uAUG-mediated translational initiations are responsible for human mu opioid receptor gene expression

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Abstract

Mu opioid receptor (MOR) is the main site of interaction for major clinical analgesics, particularly morphine. MOR expression is regulated at the transcriptional and post-transcriptional levels. However, the protein expression of the MOR gene is relatively low and the translational control of MOR gene has not been well studied. The 5′-untranslated region (UTR) of the human MOR (OPRM1) mRNA contains four upstream AUG codons (uAUG) preceding the main translation initiation site. We mutated the four uAUGs individually and in combination. Mutations of the third uAUG, containing the same open reading frame, had the strongest inhibitory effect. The inhibitory effect caused by the third in-frame uAUG was confirmed by *in vitro* translation and receptor-binding assays. Toeprinting results showed that OPRM1 ribosomes initiated efficiently at the first uAUG, and subsequently re-initiated at the in-frame #3 uAUG and the physiological AUG site. This re-initiation resulted in negative expression of OPRM1 under normal conditions. These results indicate that re-initiation in MOR gene expression could play an important role in OPRM1 regulation.

Keywords: human mu opioid receptor ● post-transcriptional regulation ● uORF

Introduction

Opioids are potent clinical analgesics but have serious limitations such as tolerance and dependence. The opioid receptors, classified into three major types (μ, δ and κ) have been characterized by molecular cloning and in numerous pharmacological reports [1, 2]. All three types of opioid receptors belong to the superfamily of G-protein-coupled receptors. The mu opioid receptor (MOR) plays roles in morphine-induced analgesia, tolerance and dependence, as indicated by pharmacological studies and analyses of MOR knockout mice [3, 4]. On binding opioids, MOR couples to G-proteins and regulates adenylyl cyclase, intracellular calcium, inwardly rectifying potassium channels, MAP kinase and other messengers, triggering a cascade of intracellular events [5]. MOR is expressed mainly in the central nervous system, with receptors varying in densities in different regions [6]. To achieve its unique spatial expression pattern, expression of MOR must be tightly regulated by mRNA processing, transport, localization and stability. MOR expression can be regulated by multiple mechanisms, including transcriptional (NRSF [7], SP3 isoforms [8], splice variants [9], epigenetic status [10], αCP3 [11], etc.) and post-transcriptional events [12]. However, the translational control of the MOR gene has not been well characterized.

Recently, it has become clear that the 5′-untranslated region (UTR) of eukaryotic mRNA is a key site for many forms of post-transcriptional regulation of gene expression, especially those sites containing at least one AUG codon upstream (uAUG) of the main open reading frame (ORF) [13, 14]. Protein translation in eukaryotes is initiated predominantly by a cap-dependent scanning mechanism in which the small ribosome sub-units, with the associated translation initiation factors, are recruited to the 5′-cap structure and scan in a 5′–3′ orientation until the first AUG is recognized. Then, the large ribosomal sub-unit joins the complex to initiate protein synthesis [15]. In higher eukaryotes, translation usually initiates at the AUG nearest the 5′-end, but this 'first AUG rule' holds true only when the AUG resides in a favourable context [16]. When the first AUG is positioned suboptimally, ribosomes can initiate at the first or second AUG *via* leaky scanning [17]. Re-initiation is another mechanism that allows ribosomes to reach

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and initiate at downstream AUGs. When the first AUG is followed shortly by an in-frame terminator codon, post-termination ribosomes apparently resume scanning and can re-initiate at downstream sites [13, 18]. Multiple proteins can be synthesized from one mRNA, not only by alternative splicing but also by the choice of a translation initiation codon. This choice, and the efficiency of translation, is controlled by mRNA-specific elements such as the GC content and the size of the 5′-UTR, and the location of potentially active uAUGs [19]. The special case in which a uAUG starts a reading frame that is fused in-frame with the principal ORF (and therefore is translated to produce a polypeptide extended at its amino-terminus) is not a distinct upstream open reading frame (uORF), but differential start-codon selection in such cases could involve mechanisms similar to those that contribute to the use of other uAUGs [20]. A uAUG that precedes a terminator codon by only a short distance, thereby creating a small uORF, should reduce (but not abolish) translation of the major ORF, because eukaryotic ribosomes can hold on to the mRNA at a terminator codon, resume scanning and re-initiate downstream [21].

The OPRM1 gene has a high GC content and contains four uAUGs between the transcription initiation sites and the physiological translation initiation codon, suggesting that the uAUGs have the potential to regulate translation. In this study, the effects of uAUGs and uORFs on the translation of major OPRM1 mRNA were analyzed by transient transfection assays, *in vitro* translation and toeprinting. Here, we report that the OPRM1 5′-UTR exerts negative effects on the expression of the downstream MOR gene. We also found that the third uAUG efficiently regulates OPRM1 gene expression, but that all four uAUGs regulated downstream MOR gene expression synergistically. This study suggests that re-initiation in the 5′-UTR at the physiological MOR AUG site could be responsible for the low level of expression of OPRM1 under normal conditions.

Materials and methods

Plasmid construction

All site-directed mutagenic PCR reactions were performed as described previously [22] using Ultra Pfu polymerase (Stratagene, LaJolla, CA, USA) and forward and reverse primers containing appropriate restriction sites at their 5′ and 3′ ends and/or appropriate mutations at the desired sequence sites. Briefly, amplified fragments were cloned into the pGL3-promoter reporter construct (Promega, Madison, WI, USA) using the corresponding restriction sites. The wild-type huAUG(+) was constructed by generating the −291 to +1 PCR fragment of the 5′-UTR of OPRM1 using HindIII-ended forward primer (5′-CAGAAGCTTGCCTCCGAATCCCGCATGGCC-3′) and NcoI-ended reverse primer (5′-AATCCATGGTACTGACGGCCGGGCGGGCAC-3′). This construct was then inserted between the HindIII and NcoI restriction sites of pGL3-promoter reporter construct. Individual uORFs in the OPRM1 5′-UTR (−291 to +1) were inactivated by introducing point mutations into the start codons using oligonucleotide-directed mutagenesis (Stratagene) according to the manufacturer's recommendation, using the following oligonucleotides: huAUG #1: 5′-TCCGAATCCCGCAcGGCCCACGCT-3′ (forward) and 5′-AGCGTGGGCCgTGCGGGATTCGGA-3′ (reverse); huAUG #2: 5′-GGGCAGGTGAcGAGCCTCTGTGAA-3′ (forward) and 5′-TTCACAGAGGC-

TAgTCACCTGCCC-3′ (reverse); huAUG #3: 5′-CGCAGAGGAGAAcTGCAGA-TGCTC-3′ (forward) and 5′-GAGCATCTGACgTTCTCCTCTGCG-3′ (reverse); huAUG #4: 5′-CGCAGAGGAGAATGTCAGAcGCTC-3′ (forward) and 5′-GAG-CgTCTGACATTCTCCTCTGCG-3′ (reverse); huAUG_Main: 5′- AGTACCACG-GAAGACGCCAAA-3′ (forward) and 5′- TTTGGCGTCTTCCgTGGTACT-3′ (reverse). Double mutations were made using the huAUG #3 construct as a template and other primers: huAUG #1, 3: huAUG #1 forward and reverse primers; huAUG #2, 3: huAUG #2 forward and reverse primers; huAUG #3, 4: huAUG #4 forward and reverse primers. For the construct in which all four uAUGs between the HindIII and NcoI site were mutated [huAUG(−)], the U within each AUG sequence was substituted with C in the forward primer and with G in the reverse primer.

SP6 promoter-controlled 5′-UTR/LUC-fused constructs were generated by cloning the luciferase gene into the pGEM-3Z vector (Promega) construct (SP6-LUC). To generate SP6–5′-UTR/LUC fused constructs [SP6-huAUG(+), SP6-huAUG #3, SP6-huAUG(−), SP6-huAUG_Main], 5′-UTR/LUC in-frame fusion constructs were digested between HindIII and NcoI and cloned into the SP6-LUC constructs. Similar constructs containing strong Kozak sequences at the first and second uAUG sites (SP6-huAUG #1S and #2S, respectively) were constructed for oligonucleotide-directed mutagenesis using the following primers: huAUG #1S: 5′-AATCCaCCATGgCCCACG-3′ (forward) and 5′- CGTGGGcCATGGtGGATT-3′ (reverse); huAUG #2S: 5′-GGCAGGTGATGgGCCTCT-3′ (forward) and 5′-AGAGGCcCATCACCTGCC-3′ (reverse).

Constructs with Human MOR 5′-UTR and an Exon fused to Flag (hMUEF) were cloned to the pCMV-Taq4A vector with the OPRM1 UTR. MOR coding regions (exons 1–4) and Flag taq were fused in-frame. First, 1.2 kb of a construct containing the OPRM1 coding region and pCMV4 [9] was digested with NcoI and SalI and cloned into the huAUG constructs [huAUG(+), huAUG #3 and huAUG(−)] using 5′-NcoI and 3′-SalI sites. These intermediate constructs were then cloned into the pCMV-Taq4A vector by ligation (5′-HindIII, 3′-SalI). All constructs were confirmed by sequencing analysis.

