Morphine Regulates Expression of \( \mu \)-Opioid Receptor MOR-1A, an Intron-Retention Carboxyl Terminal Splice Variant of the \( \mu \)-Opioid Receptor (OPRM1) Gene via miR-103/miR-107

Zhigang Lu, Jin Xu, Mingming Xu, Gavril W. Pasternak, and Ying-Xian Pan

Department of Neurology and the Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York

Received September 5, 2013; accepted December 3, 2013

ABSTRACT

The \( \mu \)-opioid receptor (MOR-1) gene OPRM1 undergoes extensive alternative splicing, generating an array of splice variants. Of these variants, MOR-1A, an intron-retention carboxyl terminal splice variant identical to MOR-1 except for the terminal intracellular tail encoded by exon 3b, is quite abundant and conserved from rodents to humans. Increasing evidence indicates that microRNAs (miRNAs) regulate MOR-1 expression and that \( \mu \) agonists such as morphine modulate miRNA expression. However, little is known about miRNA regulation of the OPRM1 splice variants. Using 3′-rapid amplification cDNA end and Northern blot analyses, we identified the complete 3′-untranslated region (3′-UTR) for both mouse and human MOR-1A and their conserved polyadenylation site, and defined the role the 3′-UTR in mRNA stability using a luciferase reporter assay. Computer models predicted a conserved miR-103/107 targeting site in the 3′-UTR of both mouse and human MOR-1A. The functional relevance of miR-103/107 in regulating expression of MOR-1A protein through the consensus miR-103/107 binding sites in the 3′-UTR was established by using mutagenesis and a miR-107 inhibitor in transfected human embryonic kidney 293 cells and Be(2)C cells that endogenously express human MOR-1A. Chronic morphine treatment significantly upregulated miR-103 and miR-107 levels, leading to downregulation of polyribosome-associated MOR-1A in both Be(2)C cells and the striatum of a morphine-tolerant mouse, providing a new perspective on understanding the roles of miRNAs and OPRM1 splice variants in modulating the complex actions of morphine in animals and humans.

Introduction

Morphine and most clinical analgesic agents act through \( \mu \)-opioid receptors (MORs). Pharmacologic studies have defined several \( \mu \) receptor subtypes including \( \mu_1 \), \( \mu_2 \), and morphine-6-glucuronide (M6G) receptors (Wolozin and Pasternak, 1981; Pasternak, 1993; Rossi et al., 1995, 1996; Reisine and Pasternak, 1996). However, a single \( \mu \)-opioid receptor gene (OPRM1) has been identified in all the species (Min et al., 1994; Giros et al., 1995; Liang et al., 1995), raising the possibility of alternative pre-mRNA splicing of the OPRM1 gene to generate multiple splice variants with diverse actions. This concept is supported by antisense mapping studies (Rossi et al., 1995, 1997), the isolation of an array of splice variants in mice, rats, and humans (Pan, 2005; Pasternak and Pan, 2013), and gene targeting studies (Schuller et al., 1999; Pan et al., 2009; Majumdar et al., 2011b).

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants R01-DA03997, R21-DA02944, R01-DA06241, R56-DA02615 and R01DA07242]; and a core grant from the National Institutes of Health National Cancer Institute [Grant CA08748] (to the Memorial Sloan-Kettering Cancer Center).

This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: HEK293, human embryonic kidney 293 cells; \(^{125}\)I-IBNtxA, \(^{125}\)I-3-iodobenzoylnaltrexamide; LNA, locked nucleic acid; M6G, morphine-6-glucuronide; miRNA, micro RNA; MOR-1, \( \mu \)-opioid receptor; OPRM1, \( \mu \)-opioid receptor; PANK, pantothenate kinase; PCR, polymerase chain reaction; PFC, prefrontal cortex; poly(A), polyadenylation; qPCR, quantitative polymerase chain reaction; RACE, rapid amplification cDNA ends; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; TM, transmembrane; 3′-UTR, 3′-untranslated region; wt, wild-type.

1521-0111/85/2/368$25.00 http://dx.doi.org/10.1124/mol.113.089292

Copyright 2014 by The American Society for Pharmacology and Experimental Therapeutics

http://molpharm.aspetjournals.org/content/suppl/2013/12/03/mol.113.089292.DC1

Morphine Regulates Expression of \( \mu \)-Opioid Receptor MOR-1A, an Intron-Retention Carboxyl Terminal Splice Variant of the \( \mu \)-Opioid Receptor (OPRM1) Gene via miR-103/miR-107

Morphine and most clinical analgesic agents act through \( \mu \)-opioid receptors (MORs). Pharmacologic studies have defined several \( \mu \) receptor subtypes including \( \mu_1 \), \( \mu_2 \), and morphine-6-glucuronide (M6G) receptors (Wolozin and Pasternak, 1981; Pasternak, 1993; Rossi et al., 1995, 1996; Reisine and Pasternak, 1996). However, a single \( \mu \)-opioid receptor gene (OPRM1) has been identified in all the species (Min et al., 1994; Giros et al., 1995; Liang et al., 1995), raising the possibility of alternative pre-mRNA splicing of the OPRM1 gene to generate multiple splice variants with diverse actions. This concept is supported by antisense mapping studies (Rossi et al., 1995, 1997), the isolation of an array of splice variants in mice, rats, and humans (Pan, 2005; Pasternak and Pan, 2013), and gene targeting studies (Schuller et al., 1999; Pan et al., 2009; Majumdar et al., 2011b).

