

Human Molybdopterin Synthase Gene: Genomic Structure and Mutations in Molybdenum Cofactor Deficiency Type B

J. Reiss,¹ C. Dorche,² B. Stallmeyer,³ R. R. Mendel,³ N. Cohen,⁴ and M. T. Zabet²

¹Institut für Humangenetik der Universität, Göttingen; ²Hôpital Debrousse, Service de Biochimie, Paris; ³Botanisches Institut der Technischen Universität, Braunschweig, Germany; and ⁴Technion-Israel Institute of Technology, Haifa

Summary

Biosynthesis of the molybdenum cofactor (MoCo) can be divided into (1) the formation of a precursor and (2) the latter's subsequent conversion, by molybdopterin synthase, into the organic moiety of MoCo. These two steps are reflected by the complementation groups A and B and the two formally distinguished types of MoCo deficiency that have an identical phenotype. Both types of MoCo deficiency result in a pleiotropic loss of all molybdoenzyme activities and cause severe neurological damage. *MOCS1* is defective in patients with group A deficiency and has been shown to encode two enzymes for early synthesis via a bicistronic transcript with two consecutive open reading frames (ORFs). *MOCS2* encodes the small and large subunits of molybdopterin synthase via a single transcript with two overlapping reading frames. This gene was mapped to 5q and comprises seven exons. The coding sequence and all splice site–junction sequences were screened for mutations, in MoCo-deficient patients in whom a previous search for *MOCS1* mutations had been negative. In seven of the eight patients whom we investigated, we identified *MOCS2* mutations that, by their nature, are most likely responsible for the deficiency. Three different frameshift mutations were observed, with one of them found on 7 of 14 identified alleles. Furthermore, a start-codon mutation and a missense mutation of a highly conserved amino acid residue were found. The locations of the mutations confirm the functional role of both ORFs. One of the patients with identified *MOCS2* mutations had been classified as type B, in complementation studies. These findings support the hypothetical mechanism, for both forms of MoCo deficiency, that formerly had been established by cell-culture experiments.

Introduction

Molybdenum cofactor (MoCo) (MIM 252150 [type A] and MIM 252160 [type B]) deficiency is a rare autosomal recessive disease with ~70 known cases found worldwide (C. Dorche and J. Reiss, unpublished data). This disorder leads to the pleiotropic loss of all molybdoenzyme activities (Duran et al. 1978; Johnson et al. 1980). The absence of an active sulfite oxidase results in severe neurological damage and early childhood death (Johnson and Wadman 1995). The most prominent clinical features are neonatal seizures unresponsive to any therapy, including massive molybdenum intake (Duran et al. 1978; Johnson et al. 1980). Molybdenum is coordinated in molybdoenzymes either in the form of a MoCo or as a component of an iron-molybdenum cofactor (Rajagopalan and Johnson 1992). The iron-molybdenum cofactor is found exclusively in the eubacterial nitrogenase (Ludden et al. 1993); all other molybdoenzymes contain the universal MoCo (Rajagopalan and Johnson 1992). This cofactor consists of the metal ion and a small organic moiety called “molybdopterin” (MPT) (Johnson and Rajagopalan 1982; Boyington et al. 1997). MPT is identical in all studied organisms, although some eubacteria form a dinucleotide from MoCo and either GTP or CTP (Rajagopalan and Johnson 1992), and some archae- or eubacteria use tungsten instead of the molybdenum ion (Huber et al. 1994; Chan et al. 1995). Free MPT is not stable, and all organisms share a conserved pathway for its biosynthesis (Mendel 1997). Complementation studies with cell cultures derived from MoCo-deficient patients suggest that group A cells are defective in the formation of precursor Z from GTP and that type B cells are defective in MPT synthase that normally converts the precursor to MPT (Johnson et al. 1989).

Elsewhere we have described the isolation of *MOCS1*, which encodes the two enzymes necessary for precursor Z formation in two consecutive open reading frames (ORFs) on a single transcript (Reiss et al. 1998b). Mutations were detected in both of these frames and together account for ~70% of the cases of MoCo deficiency (Reiss et al. 1998a). The remaining patients most

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Address for correspondence and reprints: Dr. Jochen Reiss, Institut für Humangenetik, Gosslersstrasse 12d, D-37073 Göttingen, Germany. E-mail: jreiss@gwdg.de

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likely belong to complementation group B, with a defect in the heterodimeric MPT synthase (fig. 1). The small and large subunits of this converting factor (Johnson et al. 1989) again are encoded in a single and uniform transcript, but, in contrast to the *MOCS1* transcript, the two corresponding ORFs overlap by 77 nucleotides (Stallmeyer et al. 1999 [in this issue]). We here describe both the genomic structure of the MPT-synthase gene, called “*MOCS2*” (MoCo synthesis step 2), and the identification of mutations in MoCo-deficient patients negative for *MOCS1* mutations.

