POST-TRANSCRIPTIONAL REGULATION OF MOUSE MU-OPIOID RECEPTOR THROUGH ITS 3'-UTR

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

QIFANG WU

IN PARTITAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

ADVISOR: **Horace H. Loh Ph.D**.

April 2009

Acknowledgements

First and foremost, I am deeply indebted to my advisor, Dr. Horace Loh. It was his inspiration, encouragement, support and patience that have guided me through this great journey. I am grateful to my committee chair Dr. Li-Na Wei. Her insightful comments and constructive suggestions have been invaluable to my research. I would also thank my committee members, Dr. Tim Walseth, Dr. Esam El-fakahany, and Dr. Howard Towle for their encouragement and advice.

I am thankful to Dr. Ping-Yee Law for providing his insight and suggestions to my project, Dr. Yan Zeng for the great help he gave me in the miRNA study, and Dr. Dezhi Liao from Department of neuroscience for kindly giving his expertise in the neuronal work.

I would like to thank the professors in the Department of Pharmacology, especially the three DGSs, Dr. Jonathan Marchant, Dr. Sabita Roy and Dr. Colin Campbell.

It has been my privilege to work with a group of wonderful people in the Loh/Law lab, they not only help me scientifically on a daily basis, they are also great friends to share this unforgettable journey with.

Last but not least, I would thank my friends and family, especially my parents, the ultimate source of my courage. Without their support and love, I could never have reached this point.

Abstract

Opioids are widely used in the clinic to treat moderate to severe pain. However, the development of tolerance after chronic treatment posed major medical concern. Since the cloning of opioid receptors (μ, δ, and κ or mu, delta, and kappa) in the early 90s, their spatial and temporal regulation has been intensively studied. μ-opioid receptor (MOR) is the major molecular target of opioids and understanding its regulation is critical to unravel the mechanism underlying the pharmacological effects of opioid drugs. Gene regulation can be exerted at both transcriptional and post-transcriptional levels. Early studies have demonstrated a lack of correlation between morphine treatment and MOR expression at the transcriptional level. The post-transcriptional regulation of MOR remains unclear.

In this study, we identified the major transcript of MOR (MOR1) and its contiguous 3′-UTR, which extends more than 10 kb from the stop codon. The MOR1 3′-UTR uses a single poly (A) signal 10153 bp downstream of the stop codon and is cleaved at the CA cleavage site 26 bp downstream of the poly (A) signal. The MOR1 poly (A) signal demonstrates a distinct preference to its own promoter in the luciferase assay, indicating a functional coupling between the promoter and poly (A) signal. When cloned into a reporter construct, the MOR1 3′-UTR showed a robust inhibitory effect independent of the promoter type and cellular background, which suggests a translational suppression. This is confirmed by the fact that MOR1 3′-UTR did not change the total mRNA level of the reporter gene but sharply decreased its polysome mRNA level. To identify the *cis*- and *trans*- interactions in the 3′-UTR, we predicted three putative *cis*-acting motifs (two K Boxes and one Brd Box) using the UTRscan program (http://bighost.ba.itb.cnr.it/BIG/UTRScan). The mutagenesis analysis confirmed the function of K

Box1 (3802-3807 bp downstream of the stop codon). miRNA23b was predicted to interact with the K Box motif. Over-expressing miRNA23b in NS20Y cells inhibits the luciferase activity in the plasmid with MOR1 3′-UTR, but not in the one without MOR1 3′-UTR. K Box1 mutation diminished the effect of miRNA23b, suggesting a specific interaction between miRNA23b and K Box1 in the MOR1 3′-UTR. miRNA23b represses the translation efficiency of the reporter mRNA by inhibiting its association with polysomes. Knocking down the miRNA23b in NS20Y cells induced a significant increase of MOR protein level, thereby confirming the role of miRNA23b in regulating endogenous MOR expression. Interestingly, chronic morphine treatment increases the expression of miRNA23b and subsequently decreases the polysome association of MOR1 mRNA in which the MOR receptor and MOR1 3′-UTR are required.

In summary, this study identified the 3′-UTR of MOR1 and revealed a novel type of *trans*-acting factor for MOR gene, miRNA23b that binds to the K Box in the 3′-UTR and represses translation by inhibiting the polysome mRNA association. Chronic morphine treatment increases the miRNA23b expression and inhibits the polysome association of MOR1 mRNA. This suggests a pathway that transduces the membrane signal to regulate the intracellular MOR expression as part of the post-transcriptional regulation of μ-opioid receptor.

Table of Contents

Page

List of Tables

Pages

CHAPTER V

List of Figures

Page

CHPATER IV

CHAPTER V

CHAPTER I

Introduction

Opioids and Opioid Receptors

The use of opium dates back to the third century B.C.. Stemmed from its initial use in dysenteries, many applications of opium have been appreciated during the middle Ages. Among 20 distinct alkaloids contained in opium, morphine was the first to be isolated and named after Morpheus, the Greek god of dreams. The discovery of other alkaloids followed. Soon the use of pure alkaloids rather than crude opium became popular in the medical world .

Besides their wide use as pain relievers in the clinic, opioid drugs also affect mood and rewarding behavior, and alter respiratory, cardiovascular, gastrointestinal, and neuroendocrine function. The analgesic effect of opioids arises from their ability to inhibit the ascending transmission of nociceptive information from the spinal cord dorsal horn and to activate pain control circuits that descend from the midbrain, via the dostral ventromedial medulla, to the spinal cord dorsal horn. Opioid drugs also induce undesirable side effects, such as respiratory depression, and immune inhibition. Among these, the major concerns in practice remain tolerance and dependence .

The development of tolerance after chronic use is a characteristic feature of all opioid drugs. Tolerance is defined as a state of adaptation in which exposure to a drug induces changes that result in a decrease of the drug's effects over time. Pharmacologically, tolerance can be described as a shift to the right in the dose-response curve; alternatively, a higher dose is required to maintain the same effect over long-time treatment. Pharmacokinetic changes refer to the metabolic changes of drugs in the body, for example, an accelerated elimination rate; pharmacodynamic changes reflect the alteration of drug-activated events, especially at the receptor level. In the clinic, both pharmacodynamic and pharmacokinetic changes can lead to tolerance (DuPen et al., 2007).

Major medical and social problems are associated with opioid tolerance. Numerous efforts have been made to elucidate its molecular mechanisms. Opioid drugs exert their effects by binding to three major types of surface receptors, μ (mu)-, δ (delta)-, and κ (kappa)-opioid receptors (Kieffer, 1995). Therefore, since the discovery and cloning of opioid receptors, it has been believed that the regulation of opioid receptors holds the key to understanding the mystery behind opioid tolerance and other pharmacological effects. Opioid receptors all belong to the superfamily of G-protein coupled receptors containing seven transmembrane domains. Cloning of the genes encoding three opioid receptors (Kieffer, 1995) has allowed the examination of mechanisms underlying the spatial and temporal regulation of opioid receptors.

The μ-opioid Receptor (MOR) Gene Regulation

Most pharmacological effects of opioid drugs are mediated by the μ-opioid receptor (MOR). μ-opioid receptor is widely distributed throughout the forebrain, midbrain and hindbrain. Its distribution corresponds to the roles in pain regulation and sensorimotor integration. A quantitative study conducted in selected region of the rat central nervous system showed that the medial thalamus has the highest level of MOR mRNA while the cerebellum has the lowest (Brodsky et al., 1995b).

Various physiological and neuronal activities are able to induce MOR gene expressions; for example, continuous chemical depolarization has been proven to down-regulate MOR gene expression(Simantov and Levy, 1986). *In vivo* stimuli can also regulate MOR gene expression. Animal treated with haloperidol for 7 days, which changes the striatal enkephalin levels, exhibited significantly reduced MOR expression levels in globus pallidus. It has also been reported that interleukin-1 can produce a 55-75% increase in MOR transcript in different brain

regions (Simantov, 1989). These results demonstrate that the expression of the MOR gene is temporally and spatially regulated. Therefore, understanding the MOR gene regulation is critical to unraveling the mechanism for tolerance development.

The MOR gene spans about 250 kb based on the mouse genome data bank. It contains 19 exons and generates 30 splice variants (Doyle et al., 2007a; Doyle et al., 2007b). Extensive alternative splicing occurs at the 5′-end and 3′-end. MOR1 is the dominant transcript of MOR gene and encodes the natural μ-opioid receptor protein. In the mouse genome, MOR1 includes four exons divided by three introns. The first intron is > 26 kb, the second is 0.8 kb and the third one >12 kb (Min et al., 1994). The cloning of mouse MOR gene revealed two promoters, the distal promoter (DP) (position -994/-784) and the proximal (PP) (-450/-249) (Law and Loh, 1999). They both lack a TATA box and are highly G/C rich. The PP is preferably used, accounting for 95% of the MOR gene activity in animal brain.