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants R01-DA03997, R21-DA02944, R01-DA06241, R56-DA02615 and R01DA07242]; and a core grant from the National Institutes of Health National Cancer Institute [Grant CA08748] (to the Memorial Sloan-Kettering Cancer Center).

This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: HEK293, human embryonic kidney 293 cells; \(^{125}\)I-IBNtxA, \(^{125}\)I-3-iodobenzoylnaltrexamide; LNA, locked nucleic acid; M6G, morphine-6-glucuronide; miRNA, micro RNA; MOR-1, \( \mu \)-opioid receptor; OPRM1, \( \mu \)-opioid receptor; PANK, pantothenate kinase; PCR, polymerase chain reaction; PFC, prefrontal cortex; poly(A), polyadenylation; qPCR, quantitative polymerase chain reaction; RACE, rapid amplification cDNA ends; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; TM, transmembrane; 3′-UTR, 3′-untranslated region; wt, wild-type.
splice variants. Continuous translation from exon 3a to exon 3b predicts four amino acids, VRSL in hMOR-1A and mMOR-1A and VCAF in rMOR-1A, before encountering the same TAG stop codon (Pasternak et al., 2004; Xu et al., 2013). Similar to other OPRM1 splice variants, mMOR-1A mRNA is differentially expressed in various brain regions (Xu et al., 2013). When expressed in Chinese hamster ovary cells, mMOR-1A and rMOR-1A display high μ binding affinity and selectivity (Bolan et al., 2004; Pasternak et al., 2004; Xu et al., 2013). However, they revealed marked differences in agonist-induced total G protein stimulation determined by guanosine 5′-O-(3-[35S]thio)triphosphate binding as compared with other C-terminal splice variants (Bolan et al., 2004; Pasternak et al., 2004; Xu et al., 2013), suggesting the functional significance of the C-terminal tails in agonist-induced G protein coupling and signaling transduction.

Pre-mRNA 3′-end processing in most eukaryotic genes involves cleavage and polyadenylation through conserved cis-elements in the 3′-untranslated region (3′-UTR), and is tightly coupled to transcription, splicing, and transport from the nucleus to the cytoplasm and translation as well as influencing mRNA stability (Mandel et al., 2008; Elkon et al., 2013; Tian and Manley, 2013). The complete 3′-UTR containing polyadenylation [poly(A)] signal and cleavage site of the original human and mouse MOR-1 was identified (Ide et al., 2005; Wu et al., 2005). However, little is known for the 3′-UTR of the OPRM1 splice variants including MOR-1A.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that bind to target mRNAs to regulate their stability and translation. Mu opioids such as morphine modulate expression of a number of miRNAs (Sanchez-Simon et al., 2010; Zheng et al., 2010; Wu et al., 2008, 2013; 2009; Dave and Khalili, 2010; He et al., 2010; Wang et al., 2011), and several miRNAs regulate MOR-1 expression (Wu et al., 2008, 2009, 2013; He et al., 2010). Dysregulation of miRNAs has been linked to morphine tolerance and addiction (He et al., 2010; Dreyer, 2010; Hwang et al., 2012; Tapocik et al., 2013). Yet there has been a lack of information regarding miRNA

---

**Fig. 1.** Schematic of OPRM1 gene structure and MOR-1A splice variants. MOR-1A variants from the human (A), mouse (B), and rat (C) OPRM1 genes. Exons and introns are shown by colored boxes and black horizontal lines, respectively. Promoters are indicated by arrows. Exons are numbered in the order in which they were identified. Translation start and stop points are shown by bars below and above exon boxes, respectively. The complete list of the mouse OPRM1 alternative splicing was described in the reviews by Pan (2005) and Pasternak and Pan (2013).
regulation of the OPRM1 splice variants. In the present study, we isolated the complete 3'-UTRs of hMOR-1A and mMOR-1A that contain the cleavage and poly(A) signal sites and identified a pair of paralogous miRNAs, miR-103 and miR-107, that regulate expression of hMOR-1A and mMOR-1A posttranscriptionally through a conserved miR-103/107 binding site in the 3'-UTRs. We further demonstrated that morphine altered the expression of hMOR-1A in Be(2)C cells and mMOR-1A in the striatum of morphine tolerance mice via miR-103 and miR-107.

Materials and Methods

3'-Rapid Amplification cDNA Ends and Sequencing. Total RNAs were extracted from human embryonic kidney 293 (HEK293) cells, Be(2)C cells, and mouse brain with TRI Reagent (Life Technologies, Norwalk, CT). Poly(A) plus RNA was then isolated from the total RNAs using MicroPoly(A) Purist (Ambion, Austin, TX), and used in 3'-RACE mainly following the protocol described in the 3'-rapid amplification cDNA ends (RACE) Kit (Clontech, Mountain View, CA) with some modification. Briefly, the first-strand cDNA was synthesized by using mRNA as template, 3'-RACE primer (5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC TGG AGC GGC TTT TTT TTT TTT TTT TTT TTT VN-3') and SuperScript III (Life Technologies), and used in the first-round polymerase chain reaction (PCR) with a sense primer for mouse, mA (5'-CCT AGT GTT GCTTG CAG TTC TGC ATT GGG TGT TAC AC-3'), or for human, hA (5'-CAT CCA ACC TGG TAC TGG GAA AAC CTG-3') and an antisense primer, AP1 (5'-TAT CTT AAC TGC GGA GGA GC-3'). The first-round PCR product was then used in the second-round or nested PCR with a sense primer for mouse, mb (5'-CTG CCG TTC AGT CAG TGG TGG GAG GGT ACG CAG TCT CTA GAA-3'), or for human, hb (5'-GGA AGC AAA TGG TGG TCT TGC ATT TGG TAG AGA AG-3'), and an antisense primer, AP2 (5'-ACT CAC TAT GAG GCT GGA GCC GC-3'). PCRs were performed with Platinum Taq DNA polymerase under conditions consisting of a 2-minute denaturing at 95°C and 39 cycles of amplification at 95°C for 20 seconds, 65°C for 30 seconds, and 72°C for 1 minute followed by an 8-minute extension at 72°C. PCR products from the second PCRs were separated in a 1.5% agarose gel. Amplified cDNA fragments were extracted by gel extraction kit (Qiagen, Valencia, CA) and sequenced with the primers used in PCR in both orientations.

