactive or only partly active complexes.

The glycine at position 352 is a highly conserved amino acid and the possibility that this change is a polymorphism has previously been ruled out (19). In addition, the region of MCCB encoded by exon 11 is thought to be part of the 3-methylcrotonyl-CoA binding site which gives the enzyme its substrate specificity (18). It is highly likely that replacement of 24 amino acids in this region and a missense mutation within this exon leads to failure of 3-methylcrotonyl-CoA binding with deleterious functional consequences.

The most common consequence of point mutations that affect splicing is exon skipping (16,28). This is especially true for point mutations disrupting ESEs (6,15,16,27,29). In our patient, we were only able to observed exon skipping after suppression of NMD and in our minigene expression system. The explanation for the predominance of a transcript resulting from exon skipping rather than one with inclusion of exon 10a (as in untreated fibroblast RNA) in our minigene system remains unclear. Preliminary attempts to correct exon skipping events by repairing the sequence of damaged exonic enhancers have been proven to be efficient in vitro and in vivo (30-32), and hold promise for future therapeutic use.

Cryptic exons have been shown to be activated by intronic mutations that either create or strengthen splice sites, create a new branch site, or are located within a cryptic exon (5,16,33-36). To our knowledge this is the first demonstration of an exonic point mutation disrupting an ESE that leads to skipping of the corresponding exon and at the same time activates utilization of a cryptic exon from the adjacent intron. Mutations causing inclusion of cryptic exons may be more prevalent than the current literature suggests. Potential cryptic exons are frequent in introns and in some genes greatly outnumber genuine exons but are normally not included in the mature mRNA (4). Detection of these events in disease may be overlooked because introns are often excluded from mutation analysis and because it is often impossible or impractical to utilize cDNA for mutation analysis.

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**FOOTNOTES**

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1The abbreviations used are: MCC, 3-methylcrotonyl-Coenzyme A carboxylase; MCCA, methylcrotonyl-Coenzyme A carboxylase alpha (synonymous to MCCC1); MCCB, methylcrotonyl-Coenzyme A carboxylase beta (synonymous to MCCC2); PCC, propionyl-Coenzyme A carboxylase; MCCA, methylcrotonyl-Coenzyme A carboxylase alpha subunit; MCCB, methylcrotonyl-Coenzyme A carboxylase beta subunit; ESE, exon splice enhancer; NMD, nonsense-mediated mRNA decay; RT-PCR, reverse transcriptase polymerase chain reaction

**FIGURE LEGENDS**

**Fig. 1:** Expression of pTracer constructs with MCCB wild type cDNA and both types of mutant cDNA of patient MCC019 (G352R and exon10a) in an immortalized MCCB-deficient reference fibroblast cell line. Not transfected control and patient MCC019 fibroblasts were also analyzed. Transfections were performed by electroporation and cells were harvested 48 hours later for the assay of 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) activities as well as Western blot analysis of MCCB using β-Actin as a control. Transfection with the empty vector (vector only) was used as a negative control. Values are the mean of parallel determinations form a representative experiment. For further details see Experimental Procedures.

Median control values and range (in brackets) for MCC and PCC activities in 30 different immortalized fibroblast cell lines are: MCC: 399 pmol/min/mg protein (220-683); PCC: 660 (309-840)

**Fig. 2:** Schematic diagram of the splicing defect found in MCC019. Boxes represent exons and dark horizontal lines introns.

2a, Genomic PCR of MCCB exon 11 in MCC019 showing the homozygous mutation

2b, Schematic diagram and RT-PCR products of the splicing variants

2c, RT-PCR products after treating the cells with emetine (100 µg/ml) before harvesting the RNA. Emetine is used to inhibit nonsense mediated mRNA decay (21)

**Fig. 3:** *In vivo* splicing analysis. Boxes represent exons and dark horizontal lines introns. The relevant portion of exon 11 and its 3’ flanking splice site is shown for three minigene constructs; wild type, c.1054G>A (G352R) and c.1054G>A with the optimized donor splice site (G352RΔss). Intronic sequences are shown in lower-case, mutated nucleotides are bold.

3a, Schematic diagram of the three minigene constructs and the primers used for RT-PCR analysis

3b, RT-PCR products after transfecting MCCB-deficient reference fibroblasts with the indicated minigene constructs and the corresponding exon arrangements
Figure 1

|                | untransfected | transfected MCCB-deficient reference cell line |
|----------------|--------------|-----------------------------------------------|
| control        |              |                                               |
| MCC019         | 422          | 567                                           |
| vector only    | 10           | 933                                           |
| MCCB wild type | 1            | 448                                           |
| MCCB G352R     | 62           | 431                                           |
| MCCB exon10a   | 2            | 431                                           |
|                | 1            | 2                                             |

MCC-activity (pmol/min/mg protein)

|                | MCC-activity (pmol/min/mg protein) |
|----------------|------------------------------------|
| control        |                                    |
| MCC019         | 422                                |
| vector only    | 10                                 |
| MCCB wild type | 1                                  |
| MCCB G352R     | 62                                 |
| MCCB exon10a   | 2                                  |
|                | 1                                  |

PCC-activity (pmol/min/mg protein)

|                | PCC-activity (pmol/min/mg protein) |
|----------------|------------------------------------|
| control        |                                    |
| MCC019         | 567                                |
| vector only    | 933                                |
| MCCB wild type | 448                                |
| MCCB G352R     | 431                                |
| MCCB exon10a   | 443                                |
|                | 423                                |

MCCB

- 61.3 kDa

β-Actin

- 41.7 kDa
Figure 2

a) Genomic PCR

MCC019, MCCB, c.1054G>A

(n)TATAGA(n)

b) RT-PCR

DV5020

MCC019 normal

114 105

MCC019 wt

9 10 11 12

normal

exon switching

DV5019

c) RT-PCR after emetine treatment

DV5020

MCC019 normal

114 105

MCC019 wt

9 10 11 12

normal

exon switching

DV5019

41

exon skipping

exon skipping

exon switching

exon skipping
Figure 3

a) Schematic diagram of the minigene constructs

b) RT-PCR after minigene transfection