Subjects and Methods

Subjects

MoCo deficiency comes to clinical attention through neonatal seizures unresponsive to any therapy, opisthotonos, and facial dysmorphism (Johnson and Wadman 1995). Elevated sulfite levels can be found in fresh urine samples, by means of simple test strips originally produced for control of wine or fruit juices (Merckoquant 1.10013; Merck). Other biochemical findings include hypouricemia, which is not observed in the otherwise similar phenotype of isolated sulfite oxidase deficiency (Irreverre et al. 1967), as well as abnormal sulfur and purine metabolites (Duran et al. 1978; Johnson et al. 1980). In all cases the sulfite oxidase deficiency has been tested and found to be absent in fibroblast cultures, which were also used for standard procedures of DNA isolation. In addition, EDTA samples of peripheral blood were prepared according to “salting out” procedures (Miller et al. 1988). All patients described in the present study had been previously screened for mutations in the *MOCS1* gene (Reiss et al. 1998a). For one patient (Boles et al. 1993), the complementation group was known (type B). All investigations were approved by the ethics committee of the medical faculty of the University of Göttingen.

Complementation Studies

After verification of their growth potential, fibroblast cell lines were mixed in a 1:1 ratio, grown for 7 d, trypsinized, replated, and grown for another 10 d. After cell harvest, complementation was tested by the measurement of sulfite oxidase activity in a crude extract of sonicated cells, as described by Garrett and Rajagopalan (1994).

Molecular Genetic Analysis

Isolation of the *MOCS2* cDNA (GenBank) is described in the accompanying paper (Stallmeyer et al. 1999). Unknown genomic sequences were amplified by means of a Genome walker™ kit (Clontech), with Advantage™ genomic polymerase mix (Clontech). Essen-

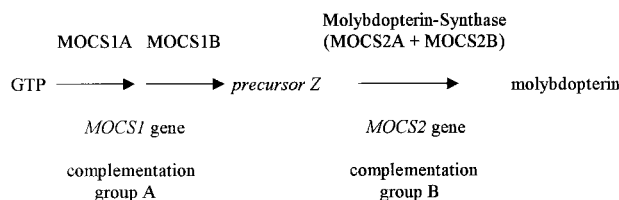


Figure 1 Schematic illustration of the MPT biosynthesis pathway. The participating enzymes are at the top; the corresponding genes and complementation groups are at the bottom.

tially, genomic DNA was digested with various restriction endonucleases prior to the ligation of universal DNA adaptors. Subsequent DNA amplification was done with a combination of one adaptor-specific and one gene-specific primer. Oligonucleotides used for PCR amplification of individual exons plus their flanking splice site–junction sequences are listed in table 1. PCR was done in an Eppendorf master cycler (30 cycles of 92°C for 1 min, 50°C for 45 s, and 63°C for 3 min), with *Taq*PCR master mix (Qiagen) including “Q-solution.” When nested PCR was done, the first round consisted of 15 cycles followed by 30 cycles after a 1:50 dilution of the template. Amplification products were column purified (Qiagen) and directly sequenced in cycles, on both strands, with the oligonucleotides used for PCR (table 1) and with Big Dye Terminator Mix (ABI). The sequence reaction products were purified by ethanol precipitation and were analyzed by electrophoresis on a 377A sequencing machine (ABI). A genomic PAC (plasmid artificial chromosome) clone was used for FISH on metaphase chromosomes after random-primed digoxigenin labeling. For radiation hybrid mapping, the Stanford G3 panel was purchased (Research Genetics) and was tested with primers F5 and R9 (table 1) in a PCR (30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min), with *Taq* Polymerase (Promega).