Northern Blot Analysis. Northern blot analysis was performed as described previously elsewhere (Pan et al., 2001) with minor modifications. Briefly, 3 µg of poly(A) plus RNA was separated on a 0.8% formaldehyde agarose gel and transferred to a GenePlus membrane. After prehybridized in Ultra hybridization buffer (Ambion) at 42°C for 2 hours, the membrane was then hybridized with 32P-labeled cDNA probes in Ultra hybridization buffer at 42°C overnight, washed sequentially with High-stringent and Low-stringent washing buffers (Ambion), and exposed to Kodak BioMax MS film (Eastman Kodak Company, Rochester, NY). Images were captured using ChemiDoc MP system (Bio-Rad Laboratories, Hercules, CA). 32P-labeled probes were generated by PCRs with following primers: mouse probe 1 (415 bp) located at exon 3a, sense primer (5'-TGC TCA AAA TTA GTG TCT TCA TCT TGC TCT CTA TC-3') and antisense primer (5'-GTT TTG AGC AAT TCG TGC ATT TGG GAG GGT ACG CAG-3'); mouse probe 2 (143 bp) located at exon 3b, sense primer (5'-GGA GTC T G A AC ACT AGA GCA AAT GCC AGC-3') and antisense primer (5'-GGC TTG GGC TGA TCT GAG TCC TGC ATT TGG GAG GGA GCC GC-3').

Plasmid Constructs. To generate pmir without poly(A) construct (pNo-3' UTR), the SV40 poly(A) sequence was deleted from pmirGLO Vector (Promega, Madison, WI) by using Chang-IT Mutagenesis kit (Affymetrix, Santa Clara, CA) with a mutagenesis primer (5'-CAT AAC CCC TTG GGG CGG CCG CTT CGA GCC GCC GTA CTC CCG GAA ATC GAA TTT TTA CAA AAT ATT AAC GC-3'). A 752 bp of hMOR-1A 3'-UTR (human PCR fragment) and a 1761 bp of mMOR-1A 3'-UTR fragments (mouse PCR fragment) were amplified by PCRs with a sense primer and an antisense primer flanking with Nhel and Xhol sites, respectively. The primers for amplifying hMOR-1A 3'-UTR were: a sense primer, 5'-GGC GTG AGC AGT AGG CAC TCT CTA GGA TTA TAT AGT ATC CTA ATC AAG CAG CAG-3', and an antisense primer, 5'-CCG CTC GAG TGG TGT TAT AGT GAA TTT TCT GTG TAG TCG TGG-3'; and for amplifying mMOR-1A 3'-UTR, a sense primer, 5'-GGC GTG AGC GTA TGT GCT TGT TAT TTT TAT GAA TTA CAA AAA ACA CAG C-3', and an antisense primer, 5'-CCG CTC GAG GGG AAT TAT ACC CTG ACC ACC C-3'. The digested PCR fragments with Nhel and Xhol sites were subcloned into pHex and Xhol sites of pmirGlo Vector (Promega) containing the original SV40 poly(A).

To generate pmir (a vector from Promega, Madison, WI) constructs with hMOR-1A (ph-wt) or mMOR-1A 3'-UTR (pm-wt) containing a wide-type (wt) miR-103/107 sequence, the human and mouse PCR fragments (see earlier) were subcloned into Nhel and Xhol sites of pmirGlo Vector (Promega) containing the original SV40 poly(A).

The wild-type miR-103/107 sequences in ph-wt and pm-wt constructs were disrupted by mutagenesis using Chang-IT Mutagenesis kit (Affymetrix) with a mutagenesis primer for mouse (M103/107-mut), 5'-GGG GAG GGA AGA CAT TAG ACA GAA GUC CGG CAG AAT GAA AGT TAC TCT CAG C-3', or for human (H103/107-mut), 5'-CAT TTT CCC CAG AAT TAT TAT AGT ACT AGC GTG GTG TAG CAG TAG CCA CCC CTC TTA TTT CTC-3', to generate pm-wt and pm-mut, respectively. The mutagenesis strategy was to not only change seed sequences, but also mutate other nonseed sequences.

To generate pcDNA3 constructs containing hMOR-1A and mMOR-1A coding region and their 3'-UTRs (ph1A/wt and hm1A/wt), the hMOR-1A and mMOR-1A 3'-UTRs were amplified with the following primers flanking with Xhol and XbaI sites (human sense primer, 5'-CCG CTC GAG GGT AGG CAC TCT CTA GGA TTA TAT AGT ATC CTA ATC AAG CAG CAG-3'); mouse sense primer, 5'-GGC TTG GGC TGA TCT GAG TCC TGC ATT TGG GAG GGA GCC GC-3').