Cell culture, DNA transfection and reporter gene assay

Human neuroblastoma NMB cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum. Transfection and reporter gene assays were carried out as described previously [23]. Briefly, cells were plated in 6-well plates at a concentration of 1×10^6 cells/well and cultured overnight before transfection. For luciferase reporter analysis of each promoter construct, 1 μg of the reporter plasmid was mixed with the Effectene transfection reagent (QIAGEN, Valencia, CA, USA) for 10 min. before being added to the well. Forty-eight hours post-transfection, cells were washed once with phosphate-buffered saline and lysed with lysis buffer (Promega). To correct for the differences in transfection efficiency, a one-fifth molar ratio of a pCH110 plasmid (Amersham, Piscataway, NJ, USA) containing the β-galactosidase gene under the SV40 promoter was included in each transfection for normalization. The luciferase and galactosidase activities of each lysate were determined according to the manufacturer's instructions (Promega and Tropix, Foster City, CA, USA respectively).

Quantification of LUC and LacZ transcripts by real-time PCR and RT-PCR

Total RNA was isolated according to the supplier's protocol (TRI Reagent; Molecular Research Center, Inc., Cincinnati, OH, USA). After quantification

of total RNA by measuring OD at 260 nm, 1 μg of RNA was treated with one unit of DNase I (Invitrogen, Carlsbad, CA, USA). Reverse transcription using oligo-dT primer was performed with the Transcription First strand cDNA synthesis kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. The first strand obtained was quantified using a real-time quantitative PCR system: a SYBR Green assay on the iCycler Optical System (Bio-Rad, Hercules, CA, USA). The following oligonucleotides were used for the amplification of 159-bp and 105-bp fragments of cDNAs, corresponding to LUC or LacZ, respectively: LUC primers, 5′-CCAGGACTG-GTTTCTGTAAG-3′ (forward) and 5′-CTTTATGTTTTTGGCGTCTTCC-3′ (reverse); LacZ primers, 5′-GCTGCATAAACCGACTACACAAA-3′ (forward) and 5′-GCCGCACATCTGAACTTCAG-3′ (reverse). After first-strand cDNA synthesis, the samples were amplified at 95°C for 30 sec, 60°C for 30 sec, 70°C for 30 sec for real-time PCR. For RT-PCR, the Qiagen one-step RT-PCR kit was used with the above primers. Relative mRNA levels were reported as the ratio of LUC mRNA to LacZ mRNA.

In vitro transcription/translation and autoradiography

Capped mRNAs were synthesized *in vitro* with the MAXIscript *In Vitro* Transcription Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Briefly, after linearization by *Sal*I digestion, DNA was gel-purified (QIAGEN). The resulting DNA was transcribed *in vitro* by SP6 RNA polymerase in the presence (*i.e.* capped) of 1 mM of the methylated cap analog m7GpppG (Ambion). After a 1-hr incubation at 37°C, samples were treated with 2 units of DNase for 15 min. at 37°C. After ethanol precipitation and a 70% ethanol wash, RNA was re-suspended in DEPC-treated water. RNA integrity was confirmed by gel electrophoresis. The amounts of RNA were analyzed by spectrophotometry and ethidium bromide visualization.

Equal amounts (0.1 μg) of RNA were added to the TnT Quick Coupled Transcription/Translation System (Promega) for translation under conditions recommended by the manufacturer. *In vitro*-translated proteins were labelled with L-[35S]-methionine (Amersham). Reactions were incubated for 60 min. at 30°C and analyzed on 10% SDS-PAGE. The gels were dried and exposed to a PhosphorImager screen overnight at room temperature. The translated peptides were detected using a Molecular Dynamic Storm 860 PhosphorImager system (Amersham).

Radioligand-binding assay

Binding assays were performed as described previously [9]. Briefly, after transient transfection with the indicated constructs, MOR gene expression was determined by a whole-cell–binding assay using [3H]diprenorphine in 25 mM HEPES buffer, 5 mM MgCl$_2$ (pH 7.6). Specific binding was defined as the difference between the radioactivities bound to the cells in the presence and absence of 100 μM of the mu-opioid antagonist, CTOP.

Primer extension inhibition (toeprinting) assay

The toeprinting assay was performed as described previously [22]. Briefly, the deoxyoligonucleotides mToe 1 (5′-TCAGTTTCTTACAAGGACAAG-3′) and mToe 2 (5′-CTTATGCAGTTGCTCTCCAG-3′) were labelled at the 5′-end by T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; Amersham). After the toeprinting reaction, the primer–mRNA complexes were incubated on ice for 15 min., whereas the reticulocyte reaction mixtures were assembled.

The ribosome binding reaction used micrococcal-nuclease-treated rabbit reticulocytes (Promega). Primer extension was initiated by adding 2 U/μl Superscript II reverse transcriptase (Invitrogen). Reactions were terminated by phenol extraction. Primer extension products were mixed with 98% formamide and 10 mM EDTA, heated for 5 min. at 90°C, and separated on 4–6% polyacrylamide sequencing gels. Sequencing ladders were generated by a fmol DNA cycle sequencing system (Promega) according to the manufacturer's protocol. Autoradiograms of the dried gels were obtained using a Molecular Dynamic Storm 860 PhosphorImager system.

Results

The 5′-UTR of OPRM1 contains four uORFs preceding the MOR initiation codon

The OPRM1 consists mainly of four exons (Fig. 1A). Several transcription initiation sites have been identified: One major site corresponds to the cDNA start positioned 216 bp upstream from the translation start codon [24], and at least three minor ones (285, 358 and 373 bp, respectively) occur upstream from the ATG start codon [25]. The 291-bp fragment of the OPRM1 5′-UTR was chosen to clone into reporter constructs for the current study (Fig. 1B). The OPRM1 5′-region contains four uAUGs (uAUG #1, #2, #3 and #4). Two of these (#2 and #4) were out-of-frame and three (#1, #2 and #4) terminate before the main initiation codon. However, #1 and #3 were in-frame, and the #3 uAUG shares the stop codon with the main ORF. Translational initiation at the first, second, third and fourth uAUGs would give rise to uORFs containing 17, 27, 463 and 8 amino acids (1.9 kD, 3 kD, 51.7 kD and 0.9 kD, respectively) (Fig. 1B and C).

In vertebrate mRNAs, initiation sites usually conform to all or part of the sequence GCC**R**CC<u>AUG</u>**G**, known as the Kozak sequence [21]. The most conserved nucleotides are the **R** (A or G) at −3 and the **G** at +4 (the A of the AUG codon is designated as +1). Strong consensus sequences contain both of these important nucleotides, whereas an adequate sequence contains only one of them, and weak sequences contain none of them. In the OPRM1 transcript, uAUG #4 is weak, whereas uAUGs #1, #2 and #3 have adequate sequences. The OPRM1 main ORF has a strong consensus sequence similar to that of the luciferase main ORF (Fig. 1C).