Results

Analysis of genomic DNA revealed six introns within the *MOCS2* gene. The genomic structure is illustrated in figure 2. The sizes of the individual exons and their splice site–junction sequences are listed in table 2. The small subunit of MPT synthase (i.e., *MOCS2A*) is encoded by exons 1–3, and the large subunit (i.e., *MOCS2B*) is encoded by exons 3–7. The overlap of these two ORFs (by 77 nucleotides) is limited to exon 3. FISH analysis of digoxigenin-labeled genomic PAC clones on metaphase chromosomes indicated a genomic map position on the long arm of chromosome 5, close to the centromere (data not shown). PCR screening of a radiation-induced cell-hybrid panel resulted in a human-

specific signal and placed the *MOCS2* locus in the region 5p11-5q11, closely linked to expressed sequence tag (EST) marker SHGC-36388 and in the vicinity of the polymorphic markers D5S660 and D5S623. This confirmed a chromosomal position at 5q11, for *MOCS2*.

Individual exons and their flanking sequences were amplified from the genomic DNA of patients in whom previous screening for *MOCS1* mutations had produced a negative result (Reiss et al. 1998a). In seven of the eight patients investigated, *MOCS2* mutations could be identified (table 3). Six of these seven patients are homozygous for a *MOCS2* mutation, and one is compound heterozygous for two different mutations affecting the same ORF. The most frequent mutation is a 2-bp deletion (726del2) removing the last nine amino acids of *MOCS2B*, of which two are conserved (Stallmeyer et al. 1999). This mutation was found homozygous in three different patients and in the compound-heterozygous patient. It therefore accounts for 50% (7 of 14) of the identified alleles. Two other frameshift mutations were detected as homozygous in one patient each: 252insC affects both *MOCS2A* and *MOCS2B* and removes conserved motifs from both proteins; 533del4 truncates *MOCS2B*, and the affected amino acid residues include the conserved motif that is also destroyed by 726del2.

Because the start codon represents the only methionine codon within *MOCS2A*, the point mutation M1I (ATG→ATA) found to be homozygous in one affected patient is expected to result in a complete loss of *MOCS2A*. In vitro translation experiments with a mutated *MOCS2A* start codon confirmed this assumption (Stallmeyer et al. 1999). In one patient heterozygous for the frameshift mutation 726del2, a point mutation affecting the first nucleotide of exon 7 was identified, resulting in the missense mutation E168K (GAA→AAA). Family analysis revealed that E168K is on the maternal chromosome and that 726del2 is the paternal allele. E168 is one of the few extremely conserved residues in

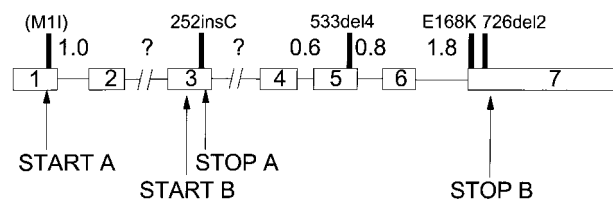


Figure 2 Genomic structure of the *MOCS2* gene, and location of described mutations. Exons are represented by open boxes and are identified by the numbers within the boxes. Between the exons the intron size (in kb) is given, if known. Exons and introns are not drawn to scale. The labeled horizontal black bars at the top indicate the locations of the mutations described in the text. Start and stop codon of the two ORFs are illustrated by the upward-pointing arrows below.

MOCS2B (Stallmeyer et al. 1999), and the amino acid substitution E168K is most likely sufficient for the impairment of the protein's enzymatic activity. The substituted G, however, contributes to the consensus value of a splice site (Krawczak et al. 1992), and an additional effect on splicing efficiency cannot be excluded.

Neither M1I (Δ *MOCS2A*) nor E168K was found in 50 healthy control individuals. We could not investigate the parents of the patients homozygous for one of the aforementioned mutations. However, no quantitative indication of hemizygosity by gross gene deletion was observed in any of the PCR amplifications. Furthermore, anamneses of families with MoCo-deficient patients have revealed a high percentage of consanguineous parents, which is known for the two homozygous patients clinically described elsewhere: Boles et al. (1993) reported on the patient with the start-codon mutation M1I, and Bonioli et al. (1996) reported on the patient with deletion 533del4. Fibroblasts from four of seven patients with *MOCS2* mutations and from the only remaining patient of our cohort who did not have *MOCS1* or *MOCS2* mutations were tested for complementation

Table 1

Oligonucleotides for PCR Amplification of Individual *MOCS2* Exons and Their Splice-Site Junctions