Cell Culture. Transfection, Morphine Treatment, and Luciferase Reporter Assay. HER293 and Be(2)C cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids, and minimum Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids, respectively, in an atmosphere of 5% CO2 at 37°C. Plasmid constructs were transfected into HER293 cells plated in 48- or 6-well plates using the Effectene reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol. Antisense locked nucleic acid (LNA) oligonucleotide against mir107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3') and antisense locked nucleic acid (LNA) oligonucleotide against miR107 (miR-107 inhibitor, 5'-GAT AGC CCT GTA CAA TG-3').

Experiments were performed on the same day of transfection.
the manufacturer’s protocol. A negative control LNA oligonucleotide (5'-GTG TAA CAC GTC TAT AGC CCC A-3') (Exiqon) was used as a control. NeuroMag reagent (OZ Bioscience, Marseille, France) was used to transfect miR-107 inhibitor into Be(2)C cells following the manufacturer’s protocol. Be(2)C cells were treated with morphine at the indicated concentrations for 48 hours in the presence or absence of control LNA oligo (5 nM) or miR-107 inhibitor (5 nM). After 48 hours of transfection, the cells were washed with phosphate-buffered saline and lysed with lysis buffer provided from Dual-Glo Luciferase Assay Kit (Promega). Cleared lysate was used to determine the luciferase activities by using the Dual-Glo Luciferase Assay Reagents in TD-20/20 luminometer (Promega). Luciferase activity was calculated by normalizing with Renilla luciferase activity obtained from the same assay.

Animal and Chronic Morphine Treatment. C56BL/6J (B6, stock no. 000664) male mice at 7 to 8 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in groups of five, maintained on a 12-hour light/dark cycle, and given ad libitum access to food and water. All animal studies were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center. Chronic morphine treatment with morphine pellet (75 mg of free base) and placebo pellet (a gift from the Research Branch of the National Institute on Drug Abuse, Rockville, MD) was performed as previously described elsewhere (Kolesnikov et al., 1993). Briefly, the pellets were subcutaneously implanted into the back of mice under oxygen/isoflurane inhalant anesthesia. All mice implanted with a morphine pellet showed morphine tolerance after the 3-day implantation, whereas mice with the placebo pellet displayed normal morphine analgesia (data not shown), as determined by the radiant heat tail-flick assay. On the fourth day, the mice were sacrificed, and the prefrontal cortex and striatum were dissected for isolating total RNA and polysome-associated mRNA.

Isolation of Polyribosome (Polysome) and RNA Extraction. The polysomal fraction was obtained after the previously described procedures had been performed (Wu and Bag, 1998) with a minor modification (Thoreen et al., 2011). Briefly, HEK293 cells or Be(2)C cells in 6-well plates were then treated with 100 μg/ml cycloheximide at 37°C for 10 minutes, washed in ice-cold phosphate-buffered saline containing 100μg/ml cycloheximide, and lysed in polysome lysis buffer (15 mM HEPES-KOH, pH 7.4, 7.5 mM MgCl2, 100 mM KCl, 2 mM dithiothreitol, and 1.0% Triton X-100) containing 100 μg/ml cycloheximide and EDTA-free protease inhibitors (Roche Applied Science, Indianapolis, IN) by homogenizing with a 26-gauge needle for 6 times at 4°C. A 1/10 volume of lysate was used for total RNA extraction by using miRNAasy Kit (Qiagen). The rest of the lysate was centrifuged at 10,000g for 10 minutes, and the supernatant was then centrifuged in an SW-41Ti rotor (Beckman Coulter, Brea, CA) at 100,000g for 1 hour. The pellet as polysome fraction was used for RNA extraction by using the miRNAasy Kit (Qiagen). Isolation of the polysome fraction from the dissected mouse prefrontal cortex (PFC) and striatum was identical to that of the cell lines except that dissected regions were immediately homogenized with a Dounce homogenizer in polysome lysis buffer containing 100 μg/ml cycloheximide and EDTA-free protease inhibitors. The RNA concentration was determined by Lowry method using bovine serum albumin as the standard. 125I-I-IBNtxA had a high affinity (KD, 0.11 nM) toward mMOR-1 when expressed in Chinese hamster ovary cells (Majumdar et al., 2011).