Extensive studies have been conducted on the MOR promoter/5′- untranslated region (5′-UTR) and its transcriptional regulation; multiple regulatory elements have been identified. The elements for the PP include an inverted GA motif with canonical SP1 binding site (Ko et al., 1998), a single stranded DNA-binding protein binding site (Ko and Loh, 2001) and an AP2/SP1(Ko et al., 2003). The elements that can activate the DP include Oct1 (Liang and Carr, 1996), IL-4 (Kraus et al., 2001), Sox 18 and Sox 21 (Hwang et al., 2003; Im et al., 2001), SP-1(Xu and Carr, 2001). A 34-bp silencing region for Pu.1-mediated suppression of the DP was also reported (Hwang et al., 2003; Law and Loh, 1999). In the 5′-UTR, there are regulatory elements such as NF-κB (Kraus et al., 2003), CREB (Lee and Lee, 2003) and neurorestrictive silencer elements (NRSE) (Kim et al., 2006; Kim et al., 2004; Kim et al., 2008; Kraus et al., 2003).

Compared to the extensive interest in the promoter and 5′-UTR, research aimed at the MOR 3′-UTR and post-transcriptional regulation has been limited. A function for the 3′-UTR was suggested by the observation of an abnormally long untranslated region in CXBK recombination-inbred mice that corresponds to a decreased level of μ-opioid receptor mRNA level (Ikeda et al., 2001). The 3′ splicing variants of the MOR gene seem to play a role in modulating MOR analgesic properties (Pasternak, 2001). In κ-opioid receptor, it has been reported that the 5′-UTR and 3′-UTR are subject to regulation at the level of mRNA splicing/processing and play a role in mRNA transport. (Hu et al., 2002; Wei et al., 2000; Wei and Loh, 2002). However, investigation of the post-transcriptional regulation of MOR had been hindered by the difficulty in identifying its lengthy 3′-UTR.

The Significance of Studying Post-transcriptional Regulation of MOR

Morphine tolerance after chronic treatment includes a series of profound changes. There are three phenomena that have been studied extensively: desensitization, which happens when the activated receptor is phosphorylated by a G-protein-receptor-coupled kinase and associates with β-arrestin. This causes uncoupling of the receptor-G-protein complex; receptor internalization, which refers to the sequestration of the receptor from the membrane to the cytosol via clathrin-coated pits and dynamin; and down-regulation, which is shown by a general decrease in receptor numbers (Binyaminy et al., 2008).

To understand the molecular mechanisms of opioid tolerance, a central problem remains that how agonist-activated signal on the cell membrane alters the receptor number or function intracellularly. Although research on MOR transcription revealed numerous factors that increase or decrease MOR transcriptional activity, the MOR mRNA level generally does not correspond to

agonist treatment, such as morphine (Brodsky et al., 1995a; Castelli et al., 1997) , indicating that MOR gene transcriptional bears little relevance to the agonist-induced tolerance. However, how the MOR mRNA is regulated after transcription has been largely unknown.

The post-transcriptional regulation can exerted by controlling the fate of the transcribed molecule, including its stability, translation efficiency and subcellular localization, etc. (Wahle and Ruegsegger, 1999). Therefore, exploring the post-transcriptional regulation of MOR is likely to advance the current knowledge of MOR gene regulation and the molecular basis for tolerance.

3'-UTR and Post-transcriptional Regulation

3'-UTR stands for the 3'-untranslated region, which is the mRNA sequence downstream the stop codon that is not translated into protein. Given the proximity of the 3'-UTR to the termination codon and poly (A) signal, it is not surprising that it offers a diversity of regulatory mechanisms.

Increasing evidence has demonstrated a major role for the 3′-UTR in regulating complex cellular functions, presumably through recognition by specific nuclear and cytoplasmic mRNA-binding proteins (i.e. *trans*-acting factors) (Jackson, 1993). The role of the 3'-UTR in altering mRNA stability is well established for various genes (growth factors, hormone receptors, cytokines, and transcription factors) (Balmer et al., 2001; Sachs and Wahle, 1993). Other functions of 3'-UTR have also been reported. For example, a repressor element in the 3'-UTR of serine protease inhibitor 2.3 gene regulates the basal transcription as well as activation during inflammation (Le Cam and Legraverend, 1995); a 288 bp sequence in the 3'-UTR of α_{2c} adrenergic receptor influences the translational efficiency of the receptor and associated signaling events (Yang et al., 1997); the deletion of the 3′-UTR sequences of β2-adrenergic receptor

resulted in a 2-2.5 fold increase in receptor expression and a shift of the mRNA distribution toward the polysomal fraction (Subramaniam et al., 2004); the 3′-UTR is also involved in controlling cell growth and differentiation in troponin, tropomysin and α-cardiac actin (Rastinejad and Blau, 1993).

Poly (A) Signal The Poly (A) signal is located at the 3′-end of 3′-UTRs. It refers to three key elements in eukaryotic cells: A highly conserved hexanucleotide motif AAUAAA located 10 to 30 bp upstream of the cleavage site; a less highly conserved U-rich or GU-rich region found downstream of the cleavage site, and the cleavage site itself, which is shown as CA in most genes. The poly (A) signal binds to the cleavage/polyadenylation machinery and directs the cleavage and polyadenylation processes at the end of transcription. The cleavage/polyadenylation machinery includes six basic members and several putative auxiliary factors. They are cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor I and II (CFIm and CFIIm, m distinguishes the mammalian protein from yeast), PAP (poly (A) polymerase), and PAPB2 (poly (A) binding protein) (Zhao et al., 1999).

In addition to directing the cleavage/polyadenylation machinery, the poly (A) signal is also involved in other activities. First, it facilitates binding of CPSF to the TFIID nucleated transcription initiation complex (Dantonel et al., 1997); second, it interacts with the 5′ cap of the mRNA to help load the 40S ribosomal subunit that is part of the translation initiation complex; third, it regulates the mRNA stability as the deadenylation is often the initial and rate-limiting step of mRNA degradation; lastly, the poly (A) signal also directs the export of mRNA from the nucleus into cytoplasm (Wahle and Stoffel, 1998).

Studies on the poly (A) signal suggested a spatial proximity between the “head” and “tail” of nucleotide sequences in the transcription and translation complex. At the transcription level, members of the cleavage/polyadenylation complex, CPSF and CstF, are copurified with RNAPolII (RNA Polymerase II) and specifically bind to the CTD (C-terminal domain) of RNAPolII. When the CTD is severely truncated, transcription as well as splicing, polyadenylation and transcription termination is inhibited. CPSF stably associates with TFIID and is recruited to the promoter by TFIID, transferred to RNA PolII at the beginning of transcription and delivered to the poly (A) signal at the end (Zhao et al., 1999). As for translation, dipeptidyl carboxypeptidase (Dcp1, i.e., the protein that removes the m^7GpppN cap) binds to the 5′-end of the transcript and also interacts with the poly (A)-binding protein (PABP); the 3′-UTR binding protein PUF-3 enhances the 5′-end dacapping. In yeast, PABP also interacts with the translation initiation factor eIF4G, which in turn interacts with the cap-binding protein eIF4E. Physical interaction between 5′-end and 3′-end of the mRNA has also been observed (Mazumder et al., 2003).

cis- and trans- interaction in 3′-UTR The function of 3'-UTR is largely mediated by the interaction between *cis*-acting elements and *trans*-acting factors. The AU-rich element (ARE), one of the most-studied *cis*-acting elements seen in 3′-UTRs, regulates the mRNA decay in various genes (Chen and Shyu, 1995; Shaw and Kamen, 1986). AREs were originally defined by the sequence AUUUA and subsequently classified into three categories. Several ARE-binding proteins have been identified in mammalian cells: Hu proteins increase the stability of mRNAs with which they interact (Keene, 1999); AUF1 (also known as hnRNP D) was first identified by its ability to promote degradation of c-myc mRNA in a cell-free mRNA decay system (Brewer,

1991); KSRP (K homology splicing regulatory protein) binds to several ARE-containing mRNAs, promoting their degradation via an exosome mediated pathway (Briata et al., 2003; Chen et al., 2001; Gherzi et al., 2004).

miRNA – Function and Regulation

In addition to *trans*-acting proteins that interact with the 3′-UTR, another category of molecules also has emerged as important regulators, microRNA (miRNA). microRNAs (miRNAs) are 21-22 nucleotide-long RNAs that silence gene expression of their target mRNAs post-transcriptionally. Since the first miRNA *lin*-4 was discovered in C. elegans in 1993 (Lee et al., 1993), at least 100 miRNA genes have been identified in invertebrates, and 500-1000 in vertebrates and plants (Eulalio et al., 2008). The recent discovery of miRNAs in the unicellular algae *Chlamydomonas reinhardtii* (Molnar et al., 2007; Zhao, 2007) has indicated a much older existence of miRNA in evolution.

miRNAs comprise 1-3% of the genome (Zhao and Srivastava, 2007) and bioinformatic predictions indicate that mammalian miRNAs can regulate ~30% of all protein-coding genes (Filipowicz et al., 2008). miRNAs feature a diverse genomic organization. Some miRNAs contain their own independent promoters and enhancers, for example, miRNA-1-1 and miRNA-133a-2 in human and mice. In contrast, ~40% of miRNAs are located within the introns of either non-protein-coding or protein-coding transcription units, like miR-208; and 10% are within exons, such as miRNA-198 (Zhao and Srivastava, 2007).