EXON ^a	PRIMER (SEQUENCE) (5'→3')		SIZE OF AMPLIFIED PRODUCT (bp)
	Forward	Reverse	
1	F27 (TGA GCC CGC GCC TGC GCC TTT)	R19 (TAG TGG GGA GGT CCG ACT GAC CA)	183
2	F17 (CAG TCA GAA AAC GAG TAT GAG TTG)	R17 (AAG TGT TAC TCATAT GCA TTC TAC)	212
3	F18 (GAG GTC TAT CAT AGC GTT GAT AC)	R18 (TCA TCT GTA TGA TCC TTC CCC)	249
4	F23 (CCC TAG GAC TTC TGT TAA GGT TTG)	R20 (ACA CTG AAC ATG GAA AGC ACA TGC)	245
5A	F16 (TTA GAA GAA TTG GTA TGC TGC TTG)	R4 (TAA CAG CCA TCC CTA TTG TCC TCC)	437
5B	F21 (CGT GCT ATT TCT TTT GTA GGG)	R21 (TCA GAG TTA GTA CAA AGA TGG)	316
6	F5 (CAC GTA GAT TCA GAG ATG ATG GTG)	R9 (CAG GTC CAC ATG CTA AAA TGT G)	213
7A	F22 (TGC CCA TGA TGT CTC TCG TCA TGA)	R22 (CTT CAG TAA ACT ATC CTG ATG TGG)	312
7B	F19 (GGA GCC ATT GAA ATA GAT GTG)	R3 (TAA TAG TTT AAC AAA GTT AAG)	224

^a Exons 5 and 7 were amplified first with the "A" primers and then with the nested "B" primers.

Table 2
Sizes of *MOCS2* Exons and Splice-Junction Sequences

Exon	Nucleotides	Length (bp)	Amino Acids	3' Junction ^a	5' Junction ^a
1	1–18	>18	1–6		CAGgtaagggtggcg
2	19–140	122	7–47	ctttactttcagGTT	TGGgtagttaaaaat
3	141–285	145	48–88, 1–33	tttccccttagATT	TAGgtatgtgagatt
4	286–413	128	34–75	tattatttctagGAA	TAGgtgagttgtaat
5	414–564	151	76–126	tcatttttaagGGA	TGGgtatgatttct
6	565–688	124	127–167	atctgttaagCTT	AAGgtaagttaagaa
7	689–1264	576	168–188	ttgttttttagGAA	

^a Reference is to the corresponding intron.

among each other and after cocultivation with fibroblasts from patients with *MOCS1A* and *MOCS1B* mutations (Reiss et al. 1998a). This confirmed that they all belong to complementation group B. Representative results are listed in table 4 and illustrate the relationship between identified mutations and the two complementation groups.

Discussion

Several assumptions and hypotheses put forward previously are confirmed by the present study: the molecular basis for the observation of two complementation groups in MoCo deficiency (Johnson et al. 1989) is two distinct *MOCS* genes on different chromosomes, one encoding enzymatic activities leading to the formation of precursor Z (Reiss et al. 1998a, 1998b) and one encoding for the small and large subunits of the heterodimeric MPT synthase, which converts the precursor to MPT. Because both of the corresponding transcripts encode two proteins, future experiments might reveal more than two complementation groups after cell fusion. This theoretical possibility, however, is unlikely if either colinear transcription or microcompartmentalization of either *MOCS1A* and *MOCS1B* or *MOCS2A* and *MOCS2B*, respectively, are indispensable for MoCo synthesis.

Fibroblasts of the only patient without identified mutations (described by Desjaques et al. 1985) are complemented by fibroblasts of complementation group A. This rules out an unidentified defect in the *MOCS1* gene. No complementation is observed after cocultivation with group B cells. The patient therefore formally belongs to group B, which leaves the possibility of an as yet unidentified disease-causing mutation in noncoding regions of the *MOCS2* gene—for example, a promoter mutation or the activation of a cryptic splice site (Krawczak et al. 1992). Another possibility is the existence of a third *MOCS* gene with disease-causing potential. Because of the observed complementation behavior, the putative gene product should either interact with MPT synthase or be involved in the insertion of molybdenum into the MPT (Johnson et al. 1989; Mendel 1997).