The third uAUG has an inhibitory effect on initiation at the main AUG

To determine whether these uORFs affect the expression of the downstream main MOR ORF, we constructed a series of plasmids (Fig. 2A) containing point mutations for each uAUGs (*i.e.* ATG changed to ACG). These wild-type and mutant constructs were transfected transiently in NMB cells that express the MOR gene endogenously. After transfection, cellular extracts were prepared and assayed for translation (*e.g.* luciferase assay, LUC/β-gal ratio) and transcription (real-time RT-PCR, LUC/LacZ mRNA) levels (Fig. 2B).
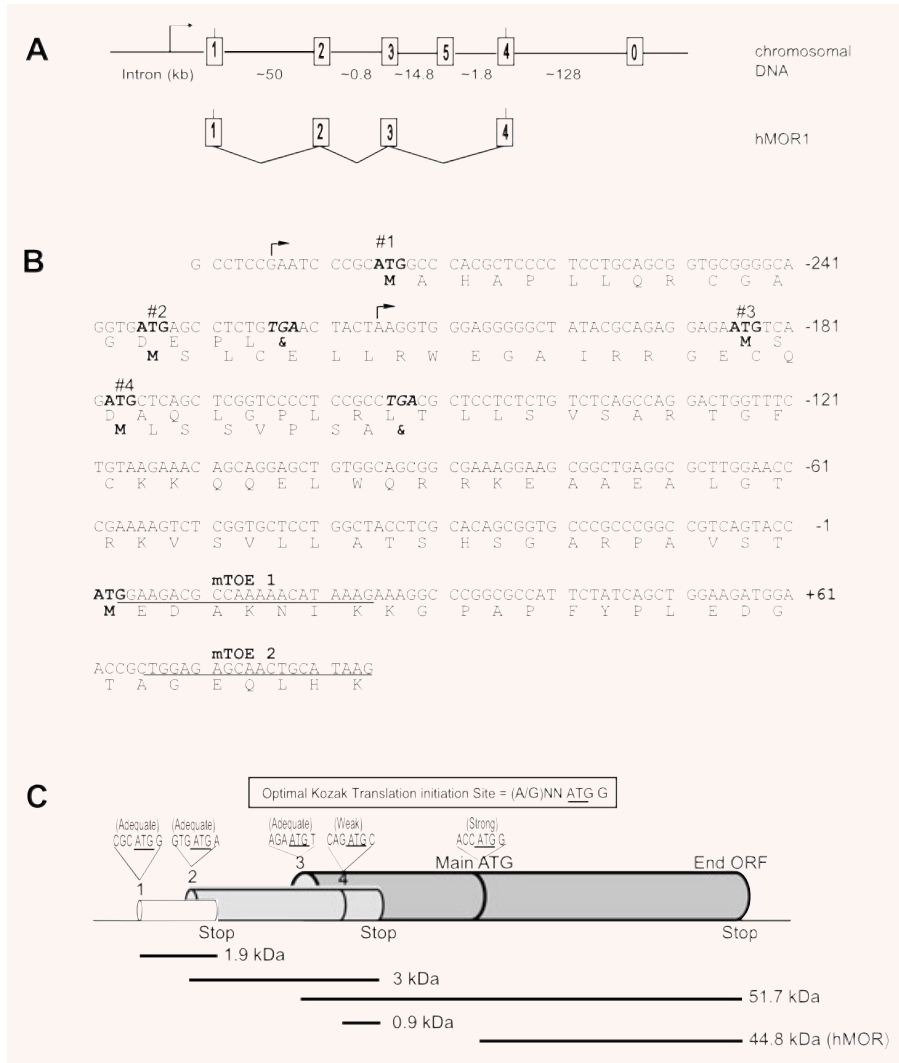
Fig. 1 Schematic of the human mu opioid receptor (OPRM1) and putative uORF sequences. (**A**) The OPRM1 structure and main transcript. Exons and introns are shown by boxes and horizontal lines, respectively. The promoter is indicated by the arrow. Exons are numbered in the order in which they were identified. Translation start and stop sites are shown by bars above the exon boxes. OPRM1 is the main MOR messenger RNA identified in humans. (**B**) Sequence of the OPRM1 5′-UTR used in this study. Four uAUG codons (#1–#4) and the main AUG are shown in bold, and the termination codon of the uORFs is italicized. Bold peptide sequences represent the uAUG and stop codons. The arrowheads above the sequence indicate the transcription initiation sites. mToe 1 and 2 indicate radiolabelled oligonucleotide primers for toeprinting. (**C**) uAUG sequences compared with the Kozak sequence. The −3 and +4 positions relative to the start codon (underlined) are represented in bold. Each uORF consists of 17, 27, 462 and 8 putative amino acids, respectively. The uAUG #3 uORF consists of 62 additional amino acids, relative to the main ORF (*i.e.* 400 amino acids) at the 5′-end.

The levels of transcripts were very similar among all constructs, indicating that the point mutations did not alter transcription levels. In contrast, LUC activity was differentially affected by the mutations of these uAUGs. In particular, mutation of the third uAUG increased LUC activity two-fold, but mutations of the other uAUGs had no significant effect. Mutating all the uAUGs [huAUG(−)] increased luciferase activity relative to mutating uAUG #3 alone but not significantly. These results suggest that the presence of the third uAUG in the MOR 5′-UTR represses LUC expression, and that the effect occurs at the level of translation.

Repression of OPRM1 translation through the uORF in the MOR 5′-UTR

We examined the initiation of peptide synthesis at the #3 uAUG by *in vitro* translation. Several constructs under the control of the SP6 promoter were generated (Fig. 3A). Using a rabbit reticulocyte lysate system, LUC synthesis should occur if ribosomes initiate translation at the uAUG codons of the uORF and/or the LUC main initiation site. The results (Fig. 3B) show that both the 5′-extended LUC and LUC proteins were expressed by the huAUG(+) construct, but only the LUC protein was expressed by either the huAUG #3 or huAUG(−) constructs. In contrast, the construct mutated at the main ORF initiation site (huAUG_Main) only expressed the 5′-extended LUC fusion protein. As expected, the 5′-extended LUC fusion protein had a molecular weight higher than the control LUC protein. These data demonstrate that the 5′-extended LUC fusion product (initiated at the #3 uAUG) is translated efficiently, but these effects were not reflected in any differences in the transcription levels (Fig. 3B, lower panel). Similar results were obtained in *in vitro* translation experiments in NMB cells transfected with the same constructs using a SV40 promoter-controlled vector (Fig. 3C).
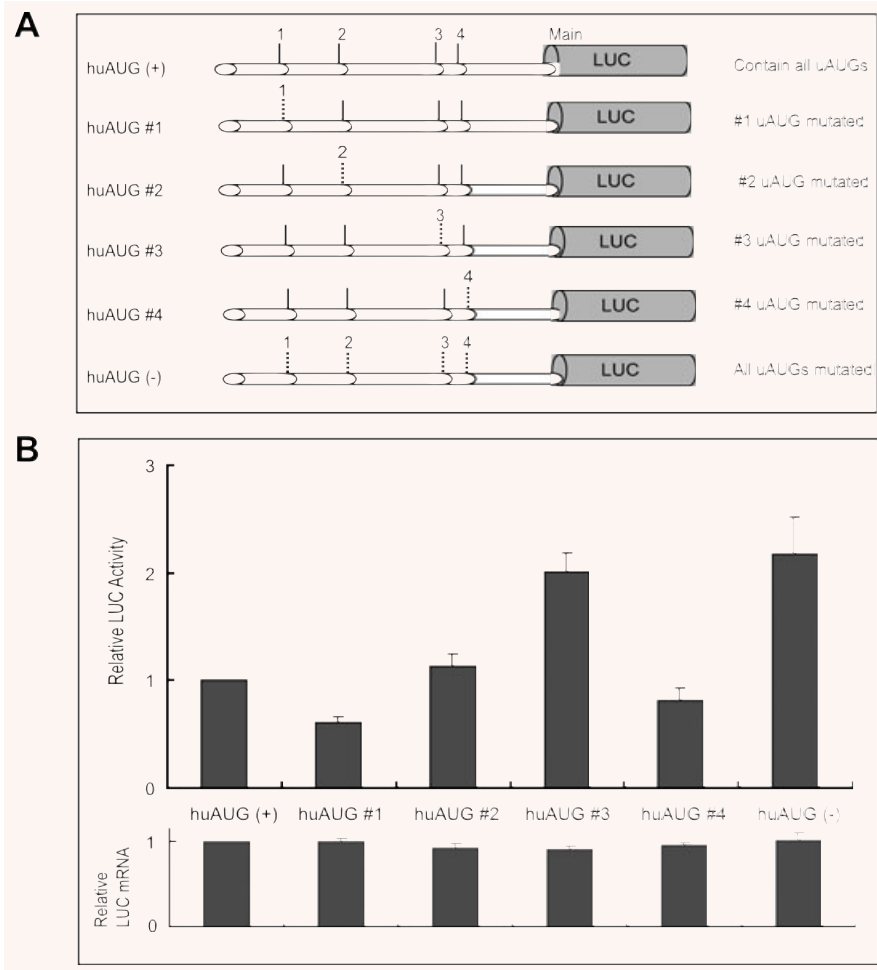
Fig. 2 Translation of the OPRM1 gene is controlled by uORFs. (**A**) Schematic representation of reporter constructs with wild-type and mutant human 5′-UTRs. Vertical dotted lines represent ATGs converted to ACGs by point mutations. (**B**) Transient transfection of each mutant construct in NMB cells. After transfection, cells were trypsinized and half were used for luciferase and β-galactosidase activity assays, whereas the other half was examined by RNA extraction and transcript quantification. Relative LUC activity and mRNA levels were determined as the ratio LUC/β-gal and LUC/LacZ. Error bars indicate the standard errors of triplicate LUC assays.

Overlapping uORFs preclude downstream initiations

As shown earlier, constructs mutated at all four uAUGs [huAUG(−)] showed only a minor increase in translation levels relative to constructs mutated at the third uAUG alone. Nevertheless, both *in vitro* translation (Fig. 3B) and transient transfection (Fig. 3C) were altered by the huAUG(−) construct. Therefore, we considered that the other uAUGs might have a synergistic effect on the effect of the #3 uAUG mutation with regard to downstream regulation. Double mutations including #3 uAUG (Fig. 4A) increased expression by the main ORF (Fig. 4B). Interestingly, the construct with in-frame mutations of both the #1 and #3 uAUGs (huAUG #1, 3) increased downstream expression nearly 3.5-fold (Fig. 4B). These effects did not result from differences in transcription levels (Fig. 4B, lower panel).