Function

miRNAs function as components of ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). The details of this dynamic assembly, usually coupled with pre-miRNA processing by DICER are still under investigation (Peters and Meister, 2007).

The key components of miRNPs are proteins of the Argonaute (AGO) family. In mammals, four AGO proteins (AGO1 to AGO4) function in the miRNA repression, but only AGO2 functions in siRNA, because its RNaseH-like P-element induced wimpy testis (PIWI) domain can cleave mRNA at the center of the siRNA-mRNA duplex (Filipowicz et al., 2008).

miRNAs participate in various cellular processes and the changes in their expression were observed in many human diseases including cancer. With just one possible exception reported (Bao et al., 2004), miRNAs control gene expression at the post-transcriptional level, by regulating mRNA translation or stability in the cytoplasm (Filipowicz et al., 2008).

In plants, miRNAs usually bind to the coding region; miRNA:mRNA interactions are rendered by nearly perfect base pairing, thereby triggering endonucleolytic mRNA cleavage by an RNAi-like mechanism (Jones-Rhoades et al., 2006). But in metazoan cells, the base pairing is usually imperfect. The critical requirement is a contiguous and perfect base pairing in miRNA nucleotides 2-8, which is called the “seed” region. In metazoan cells, the binding sites of miRNA lie in the 3'-UTR and are often present in multiple copies, assuring effective repression (Brennecke et al., 2005; Doench and Sharp, 2004; Grimson, 2007; Nielsen, 2007). When placed in the 5'-UTR or coding region, miRNAs can still exert their repressive effect, although the physiological change of the coding region sites can be marginal (Easow et al., 2007).

Numerous efforts have been made to unravel the mechanism for miRNA gene silencing. It is now clear that miRNAs inhibit protein synthesis mainly by translational repression or mRNA destabilization (Filipowicz et al., 2008).

Translational repression As mRNA translation can be divided into initiation, elongation, and termination steps, there is evidence favoring both repression-at-initiation and repression-at-post-initiation models. Recently, it is proposed that the earliest event in miRNA repression happens at the translation initiation step when the AGO proteins (components of miRISC complex) bind to the cap structure to inhibit the cap-dependent translation. Secondary effects of the inhibition then are manifested by different steps, such as mRNA degradation or proteolysis of the nascent polypeptides chains (Filipowicz et al., 2008) .

The mRNA Degradation Early studies on miRNA found the levels of miRNA-inhibited mRNAs remain almost unchanged (Seggerson et al., 2002), suggesting exclusive effect of translation repression. Recent reports showed that animal miRNAs can also induce significant degradation of mRNAs (Bagga, 2005; Behm-Ansmant, 2006; Chendrimada, 2007; Eulalio, 2007; Giraldez, 2006; Wu and Belasco, 2005). In animal cells, miRNAs form imperfect base-pairing with their targets, resulting in mRNA degradation through the general mRNA degradation machinery instead of endonucleolytic cleavage by AGO protein as in plant cells (Eulalio et al., 2008). miRNAs induce mRNA degradation by accelerating deadenylation and decapping of their targets (Behm-Ansmant, 2006; Eulalio, 2007; Giraldez, 2006; Wu et al., 2006), which requires a bevy of factors such as AGO proteins, the P body component GW182, the CAF1-CCR4-NOT deadenylase complex, the decapping enzyme DCP2, and several decapping activators including DCP1, Ge-1, EDC3, and RCK/p54 (Behm-Ansmant, 2006; Eulalio, 2007).

Regulation

The biosynthesis of miRNA involves processes happening in both nuclear and cytoplamsic compartments. Initially, DNA that encodes miRNA is transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs), which can be from a few hundreds to thousands of nucleotides in length. All pri-miRNAs have stem-loop structures which can be recognized and cleaved by the ribonuclease III (RNase III) endonuclease Drosha within the nucleus. A protein partner of Drosha, DGCR8 (DiGeorge syndrome critical region gene, also known as Pasha), is required for efficient pri-miRNA cleavage. The cleavage product, a ~70 nt stem-loop pre-miRNA, is exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, another RNase III enzyme, DICER further processes the pre-miRNA into mature miRNA (21-22 nt). The protein partners for DICER are TRBP (trans-activation response RNA-binding protein) and PKR (RNA-dependent protein kinase) (Zhao and Srivastava, 2007). The mature miRNA duplex has a protruding 2-nt 3'-end, similar to small interfering RNAs (siRNAs) operating in RNA interference (RNAi). Generally, the strand with the 5'-terminus located at the thermodynamically less-stable end of the duplex is selected to function as a mature miRNA, and the other strand is degraded. (Du and Zamore, 2005; Filipowicz et al., 2005; Kim and Nam, 2006; Rana, 2007; Sontheimer, 2005).

miRNA maturation is highly regulated. These small regulators are subject to extensive transcriptional as well as post-transcriptional regulation (Ding et al., 2008).

Transcriptional control Except for a few pri-miRNAs that are transcribed by RNApolIII, the bulk of pri-miRNAs are transcribed by RNA polII, the same enzyme that produces protein-coding mRNAs and thereby under intricate means of transcriptional regulation (Bushati and Cohen, 2007; Fabbri, 2008). Common putative regulatory motifs, like Myc, are located upstream

of miRNA genes and could act as master regulators of miRNA transcription to repress large number of mouse and human miRNA genes (Chang et al., 2008) and induce others (O'Donnell et al., 2005). Epigenetic mechanisms might also contribute to repressing certain miRNAs through histone deacetylation and DNA methylation (Fabbri, 2008).

Post-transcriptional control The processing of pri-miRNA by Drosha in the nucleus and pre-miRNA by DICER in the cytoplasm are both exquisitely regulated. A discrepancy in the quantity of mature miRNAs and their different processing intermediates (pri-miRNA) indicates a regulated maturation process (Eis et al., 2005; Suh et al., 2004).

Although the Drosha–DGCR8 'microprocessor' complex is sufficient for processing pri-miRNAs *in vitro* (Gregory et al., 2004; Han et al., 2004), cropping seems to be exquisitely modulated through accessory factors. Human Drosha was found in a larger complex that includes the DEAD box RNA helicases DDX5 (also known as P68) and DDX17 (also known as P72), but lacks DGCR8 (Gregory et al., 2004). Modulation of DDX5 and DDX17 levels exert diverse effect on different subsets of miRNAs, showing a preference for substrate (Fukuda et al., 2007).

A particularly well-studied example of regulated miRNA maturation involves the repression of *let-7* miRNA biogenesis by LIN28 and its paralog LIN28B. An intricate feedback loop functions where LIN28 or LIN28B represses *let-7* maturation and mature let-7 represses LIN28 and LIN28B accumulation (Bussing et al., 2008).

