

Human Molybdopterin Synthase Gene: Identification of a Bicistronic Transcript with Overlapping Reading Frames

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Summary

A universal molybdenum-containing cofactor (MoCo) is essential for the activity of all human molybdoenzymes, including sulphite oxidase. The free cofactor is highly unstable, and all organisms share a similar biosynthetic pathway. The involved enzymes exhibit homologies, even between bacteria and humans. We have exploited these homologies to isolate a cDNA for the heterodimeric molybdopterin (MPT)-synthase. This enzyme is necessary for the conversion of an unstable precursor into molybdopterin, the organic moiety of MoCo. The corresponding transcript shows a bicistronic structure, encoding the small and large subunits of the MPT-synthase in two different open reading frames (ORFs) that overlap by 77 nucleotides. In various human tissues, only one size of mRNA coinciding with the bicistronic transcript was detected. In vitro translation and mutagenesis experiments demonstrated that each ORF is translated independently, leading to the synthesis of a 10-kDa protein and a 21-kDa protein for the small and large subunits, respectively, and indicated that the 3'-proximal ORF of the bicistronic transcript is translated by leaky scanning.

Introduction

In eukaryotic molybdoenzymes, the catalytically active metal molybdenum is complexed by a unique cofactor, termed the “molybdenum cofactor” (MoCo), that combines with diverse apoproteins in which it is responsible for the correct anchoring and positioning of the Mo-

center within the holoenzyme (reviewed by Hille [1996] and Stiefel [1996]). The structural moiety of MoCo was identified as a unique pterin, designated “molybdopterin” (MPT; reviewed by Rajagopalan and Johnson [1992]) and has been shown to be identical in all organisms. Accordingly, the MoCo biosynthetic pathway seems to be conserved, because highly homologous proteins have been found to be involved in MoCo biosynthesis in bacteria and higher plants (reviewed by Mendel [1997]).

In *Escherichia coli*, in which the genes and proteins relevant to MoCo biosynthesis have been intensively studied, the synthesis of MoCo proceeds in three steps (reviewed by Rajagopalan [1996]): (1) conversion of GTP into the stable precursor Z; (2) conversion of precursor Z into MPT; and (3) insertion of molybdenum into MPT, thus forming MoCo. Human MoCo deficiency is a rare and fatal disease (Johnson and Wadman 1995). It is inherited in an autosomal recessive manner and results in neurologic damage leading to death in early childhood. Johnson et al. (1989) used fibroblasts from MoCo-deficient patients to define two complementation groups, which correspond to type A (MIM 252150) and type B (MIM 252160) of MoCo deficiency. It was assumed that type A patients are defective in the early steps of MoCo biosynthesis leading to precursor Z. This was recently confirmed by the identification of mutations in *MOCS1* (Reiss et al. 1998b). The *MOCS1* transcript is bicistronic, and mutations were found in both of the two consecutive open reading frames (ORFs) encoding *MOCS1A* and *MOCS1B*. Translation reinitiation was suggested as the mechanism of translation for the second ORF.

In *E. coli*, MPT-synthase is composed of two subunits, the small subunit (10 kDa) encoded by the *moaD* gene, and the large subunit (16 kDa) encoded by the *moaE* gene (GenBank; Pitterle et al. 1993). Here we describe the isolation and analysis of a human cDNA, *MOCS2*, encoding proteins homologous to the bacterial MPT-synthase, which involves two unexpected findings: (1) in humans, both subunits of MPT-synthase are again encoded by a single gene comprising two ORFs; and (2) the two ORFs overlap. To understand the translation of

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this unique genetic construct, *in vitro* mutagenesis experiments were performed. We show that both subunits of the MPT-synthase are translated independently, and provide evidence for leaky scanning as the mechanism of translation from this mRNA.

