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Translational regulation of human methionine synthase by upstream open reading frames

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Abstract

Methionine synthase is a key enzyme poised at the intersection of folate and sulfur metabolism and functions to reclaim homocysteine to the methionine cycle. The 5′ leader sequence in human MS is 394 nucleotides long and harbors two open reading frames (uORFs). In this study, regulation of the main open reading frame by the uORFs has been elucidated. Both uORFs downregulate translation as demonstrated by mutation of the upstream AUG codons (uAUG) either singly or simultaneously. The uAUGs are capable of recruiting the 40S ribosomal complex as revealed by their ability to drive reporter expression in constructs in which the luciferase is fused to the uORFs. uORF2, which is predicted to encode a 30 amino acid long polypeptide, has a clustering of rare codons encoding arginine and proline. Mutation of a tandemly repeated rare codon for arginine at positions 3 and 4 in uORF2 to either common codons for the same amino acid or common codons for alanine results in complete alleviation of translation inhibition. This suggests a mechanism for ribosome stalling and demonstrates that the cis-effects on translation by uORF2 is dependent on the nucleotide sequence but is apparently independent of the sequence of the encoded peptide. This study reveals complex regulation of the essential housekeeping gene, methionine synthase, by the uORFs in its leader sequence.

Keywords: Methionine; Upstream open reading frame; Methionine synthase; Gene regulation

Homocysteine is an intermediate in methionine metabolism and is cleared via the concerted actions of three enzymes, methionine synthase (MS), cystathionine β-synthase and betaine homocysteine methyltransferase [1]. Of these, only MS is present ubiquitiously and reclaims homocysteine to the methionine cycle via a transmethylation reaction [2]. In contrast, betaine homocysteine methyltransferase, which also catalyzes the remethylation of homocysteine, has a very restricted tissue distribution being present only in liver and kidney. Cystathionine β-synthase commits homocysteine to the transsulfuration pathway that leads to synthesis of cysteine, and is present in some but not all tissues [2].

MS is an essential gene as indicated by the embryonic lethality of the null genotype in transgenic mice [3]. It performs two significant cellular functions. First, by salvaging homocysteine to the methionine cycle, it helps maintain low intracellular levels of this nonprotein amino acid. Elevated levels of homocysteine are correlated with numerous complex diseases including cardiovascular diseases [4], neural tube defects [5] and some neurodegenerative diseases [6]. Second, by demethylating 5-CH_3-tetrahydrofolate, the methyl group donor, MS liberates tetrahydrofolate that is then used widely as a template for one-carbon transfers to support purine and amino acid syntheses. In mammals, MS utilizes methylcobalamin, a derivative of vitamin B_{12} as a cofactor.

In humans, this housekeeping gene appears to be subjected to complex regulation and the open reading frame (ORF) is framed by a 394 base-long leader sequence that precedes it and an ~3000-base long 3′ UTR that follows it. Eukaryotic leader sequences are on average 50–70 bases in length and longer leaders are invariably involved in regulation [7]. Indeed recent studies have identified regulatory elements in the 5′-leader

Abbreviations: MS, methionine synthase; ORF, open reading frame; UTR, untranslated region; uORF, upstream open reading frame; uAUG1, and 2, initiation codons for uORFs 1 and 2; IRES, internal ribosome entry site

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sequence of the MS mRNA [8]. In contrast, the role, if any, of the long 3′ UTR in mediating message stability and/or translational regulation is unknown.

B_{12} supplementation to cells in culture increases the activity of MS [9,10] and this regulation is exerted at a translational level [8,11]. Addition of B_{12} to normal medium results in a reequilibration of the MS message from the inactive ribonucleoprotein pool to the active polysomal pool [8]. The long 5′ leader sequence is predicted to be highly structured posing obvious problems for cap-dependent commencement of translation and suggests the presence of an alternative initiation strategy, i.e. via an IRES element. Indeed, studies with a bicistronic reporter construct confirmed the presence of an IRES element and localized the boundaries to 70 bases immediately upstream of the AUG [12]. Curiously, the boundaries of the B_{12} responsive element coincide with those of the IRES element (Fig. 1) and reveal that IRES-dependent translation of MS is modulated by a nutrient, i.e. the cofactor of the encoded enzyme.

Sequence analysis of the 5′-leader sequence reveals the presence of two ORFs that are upstream of the MS ORF (Fig. 1). The first, uORF1, commences at uAUG1 at position 12 and overlaps with the MS ORF, albeit in a different frame. It is predicted to encode a polypeptide that is 141 residues in length. The second, uORF2, commences at uAUG2 at position 35 and is predicted to encode a shorter polypeptide of 30 amino acids in length. It is estimated that <10% of eukaryotic mRNAs contain uORFs in their leader sequences [13] but they are prominent in oncogenes, two thirds of which have this regulatory element [14]. In addition, leader sequences containing uORFs are prevalent in other genes involved in control of cellular growth and differentiation [15]. In this study, we have evaluated the influence of the uORFs in the leader sequence of human MS and demonstrate that they are negative modulators of translation.