The strongest inhibition is caused by a uORF that overlaps the start of the downstream cistron [15, 26–28]. Indeed, the size of

the first ORF is a major limitation on re-initiation in eukaryotes: re-initiation can occur following the translation of a 'minicistron' (a small first ORF) but not following the translation of a full-length 5′ cistron [15]. Other studies have suggested that a uORF that extensively overlaps the downstream cistron is even more inhibitory than one that terminates very close to the beginning of the next coding sequence, presumably because re-initiation is very inefficient when ribosomes are forced to scan 'backwards' over large distances or additional AUG codons [29, 30].

To test this effect, we made constructs containing strong sequences at the #1 and #2 uAUGs (Fig. 4C) and examined downstream ORF initiation by *in vitro* translation (Fig. 4D). The #1 uORF overlaps the #2 uAUG and the #2 uORF overlaps the #3 uAUG site (Fig. 1C). However, improving the context at the upstream site reduces or abolishes initiation from the second site. Whether the second AUG codon resides in a strong or weak context is irrelevant: the ribosomes read the mRNA linearly, and thus the decision to stop or to bypass the first AUG is not influenced by the presence of a better initiation site downstream [15]. Also, improving
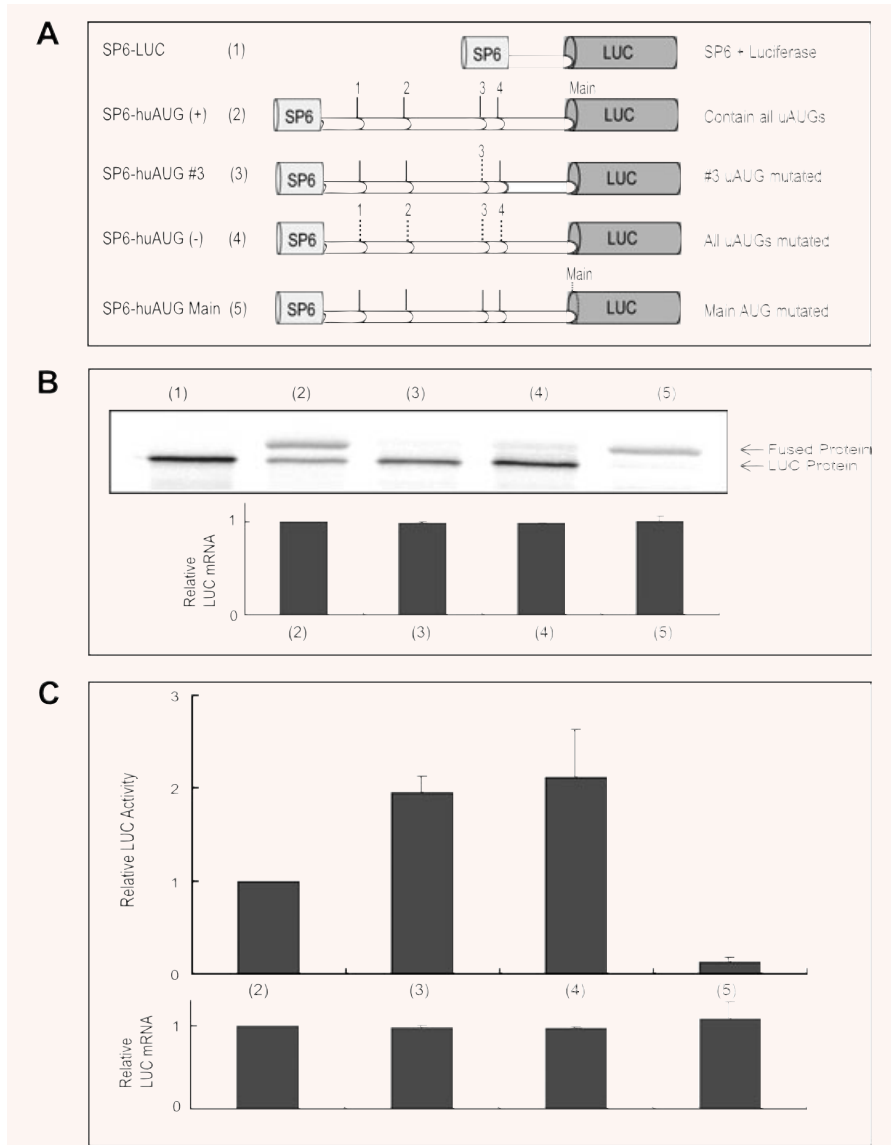
Fig. 3 The expression levels from the in-frame #3 uAUG and main initiation codons within the OPRM1 5′-UTR region. (**A**) Schematics of the constructs used. Initiation codons with vertical dotted lines indicate point mutations of ATGs to ACGs. (**B**) Representative autoradiogram of proteins translated by a coupled transcription/translation system in the presence of [^{35}S]-methionine from *in vitro* translations. The lower panel shows the relative mRNA levels from the system as quantified by real-time PCR. (**C**) Forty-eight hours after transfection with the above constructs (controlled by an SV40 promoter), cells were trypsinized and examined for luciferase and β-galactosidase activities, as well as by RNA extraction and transcript quantification. Relative LUC activity and mRNA levels are expressed as the ratio LUC/β-gal and LUC/LacZ, respectively.

the context at the #2 uAUG relative to huAUG(+) reduced downstream #3 initiation, and consequently increased initiation at the main ORF (Fig. 4D, lane 6) but only at the level of translation. In addition, the strong #1 uAUG construct only affected initiation at the overlapped #2 uAUG site. Therefore, initiation at the #3 uAUG continuously represses the downstream initiation (Fig. 4D, lane 5), relative to the huAUG(+) control.

Because human uORFs apparently can repress downstream MOR gene expression, we performed opioid radioligand-binding assays (Fig. 5A). NMB cells transfected with hMUEF #3 showed binding activity up to 2 times higher than that observed in cells transfected with the control vector [hMUEF(+)], and hMUEF(−)-transfected cells showed binding activity up to 3.5 times higher

than controls. This is even greater than the two-fold increase in luciferase activity seen in uAUG(−)-luciferase constructs (Fig. 4B). These results support the notion that uORFs can synergistically regulate MOR expression.

Toeprints correspond to ribosomes at the uAUG start codons, and ribosome re-initiation regulates OPRM1 expression