After cropping of the pri-miRNA by Drosha in the nucleus, the resulting pre-miRNA is exported into the cytoplasm, where Dicer mediates its conversion into the mature miRNA. Dicing typically seems to function efficiently, as indicated by low levels of pre-miRNAs relative to mature miRNAs (Lau et al., 2001; Lee et al., 2008). Pre-miRNA-138 is easily detectable in many tissues, whereas the mature miRNA-138 derived from this precursor accumulates only in mouse

brain and mouse fetal liver. This can be explained by the presence of a specific inhibitor for pre-miRNA-138 processing in non-neuronal tissues and cells (Obernosterer et al., 2006). Dicing of *let-7* family pre-miRNAs is also regulated. The same protein that inhibits the cropping of pri-*let-7* also represses the dicing of the pre-*let*-7 (Rybak et al., 2008). Efficient pre-miRNA processing involves additional proteins, such as AGO proteins, TRBP2 and PACT, therefore the regulation of these proteins might further modulate the Dicer activity (Diederichs and Haber, 2007; Grishok et al., 2001; Jaskiewicz and Filipowicz, 2008; O'Carroll et al., 2007).

miRNA turnover is also under regulation affecting the net amount of miRNA accumulated. miRNA processing intermediates and mature miRNA are both subject to modulated degradation. Although the degradation pathway is still largely unknown, possible examples include stabilization of miRNA122a by the DNA and RNA-binding translin (TSN) protein (Yu and Hecht, 2008) or general destabilization of mature miRNA levels by the Trim-NHL (tripartite motif, NCL-1, HT2A and LIN-41 domain) protein and Ago1 binding partner Mei-P26 (meiotic gene recovered in a P-element screen) in *Drosophila* stem cells (Neumuller et al., 2008).

This thesis work aims to explore the post-transcriptional regulation of the μ-opioid receptor (MOR1) via its 3′-UTR. In Chapter II, we identified the complete sequence of MOR1 3′-UTR and revealed its functional poly (A) signal; in Chapter III, we demonstrated the translational repression mediated by MOR1 3′-UTR; and further discovered a specific miRNA, miRNA23b, which binds to a K Box motif and accounts for part of the function of MOR1 3′-UTR. The translational suppression by miRNA23b is through a decreased polysome mRNA association rate; in Chapter IV, we showed a miRNA23b-mediated connection between morphine treatment and

translation efficiency of the MOR1 mRNA. Finally, in Chapter V, a model of the post-transcriptional regulation of μ-opioid receptor (MOR1) is proposed and discussed, based on the data obtained in this study.

Chapter II

A Major Species of Mouse Mu-opioid Receptor (MOR1) mRNA and its Promoter-dependent Functional Polyadenylation Signal

Abstract

The pharmacological effects of opioid drugs are mediated mainly by mu-opioid receptor (MOR) that is encoded by mRNA transcript named MOR1. Although several MOR mRNA splice variants have been reported, their biological relevance has been debated. In this study, we found probes of regions essential for the production of functional MOR, as well as that of the 3'-down stream region of MOR gene coding region, detected a major species of mature transcript MOR1 from mouse brain in the size of about 11.5 kb by Northern blot analyses. While exon 3 probe detected an additional 3.7 kb transcript, this transcript was not detected by other probes, ruling out its ability to produce functional MOR. The 3'-UTR of MOR1 is contiguously extended from the end of the coding region, and utilizes a single poly (A) signal (located at 10,179 bp downstream of MOR1 stop codon). The poly (A) signal (AAUAAA) is located at 26 bp upstream of the poly (A) site. Transient transfection using luciferase reporters verified the functionality of this poly (A) signal, in particular on a reporter driven by the MOR promoter. This poly (A) is much less effective for a heterologous promoter such as SV40 (simian virus-40), indicating a functional coupling of MOR promoter and its own poly (A). This report verifies MOR1 as the major mature MOR gene transcript that has the full capacity to produce functional MOR protein, identifies the 3'-UTR of MOR1 transcript, and uncovers functional coupling of MOR gene promoter and its polyadenylation signal.

Introduction

Opiate drugs are widely used as pain relievers in clinic. Beside their analgesic effects, tolerance and dependence induced by opiates have elicited major medical and social problems. Opioid drugs exert their activities by binding to three major types of surface receptors, μ (mu)-, δ (delta)-, and κ (kappa)-opioid receptors (Kieffer, 1995). They all belong to the superfamily of G-protein coupled receptors containing seven transmembrane domains. The cloning of the genes encoding three opioid receptors (Kieffer, 1995) has allowed the examination of mechanisms underlying the regulation of opioid receptor expression.

In the opioid receptor family, most pharmacological effects of opioid drugs are mediated by the mu-opioid receptor (MOR). MOR gene spans about 250 kb based on the mouse genome data bank. With PCR-based approaches, a total of 14 exons and 15 splice variants have been reported (Pan et al., 2001; reviewed in Pan, 2003). Their biological relevance has been heavily debated. Mouse MOR1 is the transcript of MOR gene that contains all necessary sequences for the production of functional MOR protein, and was defined following the initial gene structure study (Min et al. 1994) and the reports of splice variants of this gene (reviewed in Pan, 2003). It contains four essential exons divided by three introns (Min et al., 1994). The first intron is 39,520 bp, the second is 739 bp, and the third is 19,722 bp, according to the mouse genome data bank (NC000076). Extensive studies have been carried out to elucidate transcriptional regulation of MOR gene expression, primarily for the 5'-upstream region. For example, our lab has identified multiple regulatory elements in the MOR promoter region, such as Sp1 (Ko et al., 1998), single-stranded DNA-binding site (Ko and Loh, 2001), Sox (Hwang et al., 2003), PU.1 (Hwang et al., 2004), NRSF (Kim et al., 2004), and Sp3 isoforms (Choi et al., 2005a). Regulatory sequences in the 5'-UTR have also been defined by other labs, including STAT6 (Kraus et al., 2001), CREB

(Lee and Lee, 2003) and NRSE (Andria and Simon, 2001). However, studies of its 3'-UTR have been very limited.

The goal of our study is to 1) identify the mature MOR1 transcript that can produce function MOR protein, 2) obtain the complete 3'-UTR sequence of mature MOR1 mRNA; and 3) identify biologically functional poly (A) signal(s) utilized by MOR1. We found that MOR1 represents the major mature mRNA species of MOR gene that has a full capacity to produce MOR protein. We defined the length of MOR1 mRNA and revealed its 3'-UTR that is contiguously extended from the end of its coding region. Further, we identified a single 3'-end of the mature MOR1 mRNA and confirmed the biological function of a single poly (A) signal utilized by MOR gene to generated MOR1 transcript. Finally, we uncovered a functional specificity of MOR poly (A) toward its own promoter, indicating functional coupling of polyadenylation signal with the promoter activity of the MOR gene.

Materials and Methods

Plasmid construction The pGL3-promoter and pGL3-basic plasmids were purchased from Promega. The pL6 plasmid was generated by ligation of the 1.3 kb *KpnI* and *XhoI* (-1326 to +1, the translation start site was designated as +1) DNA fragment of mouse MOR into the polylinker sites of a promoterless luciferase vector, pGL3-basic (Promega). The sequence of the insertion was confirmed by sequencing. The DNA fragment flanking the poly (A) site was generated by PCR from mouse genomic DNA with a pair of primers; sense primer: 5'-AATAGGCCGGCCGCATTAGGAGCATTGCTGAG-3', antisense primer: 5'-ACGCGTCGACCCTAACTCTGGGATGGCAAG-3', the underlined nucleotides indicate the overhanging restriction enzyme sites for *Fse*I and *Sal*I, respectively. The pL6PA and pGL3pPA plasmids were constructed by subcloning this DNA fragment after digestion with *Fse*I and *Sal*I into pL6 and pGL3-promoter plasmid, respectively. The pL6 and pGL3-promoter plasmids also have been digested by *Fse*I and *Sal*I, in order to replace the original SV40 poly (A) signal with the DNA fragment flanking the MOR1 poly (A) site. The pL6N and pGL3pN plasmid was constructed by self-ligating pL6 and pGL3-promoter, respectively, after *Fse*I and *Sal*I digestion and blunt-ending at both ends to remove SV40 poly (A) signal. The sequences of four plasmids have been confirmed by restriction enzyme digestion and DNA sequence analysis.