Material and Methods

Screening of a Human Liver cDNA Library

An aliquot containing 100,000 Pfu of an adult human liver cDNA library (Stratagene), subcloned into the λ vector UNI-ZAP, was hybridized with a ^{32}P -labeled *Bgl*III/*Eco*RI fragment, representing a 5'-truncated MOCS2 cDNA clone from human fetal liver, obtained from the IMAGE consortium. Hybridization was performed according to Sambrook et al. (1989) at 65°C in 5 x SSPE, 5 x Denhardt, 1% SDS, and 1% sonicated calf thymus DNA. The filters were washed at 65°C in 2–0.1 x SSPE and 0.1% SDS. The random primed-labeling method, following Feinberg and Vogelstein (1984), was used to label the MOCS2 *Bgl*III/*Eco*RI fragment. Positive clones were verified in a second screen and transferred to p-Bluescript SK+ (pBSK) constructs by means of the *in vivo* excision system of the UNI-ZAP vector, according to the manufacturer's instructions. We determined the nucleotide sequence of the resulting plasmids using the automatic sequencer ABI Prism 310 and the DNA Sequencing Kit (PE Applied Biosystems).

Northern Blot Analysis

A human multiple-tissue northern blot (MTN, Clontech) was hybridized with the ^{32}P -labeled 1.35-kb MOCS2 *Eco*RI/*Xho*I fragment, according to the manufacturer's instructions. The random primed-labeling method was used to label the *Eco*RI/*Xho*I fragment (Feinberg and Vogelstein 1984). After hybridization, the filter was exposed to Kodak X-OMAT AR film.

Plasmid Construction

To subclone the MOCS2 cDNA from pBSK into the vector pHST07, a derivative of pHST0 (Jobling et al. 1988) optimized for *in vitro* transcription of mRNA, PCR amplification was performed to generate a *Bgl*III site in front of the 5' end of the cDNA fragment and a *Sall* site in the 3' UTR of the cDNA sequence. Primer 1 was 5'-GTCCTACGATCCAGATCTGCAGGAATTCGGCAGAGCG. Primer 2 was 5'-CTTCCAGTTCTGTCGACATTCTGTGTCAGC. The PCR fragment was cleaved by *Bgl*III and *Sall* and subcloned into the *Bgl*III/*Sall* site of pHST07 (pMOCS2). To mutate the initiator codon of the MOCS2A ORF from ATG to TTG, primer 3 (5'-AATTCGGCAGGAGATCTGTCTAGGCGGGTTGGTGCCGCTG) was used, together with primer 2 (pMOCS2A*). Primer 4 (5'-GCACGAGGAGCGGAT-

CCCCAAGCTCGACAAATTCTTGACGAACAGC) is complementary to nucleotides 202 to 247 of MOCS2, except for CAA (bold) complementary to TTG (which replaces ATG), and the mutation of the G(231) to C and of the C(236) to G, to form a *Bam*H1 site (underlined), was used with primer 1 to amplify fragment 1. Primer 5 (5'-ATGTCGAGCTTGGGGATCCGCTCCTCGTGC) is homologous to nucleotides 218–247 of MOCS2, except for the replacement of A(231) by G and A(236) by C to form a *Bam*H1 site (underlined), and was used in conjunction with primer 2 to synthesize fragment 2. PCR fragment 1 was cleaved with *Bgl*III and *Bam*HI, and PCR fragment 2 with *Bam*H1 and *Sall*, and the two fragments ligated simultaneously into the *Bgl*III and *Sall* sites of plasmid pHST07 (pMOCS2B*). In all PCR reactions, cloned Pfu DNA polymerase (Stratagene) was used according to the supplier's recommendations. PCR fragments were purified with use of the QIAquick PCR Purification Kit (Qiagen). All constructs were verified by sequencing. pMOCS2 Δ A and pMOCS2 Δ AB are 5' truncated versions of the pMOCS2 full-length clone, obtained from the screening of the adult human liver cDNA library; they lack nucleotides 1–120 (pMOCS2 Δ A) and nucleotides 1–232 (pMOCS2 Δ AB) of the full-length MOCS2 cDNA sequence, respectively.