1. Materials and methods

1.1. Materials

Eagle's minimum essential medium (EMEM) and anti-rabbit IgG antibody were purchased from Sigma. FBS (fetal bovine serum) was from HyClone. Cos-1 cells (monkey kidney fibroblast) were obtained from Dr. Charles Wood at the University of Nebraska, Lincoln. Radiolabeled [α-^{32}P] ATP (5000 mCi/mmol) was purchased from Amersham Pharmacia. Restriction enzymes were from Invitrogen or New England Biolabs. PCR reagents and enzymes were from Invitrogen or Stratagene. Primers were purchased from Fisher Scientific.

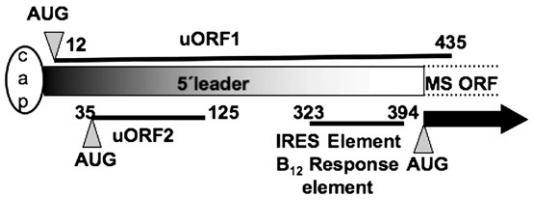

Fig. 1. A schematic representation of the 5′ leader of the human MS mRNA. The relative positions of the uORFs, the IRES element and the B_{12}-response element are indicated by solid lines. The numbers refer to the positions of nucleotides with respect to the 5′ end of the leader sequence (full length is 394 nucleotides). The MS-ORF refers to the main open reading frame shown by the thick arrow, and the cap depicts the me^{7}G cap at the 5′ end of the message.

1.2. Cell culture conditions

Cells were grown in Eagle's MEM supplemented with 10% FBS and incubated at 37 °C, 5% CO_2. For luciferase reporter studies and Western and Northern analyses of MS, cells were grown in 100 mm plates to 60–80% confluency and transfection was performed as described previously [8].

1.3. Plasmid construction

The recombinant plasmids used in this study are shown in Table 1. Primer sequences used to generate individual constructs are shown in Table 2. All PCR reactions were performed using conditions described previously [8] and the high fidelity Pfu polymerase (Stratagene). The DNA sequences of all constructs were verified by nucleotide sequence determination at the Center for Biotechnology's Genomics Core Facility (University of Nebraska-Lincoln). Mutants were generated using the Quickchange Site-Directed Mutagenesis kit (Stratagene).

1.4. Transient transfection and luciferase assays

Transfections were performed using the GeneJammer reagent (Stratagene) for Cos-1 cells. Briefly, 2–8 μg of plasmid DNA was mixed with 10–50 μl of transfection reagent according to the manufacturer's specifications, and the mixture was added to 100 mm plates containing cells at 60% confluency. Reporter gene activities (firefly and Renilla luciferase) were determined according to the vendor's protocol (Promega).

1.5. Northern analysis

Analyses were performed as previously described [8]. A ^{32}P-labeled probe consisting of a DNA fragment encoding the luciferase gene was used (1.1 and 0.9 kb fragment for Firefly or Renilla luciferase, respectively). The bands were quantified by densitometry using Quantity One software (BioRad). The luciferase band intensities were normalized versus 18S rRNA in the same samples. At least three independent experiments were performed for each sample.

1.6. In vitro transcription–translation

The plasmids or PCR fragments containing a T7 promoter upstream of the 5′ UTR were created using the primers and templates shown in Tables 1 and 2 (plasmids pBC220–pBC223). Primer BCOL20 contains the T7 promoter sequence. In vitro transcription–translation was performed according to the manufacturer's protocol (Promega).

2. Results

2.1. Mutation of the uAUGs stimulates translation of the main ORF

There are two initiation codons in the 5′ leader sequence of the MS mRNA preceding the initiation codon for the main ORF. Both uAUGs are followed by ORFs. To evaluate the role of these uORFs on the expression of MS, reporter constructs were prepared by cloning the MS 5′ leader sequence upstream of the luciferase gene (construct pSO101) and mutating uAUG1 and uAUG2 to UUG either singly or together (constructs pSO102, pSO103 and pSO104, respectively) (Fig. 2). In order to study translational effects, the reporter luciferase activities were normalized to the luciferase mRNA levels determined by Northern analysis. Elimination of either uAUG resulted in higher reporter activity (Fig. 2). Specifically, loss of uAUG1 or uAUG2 resulted in 2- or 2.5-fold increase in luciferase activity respectively, while concomitant elimination of both uAUGs