Toeprinting assays can reveal the effects of initiation context and regulated-ribosome stalling on the association of ribosomes with mRNA [31]. We therefore examined two mRNAs: the huAUG(+)
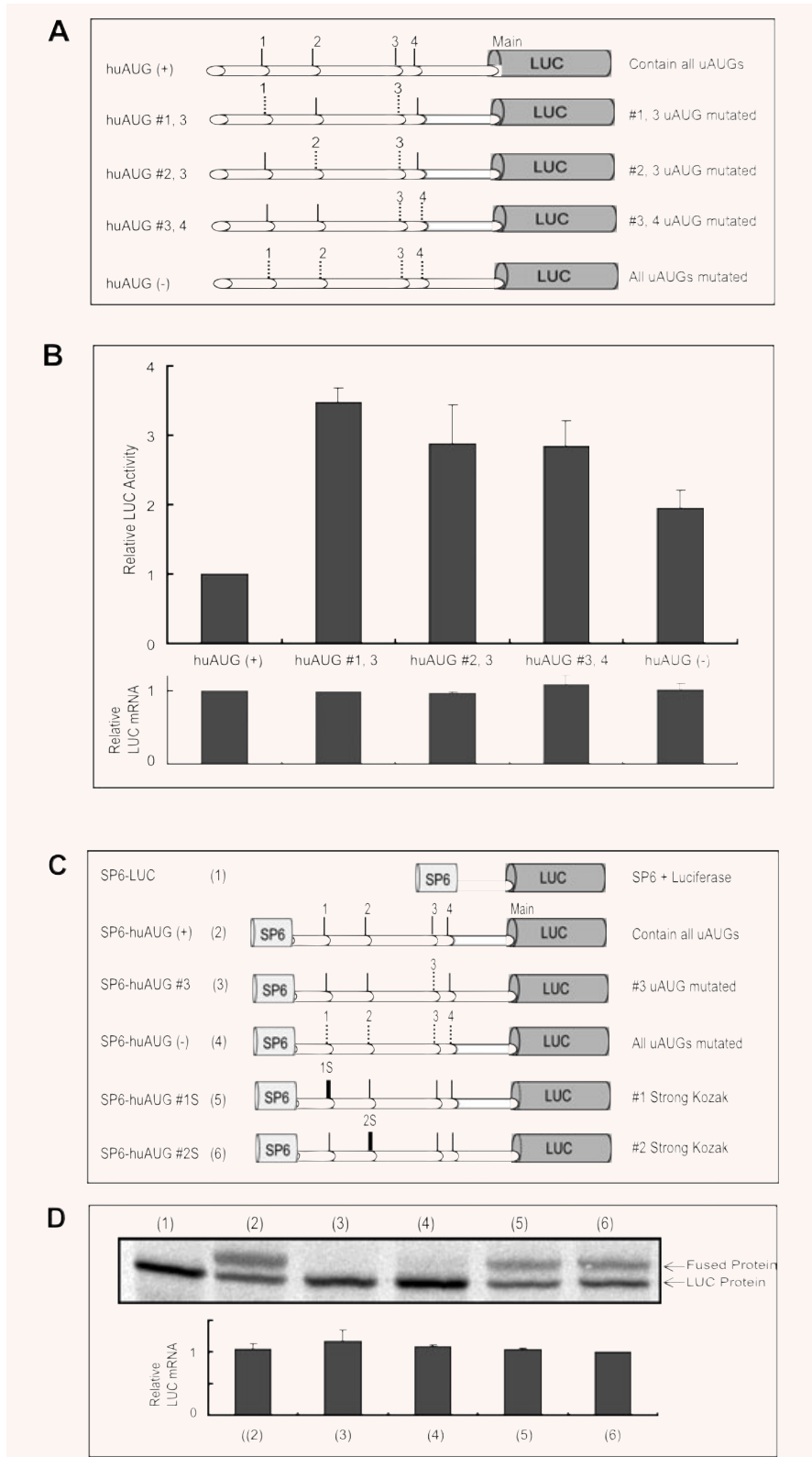
Fig. 4 Overlapping uORFs can regulate re-initiation at the main ORF. (**A**) Schematic representation of reporter constructs with wild-type and mutant human 5′-UTRs. Vertical dotted lines represent ATGs converted to ACGs by point mutations. (**B**) Relative LUC activity and mRNA levels are expressed as the ratio LUC/β-gal and LUC/LacZ, respectively. Error bars indicate the standard errors of triplicate LUC assays. (**C**) Schematics of the constructs used. Initiation codons with vertical dotted lines indicate point mutations of ATGs to ACGs. Thick lines (1S and 2S) indicate strong Kozak sequences. (**D**) Representative autoradiogram of proteins translated by a coupled transcription/translation system in the presence of [^{35}S]-methionine from *in vitro* translations. Data are mean values from triplicate determinations with standard error bars.
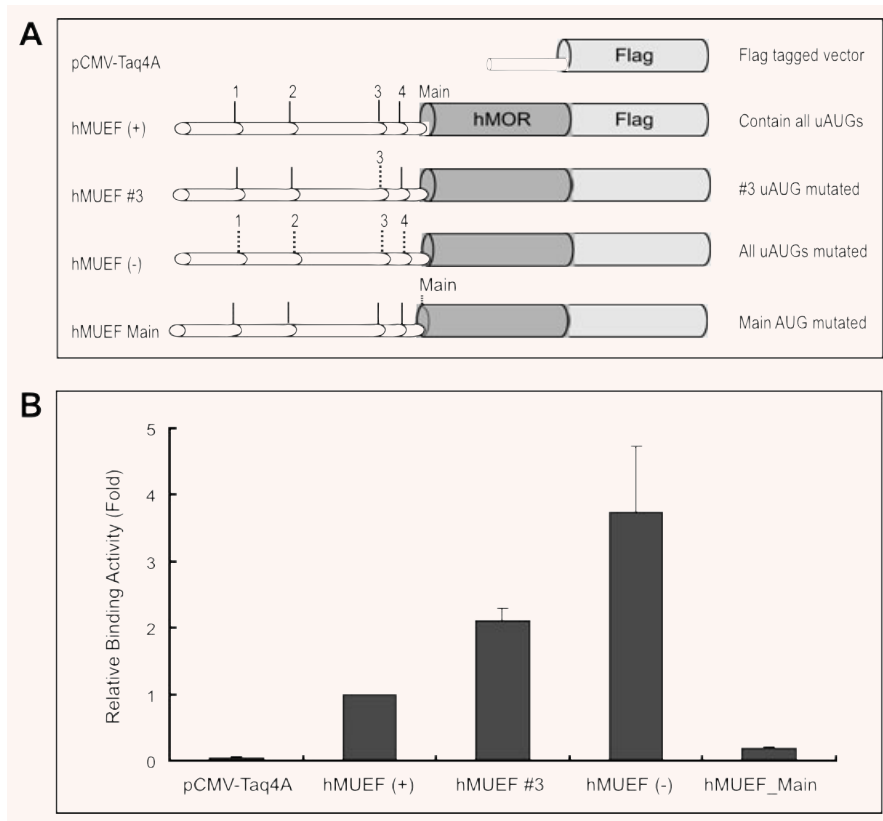
Fig. 5 The uORFs can affect the expression of human mu opioid receptor. (**A**) Structures of fused constructs. Each construct contains OPRM1 promoter and coding regions. The coding regions were fused in-frame to the Flag tag, except for the negative control vector (pCMV-Taq4A). (**B**) Radioligand-binding assay. Opioid receptor-expressing cells were transfected transiently with the above reporter DNA constructs. Data represent the binding of [^{3}H]diprenorphine to NMB cells. Mean values from representative triplicate are shown with standard error bars.

construct containing the uORFs in their wild-type initiation context, and the huAUG(−) construct mutated at all four uAUG sites.

Toeprints corresponding to ribosomes at each of the OPRM1 uORF start codons and the main ORF start codon (Fig. 1B) were observed in rabbit reticulocyte lysates by radiolabelled mToe primer 2 (to verify the scanning mechanisms) and mToe primer 1 (to elucidate the exact toeprints on each uAUG). The huAUG(+) toeprint map showed 16 nucleotides downstream of the AUG codons, indicating ribosomes with the initiation codon in their P-site [32, 33]. As shown in the preceding experiments, all the uAUG sequences were sufficient by the Kozak sequence rule except uAUG #4 (*i.e.* a weak Kozak sequence). The uAUG #1 toeprint was stronger than other uAUGs (Fig. 6A and B), illustrating the 'first AUG rule'. In higher eukaryotes, this rule holds strictly only when the 5′ proximal AUG codon resides in a favourable context [34, 35]. In all cases in which one or more OPRM1 uORFs were present, the initiation codon closest to the mRNA 5′-end showed the greatest extent of ribosome loaded during steady-state translation. Re-initiation allows ribosomes to reach and initiate at downstream AUG codons. Many studies have shown that when the first AUG codon is followed shortly by an in-frame terminator codon, post-termination ribosomes apparently resume scanning and can re-initiate at a downstream site [16].

To test the OPRM1 translational mechanism, we compared toeprints with cycloheximide added to extracts before adding RNA template (T0) or added after translation was underway (T5) (Fig. 6A). During re-initiation, scanning ribosomes load at the uORF start codon and initiate translation at each uAUG; they then re-initiate translation downstream. Adding cycloheximide at T0 traps ribosomes at the positions where they first load on the mRNA. Adding it during steady-state translation (T5) traps ribosomes where they are loaded following the primary initiation event and subsequent re-initiation events.