Northern blot analysis Total RNA was obtained from mouse brain (C57BL/6J strain) by TRI Reagent following the manufacturer's instruction (Molecular Research Center). The mRNA was isolated from total RNA using MicroPoly (A) Purist (Ambion). The Northern blot analysis was performed as described in manufacturer's manual (NorthernMax Kit, Ambion). In brief, 10 µg mRNA per lane was loaded in 1% formaldehyde agarose gel, and transferred to a Hybond-N+

membrane (Amersham). The membrane was hybridized with ^{32}P-labeled DNA probes generated by PCR with appropriate primers as described below. The cDNA as PCR template was obtained from mouse brain total RNA by reverse transcription using the First Strand cDNA Synthesis Kit (Roche). The probe 1, 2 and 3 are located at exon 1, 3'-UTR after exon 4, and exon 3, respectively (Fig. 2-1A). All probes were prepared by PCR with Taq DNA polymerase (Roche) and the above cDNA template. The PCR conditions for the probes consisted of a 2-min denaturing at 94º C, and 30 cycles of amplification at 94º C for 1 min, 57º C for 1 min, and 72º C for 1 min followed by a 10-min extension at 72º C. PCR products were separated in a 1.2% agarose gel. The sense primers were 5'-GAACATCAGCGACTGCTCTG-3', for probe 1; 5'-ATTCCCTCTCACGACGTT GT-3', for probe 2; 5'-GATTGCACCCTCACGTTCTC-3', for probe 3. The antisense primers were 5'-TCCAAAGAGGCCCACTACAC-3', for probe 1; 5'-AGGTGGGAAGAAGGAACTCTCAGA-3', for probe 2; 5'-GTGTAACCCAAGGCAATGCAG-3', for probe 3. The radiolabeled probes were produced by Random Labeling Kit (Amersham) using [α-^{32}P] dCTP.

3' RACE and DNA sequencing Total RNA was extracted from mouse brain as described in Northern blot analysis. The 3' RACE experiments were performed as described in the protocol of 3′ RACE System for Rapid Amplication of cDNA End Kit (Invitrogen). Briefly, the first strand cDNA was synthesized by SuperScript II RT from 5 µg mouse brain total RNA using anchored primer AP (5'-GGCCACGCGTCG ACTAGTACTTTTTTTTTTTTTTTT-3', included in the kit) which initiates the cDNA synthesis at the poly (A) region of mRNA. A negative control (without reverse transcriptase enzyme) was included to rule out the possible product from genomic DNA. The first round of 3' RACE-PCR was performed from 2 µl cDNA using primer A (5'-

TAGCTTAACTCGGAACTGAGT-3') and antisense primer UAP (5'-CUA CUACUACUAGGCCACGCGTCGACTAGTAC-3' included in the kit). The second round of the nested PCR used 4 µl PCR product from the first round and amplified by sense primer B (5'-CACGCTGCCTA GCTGGATTA-3'), or primer C (5'-GCATTAGGAGCATTGCTGAG-3') and antisense primer UAP. The PCR conditions consisted of a 2-min denaturing at 94º C, and 30 cycles of amplification at 94º C for 1 min, 57º C (for primer A and B) or 60º C (for primer C) for 1 min, and 72º C for 1 min followed by a 10-min extension at 72º C. PCR products were separated in a 1.2% agarose gel. The third round of the nested PCR was performed by using the PCR product from round 2 (lane 1 Fig. 2-2C; sense primer: Primer B) as template. The primer sets used are primer C and UAP. The 700 bp PCR product was also generated (lane 2, Fig. 2-2C) in the third round PCR because the PCR template from lane 1 of Fig. 2-2C contained enough amount of primer B to amplify the corresponding PCR product (700 bp). All the products from the second and third round of the nested PCR at the expected size were extracted from agarose gel by Gel Extraction Kit (Qiagen), and confirmed by DNA sequencing.

Cell culture, transfection and luciferase reporter assay Mouse neuroblastoma cell NS20Y was grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum in an atmosphere of 10% CO_2 at 37º C. For transfection, cells were plated 24 hrs prior to transfection at a density of 1.1×10^5 per well in 6-well plates. NS20Y cells were transfected with plasmids pL6, pL6PA and pL6N, pGL3p, pGL3pPA, and pGL3pN using the Effectene Transfection Reagent (Qiagen) as described by the manufacturer's manual. 320 ng of one specific reporter construct was used for each well, and 80 ng of pCH110 plasmid (Amersham) containing β-galactosidase gene was included for normalization. 48 hrs after the transfection, cells were

washed with phosphate-buffered saline and harvested by lysis buffer (Promega). The luciferase and β-galactosidase activities of each lysate were determined by a luminometer (Berthold) as described in protocol from manufacturer.

Results

Characterization of mature mouse MOR1 mRNA. In order to gain insights into the identity of MOR1, including its 3'-UTR, we employed standard high-stringency Northern blot analyses, using probes specific to several essential regions of MOR1, to analyze poly (A)-selected mRNA prepared from mouse brain (Fig. 2-1). Use of mRNA from mouse brain excludes the possible signal of immature RNA in Northern blot analysis and the potential abnormal transcripts produced in cell lines. To produce functional MOR protein, exon 1 is essential because it encodes the extra-membrane domain necessary for morphine binding. So we started with a 232 bp probe specific to exon 1 of MOR1 transcript. The Northern blot result showed one single sharp band at 11.5 kb (lanes 1 and 2 of Fig. 2-1C). This is consistent with the bands detected by the probe of exon 4 (Pan et al., 2000) and probes of other coding regions (Ikeda et al., 2001).

To reveal the identity of MOR1 3'-UTR, we first examined whether splicing occurred in the 3' downstream region of MOR gene. For this purpose, we randomly selected various 3' downstream regions of MOR gene as the probes and carried out Northern blot analyses. As shown in lanes 3 and 4 on Fig. 2-1C, a 350 bp probe, from 4,566 bp downstream of MOR1 stop codon, also detected a single band at 11.5 kb (lanes 3 and 4 Fig. 2-1C), exactly the same size as that detected by exon 1 probe. The Northern blot results suggest that the 3'-downstream region of MOR gene is likely to be contiguously transcribed following the end of the coding region, thus constituting the 3'-UTR of MOR1 transcript.

Since exon 3 is included in all reported MOR splice variants, we chose a 353 bp DNA fragments within exon 3 as the probe. As shown in lanes 5 and 6 of Fig. 2-1C, the hybridization signals appeared in two sharp bands, approximately 3.7 kb and 11.5 kb, respectively, with a similar intensity. Although five MOR splice variants have been reported to use exon 11 instead of

exon 1 (Pan et al., 2001), they are not likely to represent the 3.7 kb species since the expression levels of these alternative transcripts are much lower than that of MOR1 in mouse brain. It is possible that the 3.7 kb band represents an unknown species that lacks MOR exon 1, or it may be transcribed from a highly homologous gene other than MOR, which remains to be further investigated. As shown in Fig. 2-1B, the 18S and 28S rRNA signals were intact and comparable among all samples.

Determination of the 3'-end of MOR1 transcript To characterize the 3'-UTR sequence of MOR1, 3' RACE was used. This method allows the exact position of the cDNA end to be determined. Based on Northern blot results, the mouse MOR1 transcript is about 11.5 kb and the 3'-UTR is likely to be contiguously transcribed from this gene following the end of the coding region. Taking into consideration the 5'-UTR, the coding region and the full length of MOR1, its 3'-UTR is estimated to be around 10 kb downstream of the stop codon. We then designed three sets of primers located 8.3 kb, 9.3 kb and 9.8 kb downstream of the stop codon in the 3' RACE analyses. The predicted lengths of products would be about 1700 bp, 700 bp and 300 bp, respectively, as shown in Fig. 2-2A. Following the first round of 3' RACE-PCR, a sharp band at about 1700 bp was detected in lane 1 of Fig. 2-2B. We used the PCR product from the first round (lane 1 of Fig. 2-2B) as the template to amplify the nested PCR products with primer B or primer C as the sense primer, and UAP (provided in Kit) as the antisense primer. After the second round of the nested PCR, two sharp bands at the predicted size (700 and 300 bp, respectively) were observed in lanes 1 and 3 of Fig. 2-2C. The PCR product from lane 1 of Fig. 2-2C was further amplified by the addition of primer C, and as predicted, two bands were detected (lane 2 of Fig. 2-2C). This result supports our prediction of the position of the 3'-end of mouse MOR1 cDNA.

We extracted DNA samples from all four bands, and analyzed them by DNA sequencing (Fig. 2-2D). The result agreed with the genomic DNA information in GenBank of mouse MOR1 gene, confirming its identity as the mouse MOR1 transcript. The poly (A) tail is added after the conserved CA dinucleotide cleavage site at 10,179 bp downstream of the stop codon (Fig. 2-2D). At 26 bp upstream of this poly (A) site (10,153 bp downstream of stop codon), we found a highly conserved poly (A) signal AATAAA.

Therefore, we are able to obtain the complete sequence information of MOR1 including its 3'-UTR. The 3'-UTR is contiguously transcribed from the MOR gene following the coding region. Based upon sequence comparison, a single poly (A) signal is located at 10,153 bp downstream of the stop codon, which is likely to be used to generate the mature 11.5 kb MOR1 transcript. Taken together, this information is summarized in Fig. 2-4A.