Results

hMOR-1A and mMOR-1A Contain a Consensus Poly(A) Site in Their 3′-UTR. The initial hMOR-1A and mMOR-1A cDNA clones contained only partial 3′-UTR sequences with no information about their poly(A) and cleavage sites, the essential signals for terminating transcription, or adding poly(A) tail in most eukaryotic genes. To identify the poly(A) site and complete 3′-UTR of hMOR-1A and mMOR-1A transcripts, we used a 3′RACE approach in which poly(A)-selected RNAs purified from Be(2)C cells and mouse brain were reverse-transcribed with a oligo(T) primer flanking the unique sequences for anchoring two primers, AP1 and AP2. This allowed unbiased amplification of the 3′-UTR containing poly(A) site by subsequent nested PCRs using the combination of AP1 and AP2 antisense primers with two specific sense primers (hA, hB, mA, and mB) derived from exons 3a/3b (Fig. 2A). After two rounds of PCR, we obtained a ∼500-bp PCR fragment in the human Be(2)C cells (Fig. 2B) and a ∼850-bp PCR fragment in the mouse brain (Fig. 2D). Sequence analysis of the PCR fragments revealed a consensus poly(A) site, AAUAAA, located 15 bp and 5 bp upstream of a common cleavage site, the CA dinucleotide, in hMOR-1A and mMOR-1A, respectively (Fig. 2, C, E, and F). The total length of the 3′-UTR from the stop codon to the cleavage site is 640 bases in hMOR-1A and 1322 bases in mMOR-1A. The poly(A) and cleavage sites were flanked by U-rich and/or G-rich sequences (Fig. 2, C, E, and F). All these cis-elements including poly(A), cleavage site, and U-rich/G-rich sequences are commonly seen in a typical eukaryotic pre-mRNA 3′ end, which provides the basis of the 3′ end processing including transcript cleavage and poly(A) addition for hMOR-1A and mMOR-1A.
Fig. 2. Cloning 3’-UTRs of hMOR-1A and mMOR-1A by 3’-RACE. (A) Schematic of the 3’-RACE strategy. The 3’-RACE was performed as described in Materials and Methods. Primers are shown by arrows. (B and D) Analysis of PCR products on agarose gel. The first- and second-round PCR products of hMOR-1A (B) and mMOR-1A (D) were separated on 1.5% agarose gel and stained with ethidium bromide. The gel was imaged with ChemiDoc MP System. Lanes 1 and 3: first-round PCR products. Lanes 2 and 4: second-round PCR products. (C and E) Partial cDNA sequences of the PCR fragments for hMOR-1A (C) and mMOR-1A (E). Poly(A) signal and cleavage sites are indicated by underlined red and bold letters. (F) Alignment of the 3’-UTRs of hMOR-1A and mMOR-1A. The poly(A) signal and cleavage sites are shown by red letters, and U-rich or U/G-rich regions are indicated by black lines.
To verify the results from the 3'-RACE, we analyzed the full-length hMOR-1A and mMOR-1A transcripts using Northern blots with probes derived from regions upstream or downstream of the poly(A) sites (Fig. 3A). Probe 2 derived from the 3'-UTR upstream of the poly(A) region detected a major band of ~2 kb or ~3 kb in mRNAs from Be(2)C cells or mouse brain, respectively (Fig. 3, B and C). A probe from exon 3a (probe 1) also labeled bands with similar sizes. Thus, the lengths of hMOR-1A and mMOR-1A transcripts revealed by Northern blots were consistent with those predicted from the 3'-UTRs that were identified through the 3'-RACE. Probe 1 also hybridized several additional bands in both human and mouse associated with exon 3a, which presented in a number of additional variants. Of those, a major band of ~12 kb corresponds to the original MOR-1 transcript identified using various probes because the human or mouse exon 4 is ~10–11 kb. On the other hand, a probe derived from the region downstream of the cleavage site failed to detect any specific bands, suggesting that this region is not included in hMOR-1A and mMOR-1A transcripts and supporting the transcription termination sites identified through the 3'-RACE for hMOR-1A and mMOR-1A.

We then examined the role of the 3'-UTR in mRNA stability by using a reporter assay in which the luciferase reporter activity was measured on a construct with or without the 3'-UTR of hMOR-1A or mMOR-1A in HEK293 cells (Fig. 4A). We observed that the luciferase activity of the construct without the 3'-UTRs or poly(A) signal sequence (pNo-3'-UTR) was ~4-fold to 6-fold lower than that of the construct with the 3'-UTR (pH-3'-UTR and pM-3'-UTR, Fig. 4B). RT-PCR confirmed that the decreased luciferase activity was mainly due to a marked decrease in luciferase mRNA (Fig. 4B), suggesting that the 3'-UTR of both hMOR-1A and mMOR-1A play an important role in maintaining mRNA stability, presumably through the consensus poly(A) site in their 3'-UTR.

**miR-103/107 Reduces Expression of MOR-1A via Its Consensus Binding Site at MOR-1A 3'-UTR.** To identify potential miRNA targets in the hMOR-1A and mMOR-1A 3'-UTRs, we scanned the sequences in several computer programs including RegRNA (http://regrna2.mbc.nctu.edu.tw/) and miRBase (http://www.mirbase.org/), and identified a conserved miR-103/107 targeting site in the 3'-UTR of both hMOR-1A and mMOR-1A (Fig. 5A). The sequences of mature miR-103 and miR-107 differ by one nucleotide at position 21 (Fig. 5A). There was a perfect 7mer-seed match of miR-103/107 with a 3'-UTR region of mMOR-1A, while a 6mer-seed match was found with a 3'-UTR region of hMOR-1A.

To examine whether these predicted miR-103/107 binding sites on the 3'-UTR's can be actually targeted by miR-103/107, we first employed a mutagenesis approach to evaluate the role of the predicted miR-103/107 binding sites in a luciferase reporter assay in HEK293 cells that highly express miR-103 and miR-107 (Fig. 5, B and C). The luciferase activities of the mutant constructs (Fig. 5C) in which the predicted miR-103/107 binding site in the hMOR-1A or mMOR-1A 3'-UTR was disrupted were significantly higher than those of the wild-type (wt) constructs (Fig. 5D), suggesting that these predicted miR-103/107 sites function as a repressive element, presumably mediated through the expressed miR-103 and miR-107 in HEK293 cells.