In Vitro Transcription

We prepared and purified Plasmids by using Nucleobond Ax cartridges (Macherey Nagel), as indicated by the supplier. Plasmids pMOCS2 Δ A and pMOCS2 Δ AB in pBSK were linearized by *Xho*I and transcribed by T3 RNA polymerase (Eurogentec). Plasmids pMOCS2, pMOCS2A*, and pMOCS2B* in pHST07 were linearized by *Sall* and transcribed by T7 RNA polymerase (BRL). To produce capped transcripts, the linearized DNA (2 μg) was incubated in a 100- μl mixture containing 1 x buffer for T3 or T7 RNA polymerase (as specified by the suppliers); 10 mM DTT; 50 $\mu\text{g}/\text{ml}$ BSA (BRL); 100 U RNAsin (Promega); 0.5 mM each of ATP, CTP, and UTP; 0.2 mM GTP; and 1 mM m⁷GpppG (Pharmacia); as well as 30 U or 250 U, respectively, of T3 or T7 RNA polymerase, as required. After 1 h at 37°C, 30 U or 125 U, respectively, of T3 or T7 RNA polymerase were added. Total incubation was for 2 h at 37°C. The template DNA was then destroyed by incubation for 10 min at 37°C with 2 U RQ1 DNase (Promega), after which 2 mM EDTA (pH 8) was added to inhibit the DNase. The RNA was purified by passage through a Sephadex G-50 column, followed by phenol and chloroform extraction and ethanol precipitation. The integrity and size of the transcripts were estimated by native agarose gel electrophoresis, and the concentration was determined by A260 measurements. The

transcripts are designated by the name of the construct preceded by the prefix "t."

In Vitro Translation

Capped transcripts (400 ng), preheated for 10 min at 65°C, were incubated for 1 h at 30°C by use of 560 kBq of [³⁵S]methionine or 370 kBq [³⁵S]cysteine (Amersham) as specified, in 10 μl translation mixture containing 4 μl ribonuclease-treated rabbit reticulocyte lysate (Amersham), as described by the supplier, in the presence of 130 mM KCl and 1.5 mM Mg acetate. When indicated, 0.25 mM cap analogue m⁷GTP (Sigma) was added to the incubation mixture. Aliquots (2 μl) of the translation products were analyzed on a 16.5% acrylamide-Tricine/SDS gel system (Schägger et al. 1987). After electrophoresis, the gels were fixed, processed for fluorography with Amplify (Amersham), dried, and placed under an x-ray film. When required, the relevant bands were cut from the dried gel and their radioactivity was measured in 3 ml OptiScint "HiSafe" scintillation mixture (Wallac). The ratio of the radioactivity contained in the 21-kDa or 10-kDa protein bands in the presence versus in the absence of cap analogue was determined.

Results

Using the amino acid sequence of the *E. coli* MoaE protein, we performed a blast N search in the National Center for Biotechnology Information expressed sequence tag database (GenBank), leading to the identification of a fetal human liver cDNA clone homologous to the C-terminal part of the *E. coli* MoaE protein, the large subunit of the MPT-synthase. The clone was obtained from the IMAGE consortium (Lennon et al. 1995), and further sequence analysis revealed that it represents a 1-kb cDNA with an N-terminal truncated ORF encoding an MoaE homologous protein. To obtain a full-length cDNA clone, we screened an adult human liver cDNA library, using this 5'-truncated cDNA clone as probe. This led to the isolation of six independent cDNA clones varying from 1.35 kb–1.1 kb. Nucleotide analyses revealed that the shorter cDNAs represent 5'-truncated versions of the 1.35-kb clone. Sequence analysis of the 1.35-kb cDNA clone showed that it represents a cDNA of 1,345 bp with a full-length MoaE homologous ORF encoding a protein of 188 amino acids, with a calculated size of 20.8 kDa and 29.3% identity to the amino acid sequence of the *E. coli* MoaE protein (fig. 1). Surprisingly, 5' of the MoaE homologous ORF, a second ORF could be identified, encoding a protein of 88 amino acids with a calculated size of 9.8 kDa. Comparison of the amino acid sequence of the protein encoded by this latter ORF revealed that it shows 27.5% identity to the *E. coli* MoaD protein, the small subunit