Biological activity of MOR1 poly (A) signal To determine whether the poly (A) signal of MOR1 is biologically functional, a 430 bp genomic DNA fragment was generated by PCR, which contains the highly conserved AAUAAA sequence, poly (A) site and U-rich downstream element (Fig. 2-4B). The 430 bp fragment was used to replace the SV40 poly (A) signal in a reporter construct pL6, which utilizes the mouse MOR promoter to drive the luciferase reporter (Fig. 2-3A). This resulted in the construct pL6PA. pL6, pL6PA, and another luciferase reporter pL6N (which lacks the SV40 poly (A) signal) were transfected separately into neuroblastoma cells NS20Y, together with a galactosidase reporter (pCH110 plasmid, Amersham) as the internal control. 48 hours after transfection, the cells were lysed and the reporter activities of constructs were determined by measuring the luciferase and galactosidase activity. The relative activity of each constructs was obtained by normalizing the luciferase activity to the corresponding

galactosidase activity. The experiments were performed in triplicate and similar results were obtained from three independent experiments. As shown in Fig. 2-3A, pL6PA, the construct containing MOR1 poly (A) signal, activated the luciferase reporter activity for 12- and 3-fold as compared to pL6N and pL6, respectively. The result of luciferase assay showed that the poly (A) signal of MOR1 was much more active for the reporter gene driven by the MOR promoter (pL6PA plasmid), as compared to the heterologous SV40 poly (A) signal (pL6 plasmid).

To determine whether the activity of MOR1 poly (A) signal depends on the specific promoter usage, we fused the MOR1 poly (A) segment into pGL3-promoter (pGL3p) plasmid that is driven by SV40 promoter, generating pGL3pPA plasmid (Fig. 2-3B). Apparently, the MOR1 poly (A) signal was only half the potency of the SV40 poly (A) when fused to a SV40 promoter-driven reporter. The experiments were also performed in triplicate and similar results were obtained from two independent experiments. This indicates that the MOR1 poly (A) signal is much less active for the SV40 promoter. Taken together, the results suggest that the biological activity of MOR1 poly (A) signal is not universal; rather, it prefers the MOR promoter, indicating functional coupling of MOR promoter activity and polyadenylation signal.

Discussion

In this report, we verify that MOR1 as the major mature MOR gene transcript that has the capacity to produce functional MOR protein. The size of this mature transcript is approximately 11.5 kb in length. Many splice variants of the MOR gene have been reported (reviewed in Pan, 2003). For both strategic and technical reasons, the seemingly contradictory conclusion from this current report should not be interpreted by a direct comparison of this current study and other previous studies aiming to identify MOR variants. Strategically, our current study aims at unambiguous identification of MOR1 transcript that, as accepted by scientists in the field, has the full capacity to produce MOR protein. Accordingly, exons encoding the amino-terminal extracellular domain, the seven transmembrane, and the carboxyl intracellular domain would be minimally required to produce functional MOR protein. Second, physiologically relevant mRNA species would be those identifiable in animal tissues, in addition to cultured cell lines. Therefore, we decided to analyze only freshly prepared animal tissue that has a physiological relevance, the brain. Third, only mature mRNAs, those that carry a properly polyadenylated tail can produce protein in mammalian cells in a physiological condition. Hence, we carefully prepared poly (A)-selected RNA, which represents the pool of functional mRNAs. Strategically, studies of RNA splice variants of MOR gene aims at exploration of all possible RNA species that can be transcribed from the MOR gene, or a part of this gene. Many of these variants were detected in cell lines, not necessarily fresh tissues. Further, it remains to be verified whether all these variants contain poly (A), an essential feature of functional mRNAs in a physiological context.

Technically, we employed high stringency Northern blot analysis, which remains the standard and most reliable method to reveal the identity of specific mature transcripts of interest. However, this method is not as sensitive (compared to other detection methods such as RT-PCR

or RNase protection assays). Therefore, we can not rule out the possibility of missing some minor species using this method. On the other hand, all other detection methods, while more sensitive, can not reveal the identity of the entire transcript of interest, unless they are carried out in a manner to detect sequences spanning the predicted 5'- to 3'-ends of the poly (A)-selected mRNAs. For both strategic and technical reasons, our current study addresses very different issues than those studies exploring RNA splice variants.

The cleavage of premature RNA and addition of a polyadenylation tail are the major RNA processing events occurred to produce functional mRNA. Three core elements are involved in polyadenylation, the highly conserved AAUAAA found 10 to 35-bp upstream of the cleavage site, a less conserved U-rich or GU-rich element located within ~50-bp downstream of the cleavage site and the cleavage site itself. They are responsible for recruiting the cleavage and polyadenylation machinery to complete the final stage of RNA processing. As shown in Fig. 2-4B, these three core elements of MOR gene are well conserved between the mouse and the rat. Mouse MOR contains 42% T bases in the U-rich region in premature RNA within 50-bp downstream of cleavage site (Fig. 2-4B). The fact that the poly (A) signal used by the MOR gene to produce MOR1 transcript prefers MOR promoter suggests a functional coupling of this poly (A) to its own promoter, mostly likely through coupling event of transcription and polyadenylation. Presumably, this region can interact with the MOR promoter region, possibly through RNA polymerase II (Conne et al., 2000; Stutz et al., 1998). It will be extremely interesting to test this possibility in the future.

In opioid receptors, the regulatory function of 3'-UTR was suggested from the observation of decreased expression of MOR mRNA in CXBK recombinant-inbred mice that has an abnormally long untranslated region (Ikeda et al., 2001). Support for a role of 3'-UTR in MOR

regulation was also obtained from studying 3'-splice variants of the MOR gene in modulating the MOR analgesic properties (Koch et al., 2001). For the mouse κ-opioid receptor (KOR), it has been reported that the 5'-UTR and 3'-UTR are subjected to regulation at the level of mRNA splicing/processing, stability, translation, and KOR mRNA transport in neuronal cells (Hu et al., 2002; Wei et al., 2000). In this study, we demonstrated that the 3' downstream region of MOR gene is contiguously transcribed following the end of coding region, and it utilizes a single poly (A) signal at 10,153-bp downstream of stop codon. The discovery of un-interrupted 3'-UTR of MOR1 provides us an essential piece of information for further studies of potential regulation mediated by the 3'-UTR.

It is known that regulation by untranslated RNA sequences, such as 5'- and 3'-UTR, can affect RNA stability, translation and transport, which all involves the formation of extensive secondary and tertiary RNA structures that require specific and intact RNA sequences. Hence, the specificity of MOR1 poly (A) to its promoter detected in our reporter assay is not likely to be mediated through stabilizing or translational effects, since the majority of the 3'-UTR (approximately 10 kb) is missing in our construct that contains merely the core poly (A) signal. Therefore, the specificity of this poly (A) is likely to be due to specific functional coupling of polyadenylation signal and its own promoter activity, most probably transcription. However, the exact mechanism of this functional coupling remains to be systematically examined. Our preliminary study of the effects of this poly (A) signal on RNA stability has revealed no effect of this particular poly (A) signal on the stability of RNA produced from the MOR promoter (data not shown).

A direct link between the transcription initiation machinery and 3'-processing has been reported that involves CTD (Wahle and Ruegsegger, 1999). Possible interaction between MOR

5'-UTR and 3'-UTR has been reported for the human MOR gene (Zollner et al., 2000). Therefore, it would be interesting to examine the 3'-UTR, including secondary and tertiary structures of entire 3'-UTR and its poly (A) signal, mediated regulation of MOR gene expression in the future. Our report here provides critical information, i.e. the identity of 3'-UTR and the functionality of poly (A), for studies of MOR gene regulation by 3'-UTR and polyadenylation in the production of MOR1 transcript.