When cycloheximide was added at T0, toeprints corresponding predominantly to the start codon nearest the 5′-end of the mRNA were observed (Fig. 6A, lane 1). However, the other uAUG toeprints also showed 16 nucleotides after the uAUG site. When no uORFs were present, substantial loading of ribosomes occurred at the main AUG start codon (Fig. 6A, lanes 2 and 4). When cycloheximide was added at T5, toeprints should have revealed both the uAUGs and the main AUG. However, toeprints from these samples showed only the uAUGs (Fig. 6A, lane 3), which suggests that some other factor inhibits loading the ribosome at the main AUG initiation site. This inhibition can be explained by the #3 in-frame uORF initiation sites. Ideally, ribosomes should be present both on the uAUGs and the main AUG sites in T5 samples. However, in OPRM1, the ribosomes apparently bind only to the uAUGs sites, likely because ribosomes loaded on the in-frame #1 and #3 uAUGs translate until the ribosome complex reaches the main ORF stop codon. Overall, these
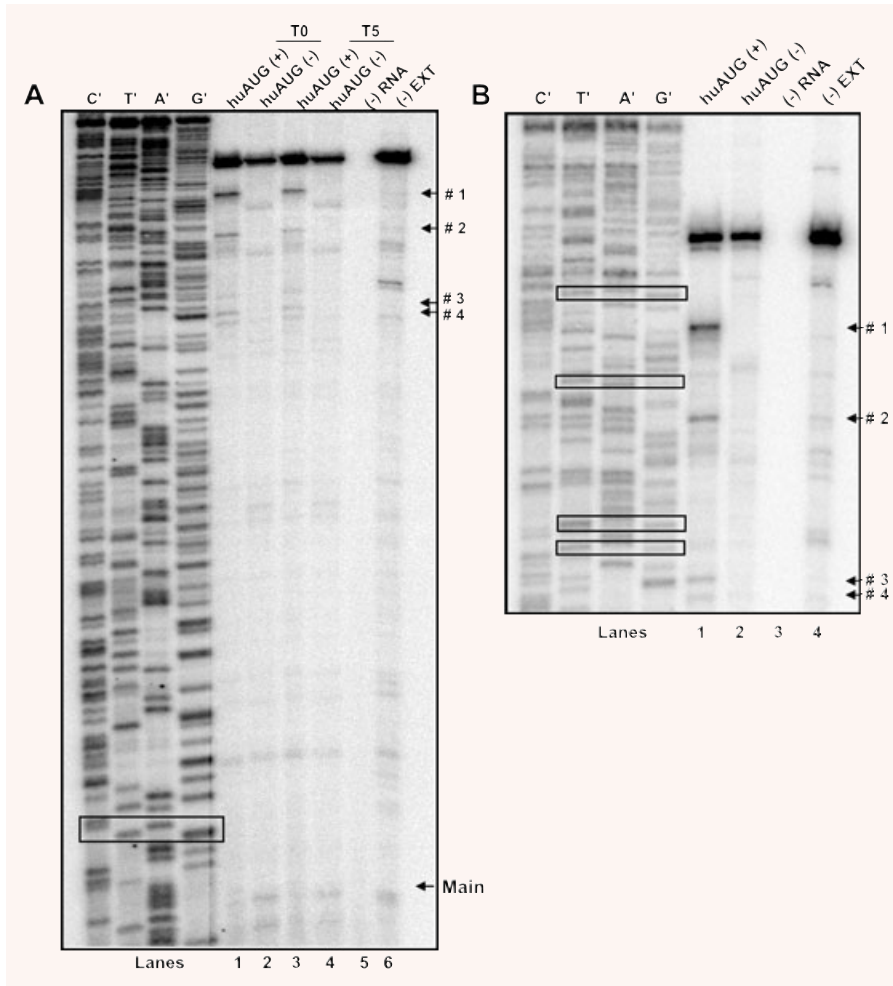
Fig. 6 Toeprinting analyses of OPRM1 containing uORFs that regulate ribosome re-initiation. The positions of the uORFs and main start codons in the DNA sequences are boxed. (**A**) Synthetic OPRM1-LUC RNA containing the wild-type uORFs [huAUG(+)] or with all four uORFs deleted [huAUG(−)] were incubated with cycloheximide either before incubation (T0) or after 5 min. of incubation at 30°C (T5). Reaction mixtures without extract (−EXT) or mRNA (−RNA) served as controls. The positions of the cDNA extension products corresponding to the mRNA 5′ end, uORF initiation and LUC initiation codons radiolabelled by the mToe 2 primer are indicated. Arrows indicate toeprints of the uAUGs and the main AUG. (**B**) Toeprint analyses of initiation at uAUGs. Radiolabelled mToe 1 primer was used for primer extension analyses and for sequencing the uAUG(+) templates. The nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is indicated above the corresponding lane (C′, T′, A′ and G′) so that the sequence of the template can be deduced directly by 5′–3′ sequence reads from top to bottom. Arrows indicate the toeprints of each uAUG and the main AUG.

data indicate that ribosomes that translate uORFs re-initiate downstream at the main start codon, that is, when the first AUG codon is followed shortly by an in-frame termination codon. Post-termination ribosomes apparently resume scanning and re-initiate at a downstream site [16]. Minor bands were also seen; these minor products might represent either authentic truncated mRNAs or false priming [31]. Figure 6B shows the relative strengths of toeprints using the mToe primer 1: Toeprints appear 16 bp downstream of each ATG sequence.

Discussion

The use of heroin and the high mortality associated with heroin intoxication continue to escalate, as does addiction to prescription opiate drugs. The rewarding effects of heroin and its psychoactive metabolites, for example, morphine, are mediated strongly *via* the mu opioid receptor [36].

To date, mu opioid receptor genes have been cloned and revealed the dendrogram (Fig. 7A) [37]. The alignment of the 5′-UTR sequences from human, monkey, guinea pig, pig, cow, rat and mouse mu opioid receptor transcripts reveals a low degree of similarity (Fig. 7B). However, human #2 uAUG is conserved in pig #1 uAUG and mouse #3 uAUG, human #3 uAUG is conserved in pig #3 uAUG only. These sequences came from their mRNA transcript (GenBank database accession no. shown in Fig. 7 legends). In addition, the alignment of the amino acid sequences for the uORFs in these species also showed a low degree of similarity (Fig. 7C). These results show that the relative positions of the uAUGs and uORFs from human and other mammal's mu opioid receptor show no significant similarities.

In this study, we have used the 291 bp of the OPRM1 5′-UTR to test the expression patterns of MOR initiated at uAUGs. The 291-bp extension of the OPRM1 5′-UTR region includes the major MOR mRNA transcription sequence (derived from the OPRM1 promoter) and contains four uORFs (Fig. 1 and Supplemental Fig. 1).
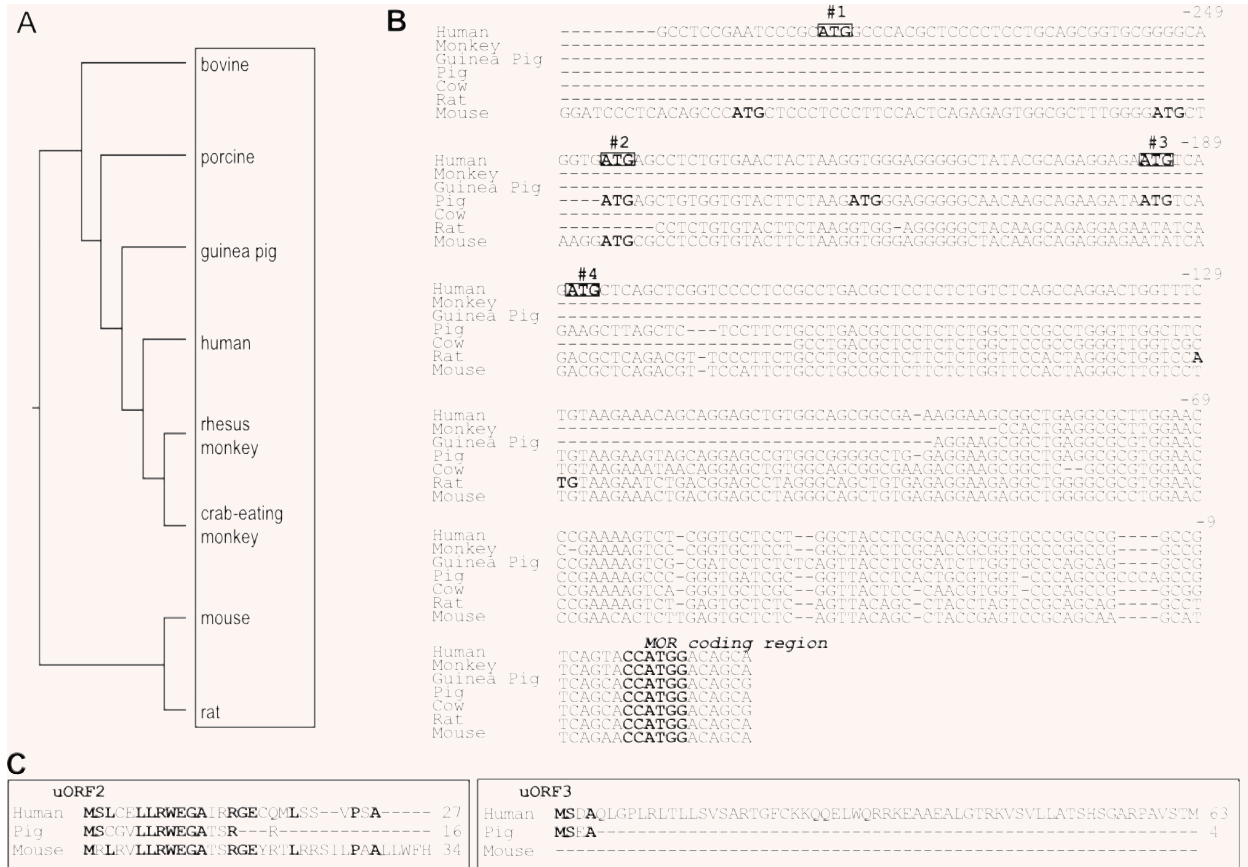
A

