Molecular mechanism of hepcidin deficiency in a patient with juvenile hemochromatosis

We describe a point mutation creating an additional ATG codon in the 5' untranslated region (UTR) of the HAMP gene, in a patient with juvenile hemochromatosis. By transient *in vitro* transfection studies, we provide evidence that the additional ATG is functional and prevents normal hepcidin production by inducing an aberrant translation initiation of the pre-hepcidin mRNA.

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Juvenile hemochromatosis is the result of mutations affecting hepcidin or hemojuvenil genes.[1,2] Recently we found, in two members of a Portuguese family, that juvenile hemochromatosis was associated with a G→A point mutation at position +14 of the 5' untranslated region (UTR) of the HAMP gene in the homozygous state.[3] This nucleotide change led to the creation of a new ATG (AUG) codon, located at positions +39→+41 out of frame with the physiological pre-hepcidin initiation site. Here we present our results of transient transfection assays confirming that this new ATG, which is embedded within a Kozak consensus sequence (GCCRCCatgG), acts as an aberrant strong translation initiation site that precludes normal hepcidin synthesis.

In order to generate a vector able to express green fluorescent protein (GFP) in the dependence of the WT (wild-type) or μ (mutated) 5'UTR of the HAMP gene, we first eliminated the ATG codon of the pEGFP-N1 vector by mutating this ATG to ATT (Quick change II Site-Directed Mutagenesis Kit, Stratagene, 200523, Clontech, USA). The wild type and mutant HAMP 5'UTR were then cloned upstream of the modified EGFP vector, ATG→ATT-pEGFP, into the multi-cloning site. We used two different 63-oligomers: one containing the unmutated sequence GTG in position +14 to +16, and one with the mutated sequence ATG corresponding to the patient's 5'UTR sequence. This resulted in the generation of the p (5'UTR WT) vector and the p (5'UTR μ) vector, respectively (Figure 1A). It should be noted that in the 5'UTR WT construct, the HAMP start ATG was in frame with the EGFP coding sequence, while in the 5'UTR μ the new ATG introduced by the mutation was out of frame with the EGFP coding sequence (Figure 1A). We then placed the EGFP coding sequence in frame with the mutant HAMP 5'UTR proximal ATG (GTG in the wild-type sequence), by a one-nucleotide deletion (Figure 1B). The two new vector constructs were called p-del (5'UTR WT) and p-del (5'UTR μ). We co-transfected 293T cells by calcium phosphate precipitation[4] with the different pEGFP vectors and with a vector coding for the red fluorescent protein DsRed2, pDS Red2-N1 (Clontech, USA), used as a marker of transfection efficiency to normalize results obtained with pEGFP vectors. We analyzed GFP expression by flow-cytometry and measured total fluorescence of GFP (percentage of GFP$^+$ cells multiplied by mean fluorescence intensity). Each experiment was performed three times.

Using the pEGFP-N1 vector we obtained 29.9±9% transfection efficiency. Mutation of the ATG codon in the pEGFP-N1 vector induced an 82% decrease of GFP protein expression [124% for pEGFP-N1 and 22% for the