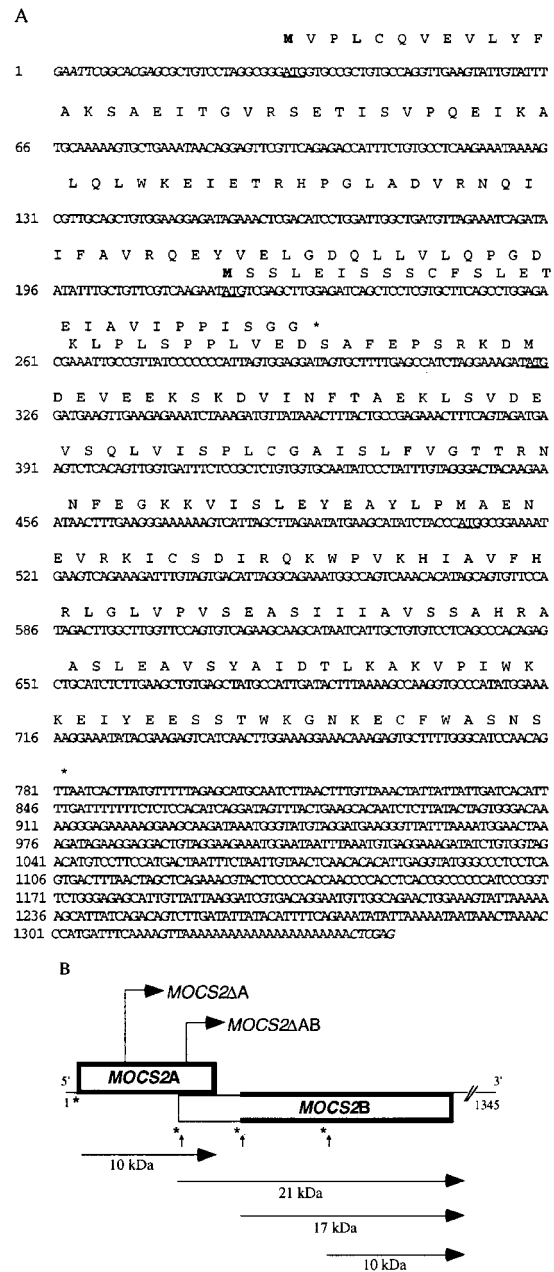


Figure 1 Nucleotide sequence (nucleotides belonging to the cDNA linker are printed in italics). The MOCS2A ORF is located at the 5' end (nucleotides 31–294). The MOCS2B ORF starts at the 3' end of the MOCS2A ORF (nucleotides 218–781). In MOCS2B, the region of homology starts after the second in-frame ATG (position 323, Met in position 36) of the full-length protein. A, Deduced amino acid sequence. The positions of the in-frame ATG codons are underlined. B, Schematic representation of the human MOCS2 cDNA clone (adult liver). Regions homologous to the *E. coli* MoaD and MoaE proteins are highlighted by a bold line (B). The positions of the in-frame ATG codons are indicated by a star below the ORFs. The calculated sizes of the resulting translation products are indicated below the horizontal arrows, the 5' ends of pMOCS2ΔA and pMOCS2ΔAB by bent arrows. CNBR cleavage sites are indicated by the small bent arrows.

of the MPT-synthase. The ORFs are arranged in different reading frames and overlap by 77 bp.

Since the isolated 1.35-kb cDNA clone represents the second human cDNA clone involved in the human MoCo biosynthesis pathway, it has been named MOCS2 (molybdenum cofactor synthesis gene 2). The human cDNA clone MOCS1 involved in step 1 of the MoCo biosynthetic pathway has been described elsewhere (Reiss et al. 1998b).

Verification of the bicistronic arrangement of the MOCS2A and MOCS2B ORFs in mammals was obtained by analyzing other cDNA clones from human brain (data not shown) or from the mouse. Figure 2 shows an alignment of the amino acid sequence of MOCS2A and MOCS2B, respectively, with different homologous proteins found in the databases. In MOCS2A, conserved amino acids are distributed over the entire sequence, including a highly conserved motif of six amino acids at the C-terminus with the consensus sequence PP(I/V/M/L)(S/A/T)GG. In the case of MOCS2B, the alignment shows that the mammalian proteins include an N-terminal extension of ~40 amino acids not found in any of the eubacterial homologues and without homology to any protein characterized in the database. This extension represents the region of the MOCS2B ORF overlapping the MOCS2A ORF. We will show below that indeed the first ATG codon of this ORF is used for the start of translation. The region with homology to the eubacterial MOCS2B homologues starts after the second in-frame ATG at position 323-325 (fig. 1).