bovine

porcine

guinea pig

human

rhesus monkey

crab-eating monkey

mouse

rat

B

```
                                                    #1                              -249
Human       ----------GCCTCCGAATCCCGCATGGCCCACGCTCCCCTCCTGCAGCGGTGCGGGGCA
Monkey      ----------------------------------------------------------
Guinea Pig  ----------------------------------------------------------
Pig         ----------------------------------------------------------
Cow         ----------------------------------------------------------
Rat         ----------------------------------------------------------
Mouse       GGATCCCTCACAGCCCATGGCTCCCTCCCTTCCACTCAGAGAGTGGCGCTTTGGGGATGCT

            #2                                              #3   -189
Human       GGTCATGAGCCTCTGTGAACTACTAAGGTGGGAGGGGGCTATACGCAGAGGAGAATGCCA
Monkey      ---------------------------------------------------------
Guinea Pig  ---------------------------------------------------------
Pig         ----ATGAGCTGTGGTGTACTTCTAAGCATGGGAGGGGGCAACAAGCAGAAGATAATGCCA
Cow         ---------------------------------------------------------
Rat         ---------CCTCTGTGTACTTCTAAGGTGG-AGGGGGCTACAAGCAGAGGAGAATATCA
Mouse       AAGGATGCGCCTCCGTGTACTTCTAAGGTGGGAGGGGGCTACAAGCAGAGGAGAATATCA

            #4                                                    -129
Human       CATGCTCAGCTCGGTCCCCTCCGCCTGACGCTCCTCTCTGTCTCAGCCAGGACTGGTTTC
Monkey      ---------------------------------------------------------
Guinea Pig  ---------------------------------------------------------
Pig         GAAGCTTAGCTC----TCCTTCTGCCTGACGCTCCTCTCTGGCTCCGCCTGGGTTGGCTTC
Cow         GACGCTCAGACGT-TCCCTTCTGCCTGCCGCTCCTCTCTGGTTCCGCCTGGGTGGCTCA
Rat         GACGCTCAGACGT-TCCCTTCTGCCTGCCGCTCCTCTCTGGTTCCACTAGGGCTTGTCCT
Mouse       GACGCTCAGACGT-TCCATTCTGCCTGCCGCTCTTCTCTGGTTCCACTAGGGCTTGTCCT

                                                              -69
Human       TGTAAGAAACAGCAGGAGCTGTGGCAGCGGCGA-AAGGAAGCGGCTGAGGCGCTTGGAAC
Monkey      --------------------------------------CCACTGAGGCGCTTGGAAC
Guinea Pig  ---------------------------------AGGGAAGCGGCTGAGGCGCGTGGAAC
Pig         TGTAAGAAGTAGCAGGAGCCGTGGCGGGGGCTG-GAGGAAGCGGCTGAGGCGCGTGGAAC
Cow         TGTAAGAAATAACAGGAGCTGTGGCAGCGGCGAACAAGCAGAGGCTC--GCGCGTGGAAC
Rat         TGTAAGAATCTGACGGAGCCTAGGGCAGCTGTGAGAGGAAGAGGCTGAGGCGCTGGAAC
Mouse       TGTAAGAAACTGACGGAGCCTAGGGCAGCTGTGAGAGAAGAGGCCTGGGAGCTGGAAC

                                                              -9
Human       CCGAAAAGTCT-CGGTGCTCCT--GGCTACCTCGCACAGCGGTGCCCGCCCG----GCCG
Monkey      C-GAAAAGTCC-CGGTGCTCCT--GGCTACCTCGCACAGCGGTGCCCGCCCG----GCCG
Guinea Pig  CCGAAAAGTCG-CGATCCTCTCTCAGTTACCTCGGGCATCTTGGTGCCCAGCAG----GCCG
Pig         CCGAAAAGCCC--GGGTGATCGC--AGGTTACCTCACTGCGTGGT-CCCAGCGCCCACGCG
Cow         CCGAAAAGTCA-GGGTGCTCCC--GGTTACTCC-CAACGTGGT-CCCAGCGGG----GGGG
Rat         CCGAAAAGTCT--GAGTGCTCTC--AGTTACAGC-CTACCTAGGTCCCGCAGCAG----GCCT
Mouse       CCGAACACTCTTGAGTGCTCTC--AGTTACAGC-CTACCGAGTCCGCAGCAA----GCAT

            MOR coding region
Human       TCAGTACCATGGACAGCA
Monkey      TCAGTACCATGGACAGCA
Guinea Pig  TCAGCACCATGGACAGCG
Pig         TCAGCACCATGGACAGCA
Cow         TCAGCACCATGGACAGCG
Rat         TCAGCACCATGGACAGCA
Mouse       TCAGAACCATGGACAGCA
```

C

```
uORF2
Human   MSLCELLRWEGAIRRGECQMLSS--VPSA----- 27
Pig     MSCGVLLRWEGATSR---R------------- 16
Mouse   MKLRVLLRWEGATSRGEYRTLRRSILPAALLWFH 34

uORF3
Human   MSDAQLGPLRLTLLSVSARTGFCKKQQELWQRRKEAAEALGTRKVSVLLATSHSGARPAVSTM e3
Pig     MSEA------------------------------------------------------- 4
Mouse   ---------------------------------------------------------
```

Fig. 7 OPRM1 5′-UTR contains no conserved uORF. (**A**) Dendrogram of mu opioid receptors based on their amino acid identity [37]. (**B**) Sequence alignment of the 5′-UTR region of the human (used in this study), monkey (GenBank database accession no. AY038989), guinea pig (GenBank database accession no. AY166606), pig (GenBank database accession no. AF521309), cow (GenBank database accession no. U89677), rat (GenBank database accession no. L13069) and mouse [22] opioid receptor mRNA. Bold nucleotide sequences are indicated uAUGs and main ATG site. The nucleotide sequences of the human uORFs are boxed (#1, 2, 3 and 4). (**C**) Amino acid sequence alignment of the uORF of human, pig and mouse opioid receptor. All uORF amino acid sequences showed before their stop codon except human uORF 3 (showed until main ORF initiation codon). The conserved amino acids are bolded.

An overlapping uORF down-modulates translation in murine IL-12 [26], and the overlapping uORF in the mRNAs of human GlyRS affects protein expression qualitatively [38]. Ribosomes that translate the uORF thereby miss the first in-frame AUG codon but proceed to re-initiate at another start codon downstream. In the OPRM1, there are also two in-frame uAUGs (#1 and #3) before the physiological AUG. Only mutations in the third uAUG site regulated downstream ORF expression, and only at the level of translation.

The results of the *in vitro* translation assays (Fig. 3B) indicated that mutating all four uAUG sites increased MOR expression relative to constructs mutated only at the #3 site. We reported previously that the Oprm1 gene has two different uAUGs that affect its downstream expression synergistically [22]. Experiments with double mutations showed that mutating both the #1 and #3 in-frame uAUG codons tremendously increased downstream ORF expression in humans as well. We also tested the ability of overlap-ping uORFs to down-modulate translation by introducing strong context sequences before the #3 in-frame uAUG (Fig. 4C and D). The natural mRNA is slightly leaky because the context flanking the #1 uAUG is not a perfect match to the Kozak sequence. In rat $A_{2A}R$ adenosine receptor mRNA, an overlapping uORF that initiates at an AUG codon in a strong context minimizes production of $A_{2A}R$ protein [15]. In OPRM1, an overlapping uORF containing a strong context can more effectively regulate downstream ORF (Fig. 4C and D), suggesting that in-frame uORFs (*e.g.* #1 and #3) in the OPRM1 could regulate the downstream ORF expression.

MOR receptor–binding assays confirmed that these uORFs mediated down-regulation of cell surface MOR expression. Indeed, the presence of the uORFs decreased expression almost 3.5-fold relative to samples lacking the uORFs (Fig. 5).

The toeprinting analysis showed that ribosomes initiate on multiple uAUGs (except for the #4 uAUG, which contains a

weak context). Therefore, the mRNAs containing 5′-UTRs of OPRM1 are translated by a re-initiation mechanism. The inefficient re-initiation mode of translation can regulate gene expression in eukaryotes in several ways [39]. First, uORFs can limit expression of potent proteins, which are required in small amounts but would be harmful if over-expressed [17]. Second, the site where translation re-initiates is dependent on the availability of eIF-GTP. This can be manipulated by kinases, which respond to growth conditions and other circumstances [40]. Third, the peptide produced by translation of a small uORF has regulatory effects [41]. In the case of MOR, uAUG/uORFs could regulate spatial and temporal expression. Future studies will determine what factors affect the re-initiation process in the OPRM1.

For downstream re-initiation to occur, the 40S sub-unit must reacquire Met-tRNA$_i$, and this appears to be an important point of control [15]. Re-acquisition of Met-tRNA$_i$ is prompted by lengthening the intercistronic domain [21]. As a rough guide, re-initiation often follows translation of a uORF of 10–12 codons; re-initiation is reduced, but not abolished, when a uORF of 13 codons was lengthened to 33 codons [16]. Although the size of the uORF might have varying effects, in general, longer intercistronic distances tend to allow more efficient re-initiation [20].