We next used an antisense LNA oligo approach to downregulate miR-103 and miR-107 and investigate the effect of
Fig. 5. Regulation of luciferase activity by miR-103/107 through a conserved miR-103/107 binding site in MOR-1A 3′-UTRs. (A) Alignment of miR-103 and miR-107 sequences with hMOR-1A and mMOR-1A 3′-UTRs. The miR-103/107 seed and aligned 3′-UTR sequences are shown by red letters. The positions of the 3′-UTRs relative to the stop codons are indicated at the 3′ ends. (B) Schematic of pmir constructs. The 3′-UTRs of hMOR-1A and mMOR-1A containing wild-type or mutated miR-103/107 (miR-103/7) binding sites were subcloned into pmir plasmid as pH-wt, pM-wt, pH-mut, and pM-mut, respectively, as described in Materials and Methods. The wild-type and mutated miR-103/7 binding sites are indicated by red and green lines, respectively. (C) Mutagenized sequences of the miR-103/7 binding sites in pmir constructs. Mutagenized sequences are indicated by green letters. S represents G or C in miR-103/7 sequences. (D) Effect of the mutation of the miR-103/7 binding site on the luciferase activity. Transfection of indicated constructs and measurement of luciferase activity are described in Materials and Methods. Fold change of luc2 activity was calculated by normalizing the values of the
the downregulation on luciferase activities with the wild-type construct. To downregulate miR-103 and miR-107 in HEK293 cells, we initially established transfection conditions, such as doses and duration, for an antisense LNA oligo against both miR-103 and miR-107 (miR-107 inhibitor). The miR-107 inhibitor efficiently downregulated expression of both miR-103 and miR-107 (Fig. 5, E and F) at 48 hours after transfection, an optimal time based upon a time course study (data not shown). The miR-107 inhibitor (7.5 nM) reduced miR-103 and miR-107 by 60% and 57%, respectively. Further increasing the dose (15 nM) did not significantly enhance the effect. We therefore used the miR-107 inhibitor at 7.5 nM in cotransfection studies with the human or mouse wild-type construct in HEK293 cells. Downregulating miR-103/107 with the inhibitor significantly increased luciferase activity of both the human and mouse constructs (Fig. 5G), suggesting that miR-103/107 functions as a repressor to regulate luciferase activity through the predicted miR-103/107 binding sites in the hMOR-1A and mMOR-1A 3'UTRs. However, the increase in luc2 activity was a modest 22% (pM-wt) and 27% (pH-wt) increase over the control oligo, similar to changes observed with the mutant constructs (Fig. 5D). This indicated that the effect is actually due to miR-103/107 on luc2 activities in this assay. However, the size of the mRNA effect was much greater using different constructs and assays (see below; Fig. 6).

**miR-103/107 Regulates MOR-1A Expression at the Post-Transcriptional Level.** MicroRNAs regulate their target genes at the translational level but also at the transcriptional level. To assess the level miR-103/107 regulation of hMOR-1A and mMOR-1A expression, we adopted an approach in which a polyribosome-associated mRNA was quantified to determine its translation efficiency while the steady-state total mRNA level from the same cells was determined in parallel. We first made a construct containing the entire coding region and complete 3'UTR of hMOR-1A (ph1A/wt) or mMOR-1A (pm1A/wt) whose expression is under the control of a cytomegalovirus promoter and a mutant construct that was identical to ph1A/wt or m1A/wt, except that the miR-103/107 binding site in the 3'-UTR was disrupted in the same way as in the luciferase constructs (Fig. 5C), as ph1A/mut or m1A/mut (Fig. 6A). When transfected into HEK293 cells, the mutant construct (ph1A/mut) increased opioid binding over 54% compared with the wild-type construct (ph1A/wt) (Fig. 6B), indicating that the miR-103/107 binding site functioned as a repressive element, in a similar manner as with the luciferase constructs (Fig. 5D).

We then determined the mMOR-1A mRNA levels in both total RNA and polyribosomal fractions in HEK293 cells transfected with the wild-type or mutant constructs by reverse-transcription qPCR. We observed no significant changes in the mMOR-1A mRNA in the steady-state total mRNAs between the wild-type and mutant constructs (Fig. 6C), suggesting that the miR-103/107 binding site did not influence the expression of mMOR-1A at the transcription and/or degradation level. However, the mMOR-1A mRNAs were increased in the polyribosomal fractions of the mutant constructs by ~190% as compared with the wild-type constructs (Fig. 6C), suggesting post-transcriptional or translational repressive effects of the miR-103/107 binding sites on mMOR-1A expression in the luciferase assays and opioid binding assays. The downregulation of miR-103/107 by the miR-107 inhibitor increased the polyribosome-associated mMOR-1A by ~125% over the control LNA oligo and did not affect on the steady-state total mMOR-1A mRNA (Fig. 6D).

**Chronic Morphine Treatment Upregulates miR-103 and miR-107 Expression in Be(2)C Cells and the Mouse Striatum.** Morphine regulates the expression of a number of miRNAs, such as miR-23b, miR-133b, and let-7, in cell lines and in animals. To investigate whether morphine can regulate miR-103 and miR-107 expression, we examined miR-103 and miR-107 expression in morphine-treated Be(2)C cells. In Be(2)C cells, morphine increased miR-107 expression in a time- and dose-dependent manner (Fig. 7, A and B). Similarly, morphine significantly upregulated miR-103 expression (Fig. 7C).

We then examined expression of miR-103 and miR-107 in a morphine-tolerant mouse model. Subcutaneous implantation of morphine pellet (75 mg free base) significantly enhanced expression levels of miR-103 and miR-107 in the striatum (Fig. 7D) in a manner similar to Be(2)C cells. This in vivo effect was region dependent, with no significant change in miR-103 and miR-107 expression in the PFC (Fig. 7D).