To determine the size of the natural MOCS2 transcript and to exclude that mRNAs representing only the MOCS2A or MOCS2B ORF are generated by alternative or trans-splicing, we performed northern blot analyses (fig. 3). In each tissue, only one transcript could be detected that correlates with a calculated size of 1.35 kb similar to the size of the characterized full-length MOCS2 cDNA clone. No indications for alternative splicing were detectable. The expression pattern follows that of MOCS1 (Reiss et al. 1998b). Exposure after MOCS2 hybridization, however, was 50 × prolonged as compared with the actin control. At such a low expression level the question of tissue distribution must be addressed cautiously.

To determine whether the proteins corresponding to the two ORFs present in tMOCS2 (transcript of wild-type MOCS2 cDNA) could be synthesized independently of each other, various transcripts were tested by in vitro translation (fig. 4). The two proteins synthesized by tMOCS2 were 10 kDa and 21 kDa, and correspond in size to MOCS2A and MOCS2B, respectively. A mutated MOCS2A start codon in transcript tMOCS2A* abolished the band at the position of 10 kDa, whereas the 21-kDa protein was synthesized in large amounts. With tMOCS2B*, carrying a mutated MOCS2B start codon,

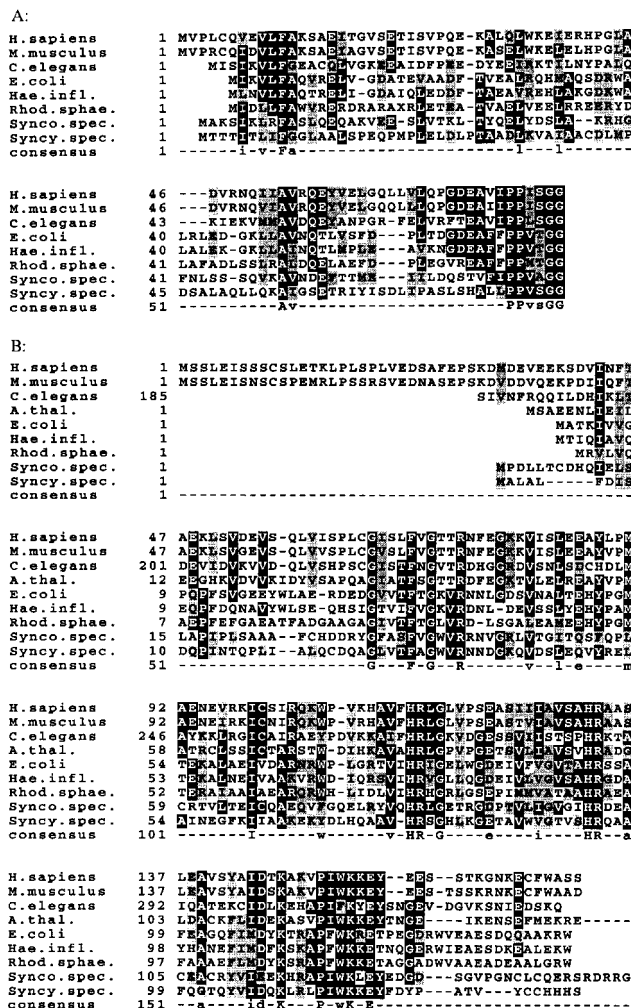


Figure 2 Multiple alignments of MOCS2A (A) and MOCS2B (B) homologous proteins. Identical amino acids are in black, similar amino acids in grey. Identical positions in all sequences are indicated by a capital letter in the consensus line, highly conserved residues in lowercase letters. Sequences of the eubacterial homologues from *E. coli*, *Haemophilus influenzae*, *Rhodobacter sphaeroides*, *Synechococcus spec. PCC 7942*, and *Synechocystis spec. PCC 6803* were obtained from GenBank, as well as the sequence of the MOCS2A and MOCS2B homologues of *Caenorhabditis elegans*. The *Arabidopsis thaliana* sequence homologous to the human MOCS2B protein is from protein Cnx6 (R. R. Mendel, unpublished results).