In conclusion, our results demonstrate that the first and third in-frame uAUGs in the 5′-UTR of the OPRM1 mRNA can function efficiently as translation initiation sites, and that the third uORF negatively affects OPRM1 expression at the level of translation. In addition, the negative effects of the uORFs act synergistically. Re-initiation is involved in the inhibition of MOR expression initiated at the physiological AUG. Such re-initiation results in weak expression of OPRM1 under normal conditions.

Acknowledgements

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Materials and methods

Real-time PCR

We performed real-time qRT-PCR in the Supporting Fig. 1 described in Materials and Methods. We used four different primer set (Supporting Fig. 1A) for each transcript [Derived from TIS 1 (-258), TIS 2 (-285), TIS 3 (-358), and TIS 4(-373)]. These primer sets are same as the previous reported primer set [wendel B., Hoehe MR, *J Mol Med*, 1998; 76: 525–32]. except for the antisense primers for the real-time PCR product size. Control PCR experiments were also performed using the corresponding templates.

Fig. S1 Expression of the transcription initiation sites (TIS)-derived MOR transcript in NMB cells analyzed by real-time qRT-PCR. Levels of MOR mRNA were determined by real-time qPCR analysis. Five micrograms of total RNA was treated with DNase I and reverse transcribed using reverse transcriptase (Roche) and primers combined with oligo(dT) and random hexamer. One-fortieth of this cDNA sample was used for real-time qRT-PCR analysis of gene expression, using the Quantitect SYBR Green PCR kit (QIAGEN) in an iCycler (Bio-Rad). The relative expression of mRNA was calculated using the comparative threshold cycle method as described in Materials and Methods after normalization against β-actin as an internal control. Primer sequences are shown in panel A.

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References

1. **Min BH, Augustin LB, Felsheim RF, *et al.*** Genomic structure analysis of promoter sequence of a mouse mu opioid receptor gene. *Proc Natl Acad Sci USA.* 1994; 91: 9081–5.

2. **Wei LN, Loh HH.** Regulation of opioid receptor expression. *Curr Opin Pharmacol.* 2002; 2: 69–75.

3. **Kieffer BL.** Recent advances in molecular recognition and signal transduction of active peptides. *Cell Mol Neurobiol.* 1995; 15: 615–35.

4. **Law PY, Wong YH, Loh HH.** Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol.* 2000; 40: 389–430.

5. **Maldonado R, Saiardi A, Valverde O, *et al.*** Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature.* 1997; 388: 586–9.

6. **Mansour A, Fox CA, Akil H, *et al.*** Opioid receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* 1995; 18: 22–9.

7. **Kim CS, Hwang CK, Choi HS, *et al.*** Neuron-restrictive silencer factor (NRSF) functions as a repressor in neuronal cells to regulate the mu opioid receptor gene. *J Biol Chem.* 2004; 279: 46464–73.

8. **Choi HS, Hwang CK, Kim CS, *et al.*** Transcriptional regulation of mouse mu opioid receptor gene: Sp3 isoforms (M1, M2) function as repressors in neuronal cells to regulate the mu opioid receptor gene. *Mol Pharmacol.* 2005; 67: 1674–83.

9. **Choi HS, Kim CS, Hwang CK, *et al.*** The opioid ligand binding of human mu-opioid receptor is modulated by novel splice variants of the receptor. *Biochem Biophys Res Commun.* 2006; 19: 1132–40.

10. **Hwang CK, Song KY, Kim CS, *et al.*** Evidence of endogenous mu opioid receptor regulation by epigenetic control of the promoters. *Mol Cell Biol.* 2007; 27: 4720–36.

11. **Choi HS, Kim CS, Hwang CK, *et al.*** Novel function of the poly(C)-binding protein alpha CP3 as a transcriptional repressor of the mu opioid receptor gene. *FASEB J.* 2007; 21: 3963–673.

12. **Wei LN, Law PY, Loh HH.** Post-transcriptional regulation of opioid receptors in the nervous system. *Front Biosci.* 2004; 9: 1665–79.

13. **Morris DR, Geballe AP.** Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol.* 2000; 20: 8635–42.

14. **Peri S, Pandey A.** A reassessment of the translation initiation codon in vertebrates. *Trends Genet.* 2001; 17: 685–7.

15. **Kozak M.** Pushing the limits of the scanning mechanism for initiation of translation. *Gene.* 2002; 299: 1–34.

16. **Kozak M.** Constraints on reinitiation of translation in mammals. *Nucleic Acid Res.* 2001; 29: 5226–32.

17. **Kozak M.** An analysis of vertebrate mRNA sequences: intimations of translational control. *J Cell Biol.* 1991; 115: 887–903.

18. **John E, McCarthy G.** Posttranslational control of gene expression in yeast. *Microbiol Mol Biol Rev.* 1998; 62: 1492–553.

19. **Meijer HA, Thomas AA.** Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. *J Biochem.* 2002; 367: 1–11.

20. **Sachs MS, Geballe AP.** Downstream control of upstream open reading frames. *Genes Dev.* 2006; 20: 915–21.

21. **Kozak M.** Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. *Mol Cell Biol.* 1987; 7: 3438–45.

22. **Song KY, Hwang CK, Kim CS, *et al.*** Translational repression of mouse mu opioid receptor expression *via* leaky scanning. *Nucleic Acid Res.* 2007; 35: 1501–13.

23. **Kim CS, Choi HS, Hwang CK, *et al.*** Evidence of the neuron-restrictive silencer factor (NRSF) interaction with Sp3 and its synergic repression to the mu opioid receptor (MOR) gene. *Nucleic Acid Res.* 2006; 34: 6392–403.

24. **Wang JB, Johnson PS, Persico AM, *et al.*** cDNA and genomic clones, pharmacologic characterization and chromosomal assignment. *FEBS Lett.* 1994; 338: 217–22.

25. **Wendel B, Hoehe MR.** The human mu opioid receptor gene: 5' regulatory and intronic sequences. *J Mol Med.* 1998; 76: 525–32.

26. **Babik JM, Adams E, Tone Y, *et al.*** Expression of IL-12 is regulated by translational control of the p35 subunit. *J Immunol.* 1999; 162: 4069–78.

27. **Cao J, Geballe AP.** Translational inhibition by a human cytomegalovirus upstream open reading frame despite inefficient utilization of its AUG codon. *J Viol.* 1995; 69: 1030–6.

28. **Kos M, Denger S, Reid G, *et al.*** Upstream open reading frames regulate the translation of multiple mRNA variants of the estrogen receptor alpha. *J Biol Chem.* 2002; 277: 37131–8.

29. **Abastado JP, Miller PF, Jackson BM, *et al.*** Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for GCN4 translational control. *Mol Cell Biol.* 1991; 11: 486–96.

30. **Peabody DS, Berg P.** Termination-reinitiation occurs in the translation of mammalian cell mRNAs. *Mol Cell Biol.* 1988; 6: 2695–703.

31. **Sachs MS, Wang Z, Gaba A, *et al.*** Toeprint analysis of the positioning of translation apparatus components at initiation and termination codons of fungal mRNAs. *Methods.* 2006; 26: 105–14.

32. **Wang Z, Sachs MS.** Arginine-specific regulation mediated by the *Neurospora crassa arg*-2 upstream open reading frame in a homologous, cell-free *in vitro* translation system. *J Biol Chem.* 1997; 272: 255–61.

33. **Pestova TV, Hellen CU, Shatsky IN.** Canonical eukaryotic initiation factors determine initiation of translation by internal ribosome entry. *Mol Cell Biol.* 1996; 16: 6859–69.

34. **Kozak M.** Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell.* 1986; 44: 283–92.

35. **Kozak M.** Recognition of AUG and alternative initiator codons is augmented in position +4 but is not generally affected by the nucleotides in positions +5 and +6. *EMBO J.* 1997; 16: 2482–92.

36. **Drakenberg K, Nikoshkov A, Horváth MC, *et al.*** μ Opioid receptor A118G polymorphism in association with striatal opioid neuropeptide gene expression in heroin abusers. *Proc Natl Acad Sci USA.* 2006; 103: 7883–8.

37. **Waldhoer M, Bartlett SE, Whistler JL.** Opioid Receptors. *Annu Rev Biochem.* 2004; 73: 953–90.

38. **Mudge SJ, Williams JH, Eyre HJ, *et al.*** Complex organization of the 5'-end of the human glycine tRNA synthetase gene. *Gene.* 1998; 209: 45–50.

39. **Kozak M.** Regulation of translation *via* mRNA structure in prokaryotes and eukaryotes. *Gene.* 2005; 361: 13–37.

40. **Clemens MJ.** Initiation factor eIF2a phosphorylation in stress responses and apoptosis. *Prog Mol Subcell Biol.* 2001; 27: 57–89.

41. **Fang P, Spevak CC, Wu C, *et al.*** A nascent polypeptide domain that can regulate translation elongation. *Proc Natl Acad Sci USA.* 2004; 101: 4059–64.