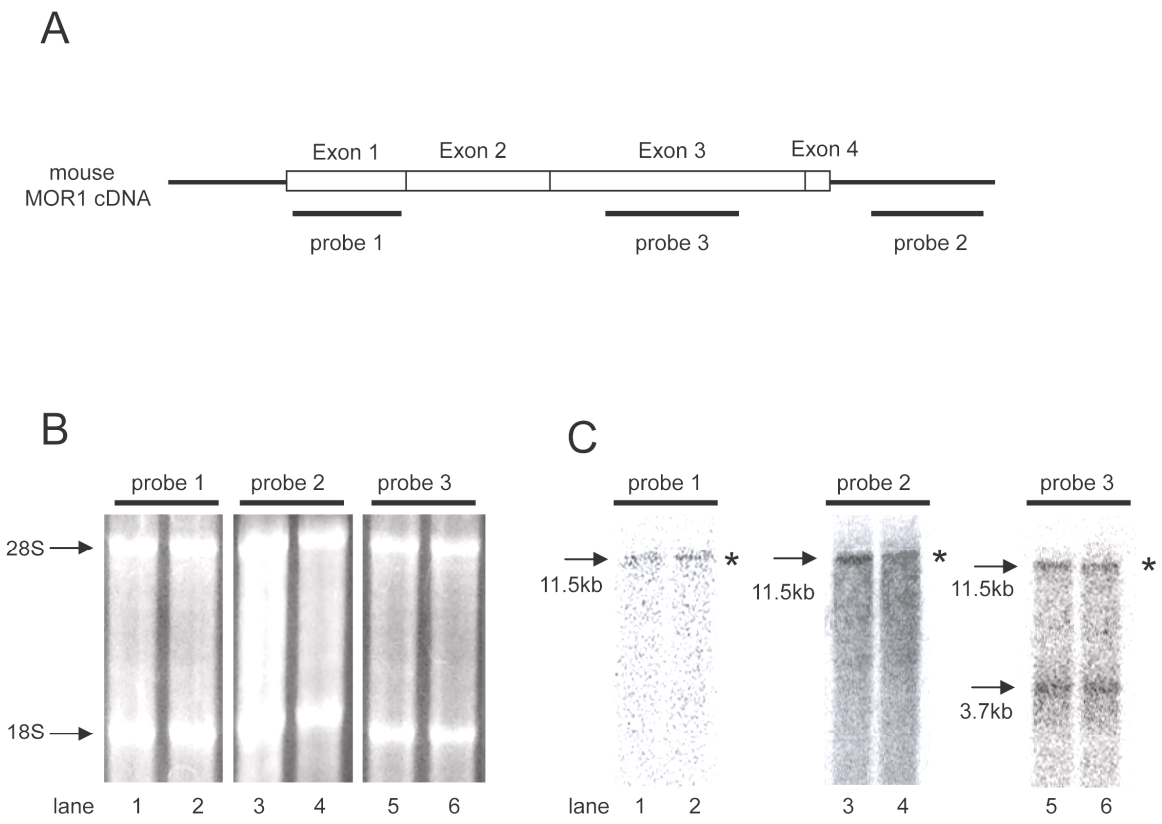
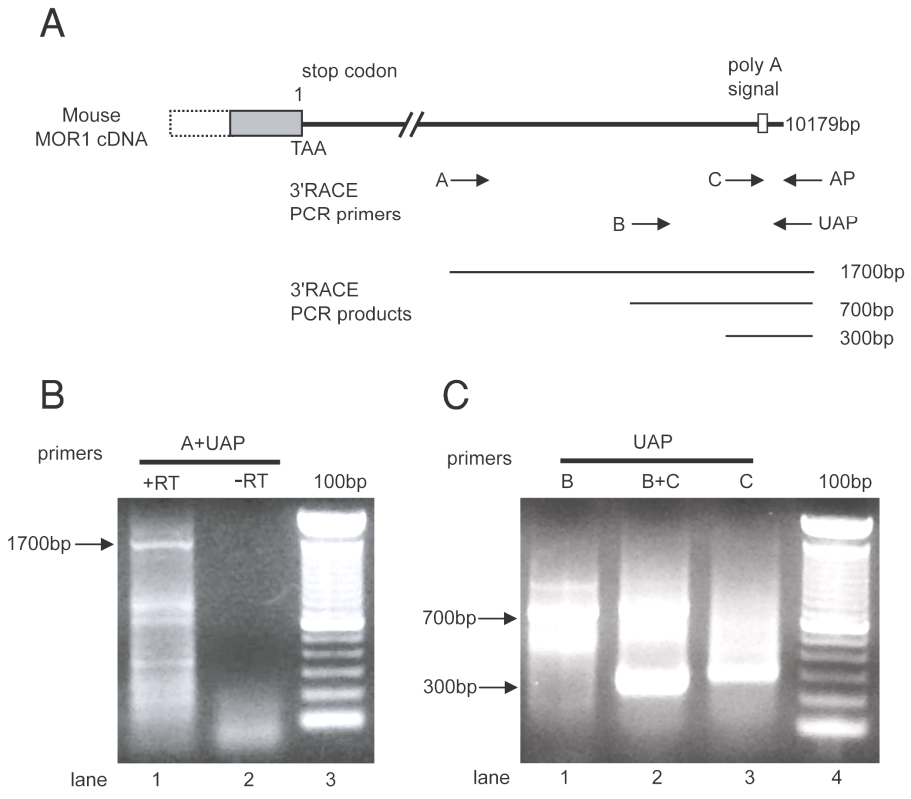

A
mouse
MOR1 cDNA
Exon 1
Exon 2
Exon 3
Exon 4
probe 1
probe 3
probe 2
B
probe 1
probe 2
probe 3
28S
18S
lane 1 2 3 4 5 6
C
probe 1
probe 2
probe 3
11.5kb
11.5kb
11.5kb
3.7kb
*
*
*
lane 1 2 3 4 5 6

Figure 2-1

Figure 2-1. Northern blot results of mouse brain mRNA using different probes. **A**. The structure of the MOR1 cDNA and the positions of probes. **B**. The denaturing RNA gel stained with ethidium bromide, showing the integrity of mRNA samples. The same amounts (10 μg) of mRNA were loaded in two wells for duplicating results. **C**. Northern blots were hybridized with probe 1, 2, and 3, separately as indicated. Northern hybridization was performed in duplicate lanes with same results. Asterisks represent single mature MOR1 mRNAs.



D DNA sequencing result

```
 9968   TTTGCAGCATTTCTACTTGACTGCTTATAATATTGTACTTAAATATATAT

10018   ATATATACACATATATATGTAAACTTTTGCACTGTGTGACTGTTGATGTG

10068   CCAGAATCCACAAACATTTTGGGCTGTATTGTGATTTCTTGCATGCAGTG

10118   TGTGATTATAAAAATTATAATATATTTATCATAACTAATAAAGAAAACCN

10168   ACTTGTAAAGCAAAAAAAAAAAAAAAAAAAANNNCCCCCCCCNNNNNCNNN
```

location from stop codon: 10179bp

Figure 2-2

Figure 2-2. 3' RACE, the nested PCR and DNA sequencing result. **A**. Locations of 3' RACE-PCR primers are shown corresponding to MOR1 3'-UTR. The sensed primers used in PCR reactions are primers, A, B, and C. The antisense primers (AP and UAP) are provided in the kit (Invitrogen). Expected sizes for 3' RACE PCR products are also shown. **B**. The 3' RACE-PCR product of first round PCR was separated in 1.2% agarose gel. Lane 1: the PCR product from cDNA which was reversely transcribed (+RT) using primer AP from 5 µg total RNA of mouse brain. Lane 2: the negative control. No reverse transcriptase (-RT) enzyme was added in the reverse transcription step. The 1700 bp band in lane 1 conforms to the predicted size. **C**. The second and third round of the nested PCR. Lane 1 and 3: Second round PCR product. First round PCR product (lane 1 of Fig. 2-2B) was used as template, and sense primers for lane 1 and 3 are primer B and C, respectively. Lane 2: The third round PCR product. Second round PCR product from lane 1 of Fig. 2-2C was used as the template and sense primer is primer C. 700 bp PCR product in lane 2 for the third-round PCR was generated because the template contains the remaining primer B. All the PCR amplification used primer UAP as antisense primer. **D.** The DNA sequencing result. All the PCR products represented by the four bands in Fig. 2-2C have been analyzed by DNA sequencing. The identity of DNA fragments was checked by comparing to the mouse genomic sequence. The nucleotides near the poly (A) signal site are shown here. The <u>AATAAA</u> represents the poly (A) signal site, <u>CA</u> is identified as cleavage site where the poly (A) tail is added. Numberings are indicated as the locations from MOR1 stop codon.