the 10-kDa protein was synthesized as efficiently as with tMOCS2. No band was detected at the level of the 21-kDa protein. In tMOCS2ΔAB, which begins within the MOCS2B ORF, both bands were absent. Transcript tMOCS2ΔA, which begins within the MOCS2A ORF, yielded only the 21-kDa protein. To further verify that the 21-kDa protein was synthesized from the first AUG present in the corresponding ORF, the protein after SDS-PAGE purification was submitted to CNBr treatment, which cleaves after methionine residues. This cleavage

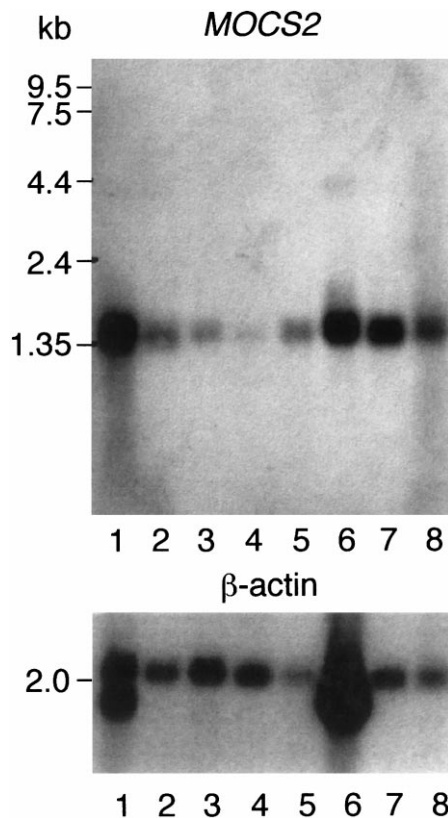


Figure 3 Northern blot analysis of the human *MOCS2* gene. Results of reprobing with human β -actin are shown at the bottom. Poly A+ mRNA was derived from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). The sizes of the mRNA markers are indicated to the left.

yielded the expected three peptides of 3.8 kDa, 6.9 kDa, and 10.1 kDa (data not shown).

If the 21-kDa protein is produced from capped *tMOCS2* by leaky scanning, its synthesis should be hindered by the addition of cap analogue (m^7GTP) to the same extent as synthesis of the 10-kDa protein. This would normally not be the case if synthesis of the 21-kDa protein occurred by internal initiation. As shown in figure 5, translation of capped *tMOCS2* in the presence of cap analogue dramatically reduced the amount of the two proteins synthesized.

Discussion

With ~30% identity, the degree of homology between the human proteins and their *E. coli* homologues is as high as between different eukaryotic proteins involved in MoCo biosynthesis characterized hitherto and their *E. coli* homologues (Hoff et al. 1995; Stallmeyer et al. 1995; Reiss et al. 1998b). Furthermore, an alignment of the human *MOCS2A* and *MOCS2B* amino acid se-

quences with the homologous proteins of eubacterial and eukaryotic origin demonstrates that, within the mammalian sequences, the same amino acids are conserved as within the sequences of the corresponding eubacterial *MoaD* and *MoaE* proteins.

The bicistronic structure of the mammalian *MOCS2* gene contrasts with the situation in *Arabidopsis thaliana* and *Caenorhabditis elegans*, in which the small and large subunits of the MPT-synthase are encoded by separate genes (Wilson et al. 1994; reviewed by Mendel [1997]). In principle, two mechanisms could enable translation initiation at a 3'-located ORF in eukaryotic mRNAs. The first possibility is through leaky scanning (Kozak 1989), in which 40S ribosomal subunits bind at the capped 5' end of the mRNA and scan downstream to the first AUG codon. If this AUG is in an unfavorable codon context, it might be bypassed by some 40S subunits that continue scanning downstream to initiate at the next available AUG in a more favorable context. As opposed to the situation in viral mRNAs, in eukaryotes, leaky scanning of bicistronic mRNAs is rare and is generally used by some ribosomes to bypass short nonoverlapping 5'-prox-