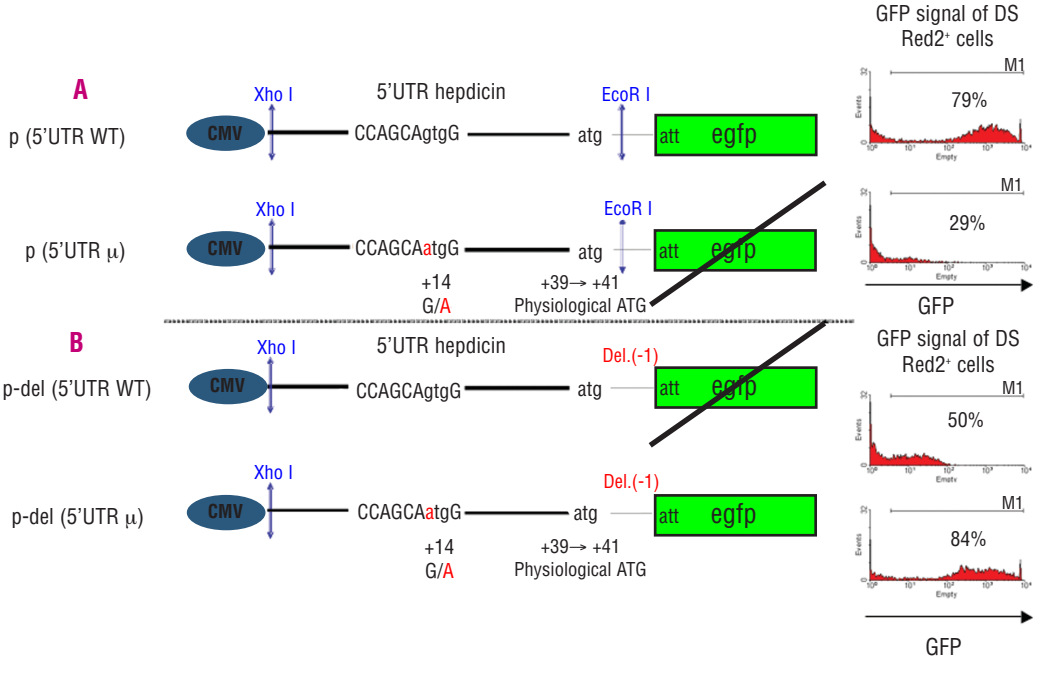

Figure 1. Schematic representation of the pEGFP-N1 constructs and the GFP signal analyzed by flow-cytometry. This signal is that determined for the transfected DS Red2$^+$ cells in one representative experiment out of three. A: pEGFP-N1 constructs containing the wild type/mutated 5'UTR region of the HAMP gene. Note that the normal hepcidin initiation codon is in frame with the EGFP coding sequence. B: pEGFP-N1 constructs containing the wild type/mutated HAMP 5'UTR and one nucleotide deletion at the EGFP gene. In this construct the normal hepcidin initiation codon is out of frame with EGFP coding sequence. Note that results in histograms represent the percentage of GFP$^+$ cells. The total fluorescence of GFP is represented in Figure 2. CMV: promoter cytomegalovirus; EGFP: coding sequence of the enhancer green fluorescent protein.

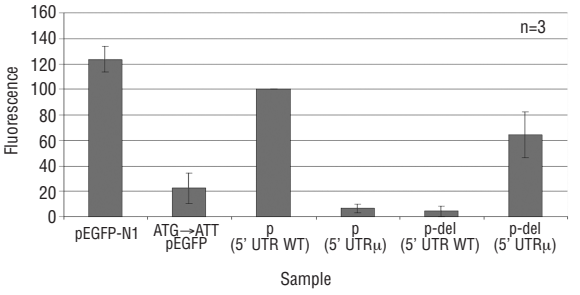

Figure 2. Total fluorescence of GFP (percentage of GFP$^+$ cells multiplied by the mean fluorescence intensity) normalized according to transfection efficiency (DSRed2 signal).

ATG→ATT-pEGFP vector, respectively (Figure 2)]. The insertion of the mutant HAMP 5'UTR in this vector as compared with the insertion of the wild-type HAMP 5'UTR led to a 93% decrease in GFP expression (Figure 1A and Figure 2) which means that the residual activity is about 10%. When the EGFP coding sequence was put in frame with the mutant HAMP 5'UTR proximal ATG (GTG in the wild-type sequence, Figure 1B), GFP expression was restored to levels obtained when its coding sequence was in frame with the physiological hepcidin ATG of the wild type HAMP 5'UTR (Figure 1B). Figure 2 summarizes the results obtained. This confirms the functionality of the new ATG out of frame with the GFP sequence and present in the mutant HAMP gene.

Mutations of hepcidin are a rare cause of juvenile hemochromatosis. We demonstrated, by *in vitro* experiments, that the G→A mutation in the 5'UTR, position +14 from the cap site of the HAMP gene, previously described by us and by Porto *et al.*, is correlated with a severe decrease in hepcidin production when present in the homozygous state.[3,5] This mutation creates a new ATG site located at a relatively short distance from the normal ATG (25 nucleotides upstream). As it is embedded in a favorable flanking nucleotide sequence (the so-called Kozak consensus sequence) with a G at positions –3 and +4, and it is out of frame from the normal iniation codon of the hepcidin gene, this mutation leads to a marked decrease of normal hepcidin synthesis. Our findings also support the proposition that mutations restructuring the 5'UTR upstream of the ATG codon may greatly perturb normal translation of mRNA leading to various diseases including tumorigenesis.[6-9] This region should, therefore, be systematically explored when investigating the molecular mechanism of an inherited or an acquired disease. Our *in vitro* experiments show a residual activity of the physiological ATG (~10%). This result is in accordance with the very low level or absence of hepcidin detected in the urine of the patient described by Porto *et al.*[5] and in our two patients.[3]

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