**Chronic Morphine Treatment Downregulates Polyribosome-Associated MOR-1A via miR-103/107 in Be(2)C Cells and the Mouse Striatum.** We next examined the effect of morphine on expression of endogenous hMOR-1A in Be(2)C cells. Morphine treatment significantly decreased the polyribosome-associated hMOR-1A mRNA in Be(2)C cells (lanes 5 and 6, Fig. 8A), suggesting that morphine inhibits the hMOR-1A expression at the translational or post-transcriptional level, leading to translation of less hMOR-1A protein. The decreased polyribosome-associated hMOR-1A by morphine was clearly not due to the change of the steady-state total hMOR-1A mRNA level. On the contrary, the steady-state total hMOR-1A mRNA level was actually increased by morphine (lanes 1 and 2, Fig. 8A), suggesting that morphine differentially regulates hMOR-1A expression at transcription and/or degradation levels.

To examine whether the effect of morphine on the polyribosome-associated hMOR-1A mRNA was mediated through miR-103/107 in Be(2)C cells, we used the miR-107 inhibitor to downregulate miR-103 and miR-107 in morphine-treated Be(2)C cells. The miR-107 inhibitor effectively reduced both miR-103 and miR-107 by 75% and 85%, respectively, in Be(2)C cells when compared with the control LNA oligo (Fig. 8B). The miR-107 inhibitor did not affect the levels of steady-state total hMOR-1A mRNA (lanes 3 and 4, Fig. 8A). However, mutant constructs with those of the wild-type constructs. ***P < 0.001, compared with pH-wt; **P < 0.01 (Student’s t test) compared with pM-wt. (E and F) Effect of miR-107 inhibitor on the expression of miR-103 (E) and miR-107 (F) in HEK293 cells. Transfection of miR-107 inhibitor into HEK293 cells using indicated concentrations and determination of miR-107 level by reverse-transcription qPCR was as described in Materials and Methods. Fold inhibition by miR-107 inhibitor was calculated by normalizing the levels with inhibitor with those with a control LNA oligo. *P < 0.05; **P < 0.01; ***P < 0.001 (Student’s t test) compared with control LNA oligo. (G) Effect of miR-107 inhibitor on the luciferase activity in HEK293 cells. miR-107 inhibitor or control LNA oligo was cotransfected with pH-wt or pM-wt construct into HEK293 cells as described in Materials and Methods. Fold change of luc2 activity was calculated by normalizing the values of the miR-107 inhibitor with those of the control LNA oligo. **P < 0.01; *P < 0.05 (Student’s t test) compared with control LNA oligo.
Interestingly, computer modeling suggested several miR-103/107 binding sites in the striatum (Fig. 7D), these data suggested that the in vivo effect of morphine on modulating the polyribosome-associated hMOR-1A mRNA in the striatum is most likely acted through miR-103/107 as it did in Be(2)C cells.

Discussion

The 3′-UTR plays crucial roles in many processes of gene regulation such as transcription, splicing, transport, translation, and mRNA stability (Mandel et al., 2008; Elkon et al., 2013; Tian and Manley, 2013). To better understand gene regulation of the OPRM1 splice variants, we isolated the complete 3′-UTR for one of the dominant C-terminal splice variants in both human (hMOR-1A) and mouse (mMOR-1A) by 3′-RACE, enabling us to explore the functions of the 3′-UTR. The 3′-RACE and Northern blots indicated that a conserved major poly(A) signal and its associated cleavage site were used to terminate transcription of both hMOR-1A and mMOR-1A mRNA, although the lengths of the 3′-UTRs differed between hMOR-1A (~0.6 kb) and mMOR-1A (~1.3 kb). These cis-elements, together with the flanking U-rich and/or G-rich regions, are major components in pre-mRNA 3′ end processing of most eukaryotic genes, and play important roles in regulating expressions of hMOR-1A and mMOR-1A mRNAs.

One major function of polyadenylation is to protect mRNA from degradation. Like the 3′-UTRs of other genes and the original mOR-1 from the same gene, the 3′-UTRs of hMOR-1A and mMOR-1A greatly enhanced the stability of the luciferase mRNA in HEK293 cells using the luciferase reporter assay in which the expression of the constructs was under the control of an exogenous PGK promoter, through the conserved poly(A) signal and cleavage sites. Pre-mRNA 3′ end processing is influenced by promoter activity. When we replaced the PGK promoter with a ~2 kb endogenous exon 1 promoter in the same reporter constructs, we observed much lower luciferase activity in HEK293 cells compared with the PGK promoter (data not shown), presumably because of the
limitations of the non-neuronal cell type we used and/or the exogenous luciferase coding sequences. It will be interesting to further explore the functional relationships between the endogenous promoter and 3' end processing in expression of hMOR-1A and mMOR-1A mRNAs in neuronal cells.

The poly(A) sites of hMOR-1A and mMOR-1A can be considered as an intronic polyadenylation site because they are located within the intron of other splice variants. These variants have alternative downstream exons that presumably contain their own poly(A) sites. A major poly(A) site was already identified in exon 4 mainly for terminating the transcription of the original mMOR-1 and hMOR-1. In addition to the poly(A) sites of hMOR-1A and mMOR-1A reported in our present study, we also identified a poly(A) site in the 3' UTR of the mMOR-1Bs (unpublished observation). We believe that the other splice variants, such as mMOR-1C, mMOR-1D, mMOR-1E, hMOR-1Bs, hMOR-1X, hMOR-1Y, and hMOR-1O, have their own poly(A) sites. This raises intriguing questions as to how these poly(A) sites spanning over 100 kb from exon 3 to exon 7 (mouse) or exon O (human) or exon 8 (Fig. 1) are used in the context of transcription and splicing.