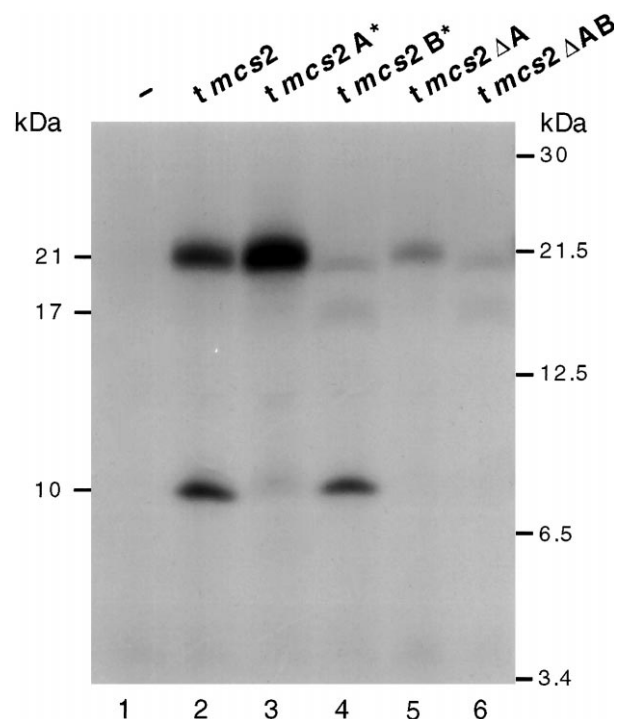


Figure 4 Fluorogram of the in vitro translation products of capped transcripts in a rabbit reticulocyte lysate with [^{35}S]methionin. Lane 1, Translation in the absence of transcript. Lanes 2-6, Translation in the presence of *tMOCS2*, *tMOCS2A**, *tMOCS2B**, *tMOCS2ΔA*, and *tMOCS2ΔAB*, respectively. The products were analyzed on a 16.5% polyacrylamide-Tricine/SDS gel system. The sizes of the products synthesized (left) and the sizes and positions of [^{14}C] protein markers (Amersham) run on a parallel lane (right) are given.

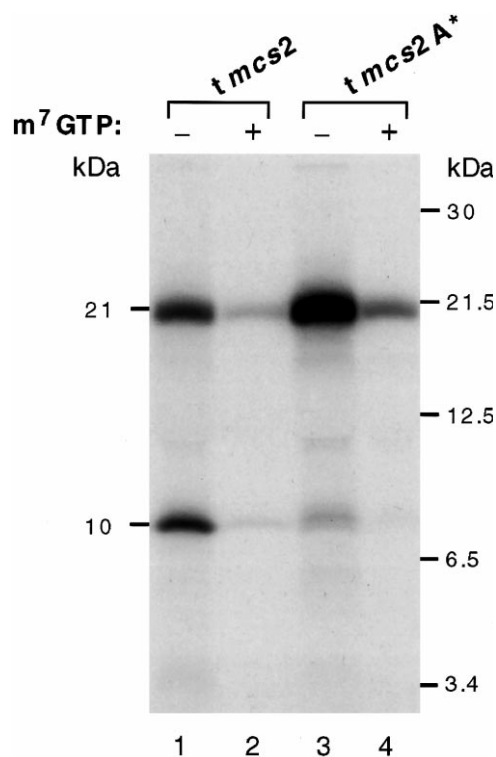


Figure 5 Effect of the cap analogue m^7GTP on the translation of capped transcripts. Translation in a rabbit reticulocyte lysate with [^{35}S]methionine of the capped $tMOCS2$ (lanes 1, 2) or $tMOCS2A^*$ (lanes 3, 4) was carried out in the absence (-; lanes 1, 3) or in the presence (+, lanes 2, 4) of m^7GTP . The sizes of the products analyzed on a 16.5% polyacrylamide-Tricine/SDS gel system are indicated to the left, and the sizes and positions of [^{14}C] protein markers run on a parallel lane are given to the right of the fluorogram.

imal ORFs and reach the major ORF (reviewed by Geballe [1996]). Because the AUG of the $MOCS2A$ ORF is contained within a rather favorable context (GGCGGGAUGGUG), the reason why some ribosomes bypass this initiation codon is unclear.

The other mechanism that allows translation of 3'-located ORFs is a cap-independent mechanism, characterized by internal ribosome initiation facilitated by the presence of an internal ribosome entry site (IRES) within the 5' UTR of the mRNA. IRES-dependent translation initiation has been described for picornavirus RNA (Jang et al. 1988; Pelletier and Sonnenberg 1988) and some eukaryotic cellular mRNAs (reviewed by Iizuka et al. [1995]), but can be ruled out for $MOCS2B$ expression because it is sensitive to cap analogue.