The identification of alterations—which, by their nature and position, can clearly be assumed to be disease causing—in both ORFs of the bicistronic *MOCS2* gene confirms not only that both proteins encoded by these overlapping ORFs are expressed but, furthermore, that the expression of both ORFs is indispensable for a normal phenotype. It is counterintuitive that the twofold observation of an unusual architecture such as a bicistronic eukaryotic transcript in both *MOCS1* (Reiss et al. 1998b) and *MOCS2* (present study) should be coincidental. The fusion of genes for the sake of synchronized expression and microcompartmentalization appears to be a frequent event in eukaryotic evolution (Amy et al. 1992; Davidson et al. 1993), and the transcription of both *MOCS1* and *MOCS2* seems to proceed by the usual means. In both cases, however, the fusion of the two encoded proteins into one multicatalytic enzyme has not happened. Because the mode of the unusual translation of a second ORF in case of *MOCS1* with two consecutive ORFs seems to be a reinitiation (Reiss et al. 1998b) but, in the case of *MOCS2* with two overlapping ORFs, leaky scanning (Stallmeyer et al. 1999), the mode of expression of the second ORF seems not to be correlated with the phenomenon of multiple bicistronic transcripts in one biochemical pathway.

Another common feature of *MOCS1* and *MOCS2* might be important in this context. Both proteins encoded by the first ORF—*MOCS1A* and *MOCS2A*—end with a highly conserved and identical motif: glycine-glycine-stop. Again, a coincidence is unlikely, and the

Table 3
Genotypes of Patients with Identified *MOCS2* Mutations

Genotype	Exon	Ethnic Origin	Affected Proteins
M11/M11	1	Coptic Egyptian	<i>MOCS2A/MOCS2A</i>
252insC/252insC	3	Portuguese	<i>MOCS2A/MOCS2A</i> , <i>MOCS2B/MOCS2B</i>
533del4/533del4	5	Italian	<i>MOCS2B/MOCS2B</i>
E168K/726del2	7	German	<i>MOCS2B/MOCS2B</i>
726del2/726del2	7	French	<i>MOCS2B/MOCS2B</i>
726del2/726del2	7	Portuguese	<i>MOCS2B/MOCS2B</i>
726del2/726del2	7	English	<i>MOCS2B/MOCS2B</i>

Table 4**Enzymatic Activity of Cultured and Cocultured Fibroblasts**

Cell Lines (Identified Mutations)	Sulfite Oxidase Activity ^a (μkat/kg protein)
Wild type 1	3.1
Wild type 2	7.7
MOCS1A (722delT/722delT) + MOCS1A (G324E/G324E)	ND
MOCS1A (722delT/722delT) + MOCS1B (1523del2/1523del2)	ND
MOCS1A (722delT/722delT) + MOCS2B (756del2/756del2)	4.1
MOCS1B (1313insG/T182P) + MOCS2B (756del2/756del2)	3.9

^a ND = not detectable.

importance of these extremely conserved residues is further emphasized by the observation of a corresponding *MOCS1* mutation of the penultimate glycine codon (G384S) in a MoCo-deficient patient (Reiss et al. 1998a). The importance of a C-terminal glycine-glycine motif has already been highlighted, by Pickart et al. (1994), for ubiquitin, in which the terminal glycine seems to be necessary for a transthiolation. Taylor et al. (1998) have pointed out that both *thiS* and *moaD*, the bacterial homologue of *MOCS2A*, carry two C-terminal glycine residues probably also involved in a transthiolation. In summary, these data suggest that the evolutionarily favored fusion of MoCo biosynthesis enzymes is stuck twice on the level of a fused transcript, because of the necessity of a free C-terminal motif for the enzymatic activities of both *MOCS1A* and *MOCS2A*.

All described *MOCS2* mutations affect one or more highly conserved motifs. As observed in the *MOCS1*-mutation screening (Reiss et al. 1998a), no missense mutation of a less conserved residue was identified. This parallels the absence of mild or partial forms of MoCo deficiency and supports the hypothesis of a qualitative “yes or no” mechanism—rather than quantitative kinetics—for MoCo function; that is, this function is either completely abolished or sufficient for a normal phenotype. This theory may provide hope for a successful gene therapy in this hitherto incurable disease (Reiss et al. 1998; Wolff 1997). Precursor-producing cells seem to be capable of feeding their precursor-deficient neighbor cells, but no such effect can be expected for excretion and uptake of MPT synthase (Johnson et al. 1989). Therefore, *MOCS2* supplementation, in comparison with *MOCS1* delivery, might require a larger number of cured cells, for a successful gene therapy.

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Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Web/Genbank> (for *MOCS2* cDNA [AF091871])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim> (for MoCo deficiency, types A [MIM 252150] and B [MIM 252160])

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