Growing evidence indicates that poly(A) sites play an important role in alternative splicing (Tian et al., 2007; Licatalosi and Darnell, 2010; Vorlova et al., 2011). Particularly, interconnection between polyadenylation and U1 small nuclear ribonucleoprotein (snRNP) is one of the key mechanisms to determine the usage of 5' splice sites (Gunderson et al., 1998; Fortes et al., 2003; Goraczniak et al., 2009; Kaida et al., 2010). In the future, we hope to investigate the interaction between the poly(A) sites of hMOR-1A and mMOR-1A and U1 snRNP, and how these interactions influence the expression of hMOR-1A and mMOR-1A mRNAs. This interaction may contribute, at least partly, to the differential expression of MOR-1A mRNAs in various brain regions (Xu et al., 2013).

MicroRNAs primarily target the 3'-UTR to regulate gene expression at both translation and/or transcription levels, although 5'-UTR, coding, and intronic regions also can be involved. Our studies reveal that miR-103/107 targets a conserved miR-103/107 binding site in the 3'-UTR of hMOR-1A and mMOR-1A that was initially predicted from the computer models. This conclusion is based upon the observations that

Fig. 7. Effect of chronic morphine on miR-107 expression in Be(2)C cells and morphine-tolerant mice. (A) Effect of morphine on miR-107 expression at various times in Be(2)C cells. Morphine treatment (3 μM) at indicated times and miR-107 expression determined by reverse-transcription qPCR were described in Materials and Methods. Expression of miR-107 is indicated by fold changes calculated by normalizing with the level of 0 hours. One-way analysis of variance (ANOVA) was used for analyzing statistical significance. *P < 0.05; ***P < 0.001 compared with 0 hours; **P < 0.01 compared with 12 hours; ***P < 0.01 compared with 24 hours. (B) Effect of morphine on miR-107 expression at various concentrations in Be(2)C cells. Expression of miR-107 was determined by reverse-transcription qPCR in Be(2)C cells treated with indicated concentrations of morphine for 48 hours. Fold changes were calculated by normalizing with the level of 0 μM. ***P < 0.001 compared with 0 μM; **P < 0.01 compared with 0.3 μM; *P < 0.05 compared with 0.9 μM. (C) Effect of morphine on miR-103 expression in Be(2)C cells. Be(2)C cells were treated with 3 μM of morphine for 48 hours, and miR-103 expression was determined by RT-qPCR as described in Materials and Methods. *P < 0.01 compared with no morphine treatment (control). (D) Effect of morphine on miR-103 and miR-107 expression in the PFC and striatum of the morphine tolerant mouse model. Expression of miR-103 and miR-107 was determined by RT-qPCR in the PFC and striatum of a morphine tolerant mouse model implanted with s.c. morphine pellet (75 mg/mouse) or placebo pellet (control), as described in Materials and Methods. Student t test was used to analyze statistical difference. *P < 0.05 compared with control.
disrupting the miR-103/107 binding sequences in the 3′-UTRs by mutagenesis significantly increases the exogenous luciferase activity in the luciferase assay and hMOR-1A receptor expression in opioid binding assay in HEK293 cells, and that downregulating miR-103/107 in HEK293 cells with a miR-107 inhibitor enhances the luciferase activity of the 3′-UTR constructs containing the miR-103/107 binding sites. We further illustrated that miR-103/107 mainly functions at the post-transcriptional or translational level to regulate MOR-1A expression, as shown by the increase in polyribosome-associated MOR-1A mRNA when the mutant construct and miR-107 inhibitor were used, without affecting steady-state mRNA levels. Previous studies indicated that several miRNAs such as miR-23b and let-7 inhibit the expression of the original mMor-1 at the post-transcriptional level through their binding sites located at the 3′-UTR (Wu et al., 2008, 2009; He et al., 2010). Our study shows for the first time that a pair of paralogous miRNAs, miR-103 and miR-107, can regulate the expression of a C-terminal splice variant at the post-transcriptional level through its 3′-UTR, as determined by measurement of polyribosome-associated mRNA.

Determined variant OP RM1 splice variants presumably have diverse 3′-UTRs, leading to the question of the role of miRNAs on their expression. Extending the current studies to other variants may prove revealing.

Mature miR-103 and miR-107 have identical sequences except for one nucleotide at the 3′-end, and regulate overlapping targets (Trajkovski et al., 2011; Chen et al., 2012;
Zhang et al. (2012). They are widely expressed in different tissues such as brain, liver, lung, and heart (Miska et al., 2004; Baskerville and Bartel, 2005; Wang and Wang, 2006). Both miRNAs are transcribed from the introns of the pantothenate kinase family (PANK) genes that encode key regulatory enzymes in the biosynthesis of coenzyme A (Wilfred et al., 2007). Disregulation of miR-103 and miR-107 occurs in a number of diseases, including metabolic disorders (Trajkovski et al., 2011), cancer (Rottiers and Naar, 2012; Chen et al., 2012, 2013), and neuropathic pain (Faverouex et al., 2011).


Xu J, Chen L, Bolan E, Gilbert AK, Pasternak GW, and Pan Y-X (2013) Characterizing three alternatively spliced mu opioid receptor variants: mMOR-1A, mMOR-1O and mMOR-1P. Synapse, in press.


Address correspondence to: Dr. Ying-Xian Pan, Department of Neurology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. E-mail: pany@mskcc.org.