Another scenario that needed to be envisaged was frameshifting. A +1 frameshift event would lead to the synthesis of a $MOCS2A/B$ frameshift protein of 27 kDa, in addition to $MOCS2A$. A first examination of the level of $MOCS2A$ and $MOCS2B$ synthesized indicates that they are produced in similar amounts, which is an ar-

gument against frameshifting, the efficiency of which is generally <30% in eukaryotic RNA viruses (discussed by ten Dam [1995]) and 19% in the case of mammalian ornithine decarboxylase antizyme (Matsufuji et al. 1995). Analysis of the peptides obtained from a CNBr cleavage of the 21-kDa $MOCS2B$ protein, synthesized in vitro in the presence of [^{35}S]cysteine, clearly demonstrated that frameshifting can be ruled out as a mechanism leading to the translation of the 3'-located ORF.

Whereas in eubacteria functionally related proteins are often encoded from bicistronic or polycistronic mRNAs, eukaryotic mRNAs are generally monocistronic. Examples of eukaryotic bicistronic mRNAs are nearly exclusively known for viral systems. In very few cases bicistronic mRNAs have been described in eukaryotic cellular systems (Lee 1991; Andrews et al. 1996; Ritchie and Wang 1997). The mechanism leading to the translation of the 3'-located ORF is unknown. Interestingly, the human $MOCS1$ gene catalyzing the first step of the molybdenum cofactor biosynthesis is also bicistronic (Reiss et al. 1998b). Nevertheless, the two $MOCS1$ ORFs are arranged in tandem, and the mode of $MOCS1B$ expression is presumed to be translation reinitiation. Our in vitro experiments with $MOCS2$ suggest leaky scanning as the mode of translation for the second protein. Thus, the mode of translation does not appear to be the explanation for the unusual architecture of the $MOCS$ genes.

We have already reported that mutations leading to MoCo deficiency type A were found in both ORFs of the $MOCS1$ transcript and that all identified mutations were "severe" mutations (i.e., frameshift mutations; Reiss et al. 1998a, 1998b). Similar results are described for MoCo deficiency type B and the $MOCS2$ gene in the accompanying paper by Reiss et al. (1999 [in this issue]). These observations support the idea that in vivo expression of both ORFs is necessary for a normal phenotype. The absence of leaky mutations—which would allow for a partial enzyme activity—in the patients is in agreement with a very low expression level and perhaps also the possibility of unusual and otherwise inefficient ways of expression, such as translation reinitiation or leaky scanning.

For essential metabolic pathways, the evolution of a multifunctional eukaryotic fusion protein comprising the functions of two separate *E. coli* proteins has already been demonstrated, for example, for MoCo biosynthesis in plants (Stallmeyer et al. 1995), and in *Aspergillus nidulans* (Unkles et al. 1997) for fatty acid synthesis (Amy et al. 1992) and for pyrimidine biosynthesis (Davidson et al. 1993). One reason for the development of such gene fusions might be the colinear regulation of gene expression and also the guaranteed proximity of the interacting proteins, thus leading to an evolutionary advantage for the organism. The latter aspect of micro-

compartmentalization can be expected to be of greater importance for low substrate concentrations, such as in MoCo biosynthesis.

Acknowledgments

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Electronic-Database Information

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

GenBank, <http://www.ncbi.nlm.nih.gov/> (for *E. coli* MoaE [X70420], *Haemophilus influenzae* [U32840], *Rhodobacter sphaeroides* [U29587], *Synechococcus spec.* PCC 7942 [X99625], *Synechocystis spec.* PCC 6803 [D64003], *Homo sapiens* MOCS2A and MOCS2B [AF091871], MOCS2A and MOCS2B homologues of *Caenorhabditis elegans* [U88180, U21322], and *Mus musculus* [AF091872])
 IMAGE, <http://www-bio.lnl.gov/bbrp/image/image.html> (for human fetal liver clone 109399 [T70309])
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for MoCo deficiency type A [252150] and type B [252160])

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