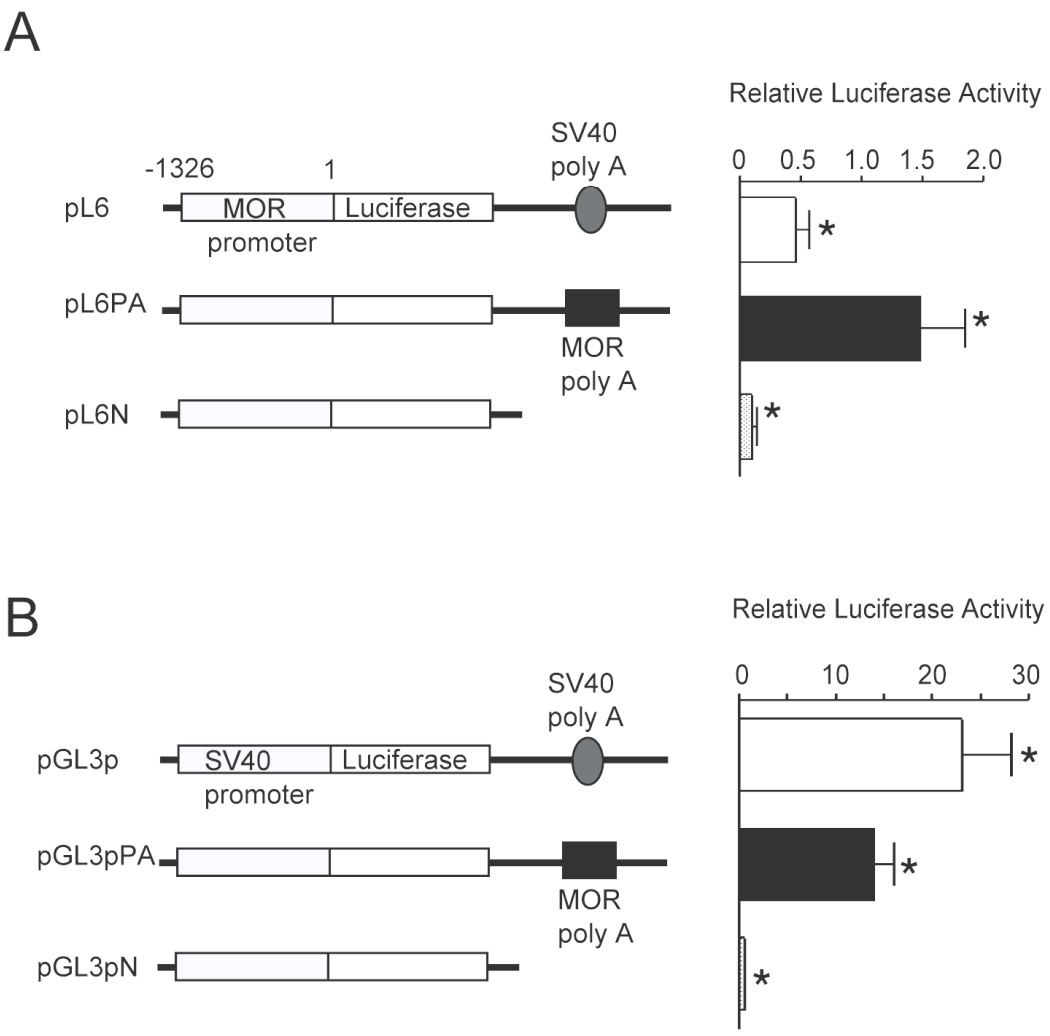
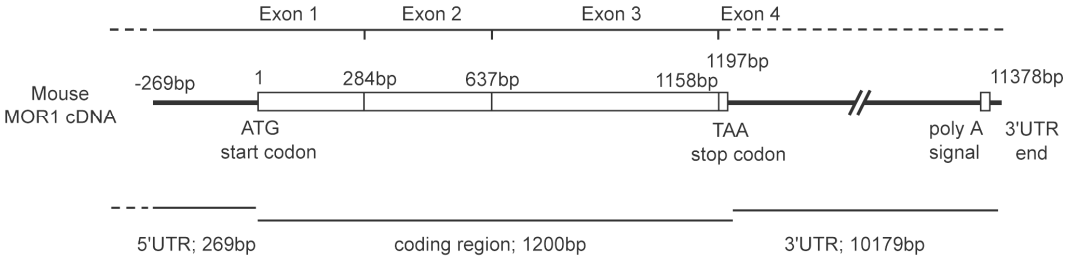

A
Relative Luciferase Activity
SV40
poly A
-1326
1
0
0.5
1.0
1.5
2.0
pL6
MOR
Luciferase
promoter
pL6PA
MOR
poly A
pL6N
*
*
*
B
Relative Luciferase Activity
SV40
poly A
0
10
20
30
pGL3p
SV40
Luciferase
promoter
pGL3pPA
MOR
poly A
pGL3pN
*
*
*

Figure 2-3

Figure 2-3. Transient transfection assay demonstrating the biological activity of the poly (A) signal. **A**. Analysis of homologous MOR promoter reporters including pL6 and its derivatives. Left-hand side showed the map of reporter constructs used in these transient transfection experiments. Gray box, MOR promoter; blank box, luciferase reporter; filled black box, MOR1 poly (A) signal; filled oval box, SV40 poly (A) signal. Right-hand side showed relative activity of the reporter constructs (luciferase activity/galactosidase activity) with the means of three independent experiments. Each experiment was performed in triplicate. Error bars indicate the range of standard errors (SEM); *, significant differences among pL6, pL6PA, and pL6N (p<0.05). **B.** Analysis of heterologous SV40 promoter reporters including pGL3-promoter (pGL3p, Promega) and its derivatives. Figure legends are the same as in panel A except use of the SV40 promoter as indicated. Two independent experiments were performed in triplicate for this SV40 promoter reporter assay. The resulting values for both panels are reported as means ± SEM and analyzed by one-way ANOVA test. Significance was set at $p < 0.05$.

A



B

gene	location from stop codon	homology of genomic DNA sequences
mMOR1	10075	CCACAAACATTTTGGGCTGTATTGTGATTTCTTGCATGCAGTGTGTGATTATAAAAATTA
rMOR1	10349	CCACAAACATTTTGGGCTGTATTGTGATTTCTTGCATGTGATGTATAATTATA-------
		************************************ *** * ******
mMOR1	10135	TAATATATTTATCATAACT*AATAAA*GAAAACCTACTTGTAAAGCAAATATAACTCATGTC
rMOR1	10402	----------TATGATT*AATAAA*GAAAACCTACTTGTAAAGCAAATAAAGCTTTTGTC
		** * ********** * ** ****
mMOR1	10195	TTCTTTATTCTCGCATGATAACTGCTCTGTTTG-----AATAGTCTCATGAGGGCCTAAG
rMOR1	10450	TTTTTTATTCTCATATC-TAACTGATCTGTCTGGAGTTAATAGTCTCATGAGAACCTGAG
		** ********* ** ****** ***** ** ********** *** **

U-rich region
42 % in 50 bp

Figure 2-4

Figure 2-4. The entire mRNA structure of mouse MOR1 gene. **A**. The cDNA structure of mouse MOR1. Translational start site was designated as 1. Open boxes are coding exons, except small open box at 3′ end indicates poly (A) signal. **B**. Homology analysis of genomic DNA sequences near MOR1 poly (A) signal between mouse (mMOR1) and rat (rMOR1) species. Genomic sequences of mouse MOR1 and rat MOR1 are from GenBank accession numbers, *Mus musculus* NC000076 and *Rattus norvegicus* NW047550.1, respectively. Numberings are indicated as the locations from MOR stop codon. The poly (A) signal and cleavage site (CA) are thick-underlined. U-rich region at downstream of poly (A) tail is thin-underlined. The U-rich region contains highly 42 % of U (as T base in genomic DNA) within 50 bp downstream of poly (A) site. Major transcript size of rat MOR1 is very similar to the size of mouse MOR1 transcript as reported previously (Brodsky et al., 1995b; Fukuda et al., 1993).

Chapter III

Post-transcriptional Regulation of Mouse Mu-opioid Receptor (MOR1) via its 3´-UTR — A role for miRNA23b

ABSTRACT

Expression of the mu opioid receptor (MOR1) protein is regulated temporally and spatially. Although transcription of its gene has been studied extensively, regulation of MOR1 protein production at the level of translation is poorly understood. Using reporter assays, we found that the MOR1 3′ untranslated region (3′UTR) represses reporter expression at the post-transcriptional level. Suppression by the 3′UTR of MOR1 is mediated through decreased mRNA association with polysomes, which requires miRNA23b, a specific miRNA that is expressed in mouse brain and NS20Y mouse neuroblastoma cells. miRNA23b interacts with the MOR1 3′UTR via a K Box motif. By knocking down the miRNA23b in NS20Y cells, we confirmed that miRNA23b inhibits the endogenous MOR1 protein expression. This is the first study reporting a translationally repressive role for the MOR1 3′UTR. We propose a mechanism in which miRNA23b blocks the association of MOR1 mRNA with polysomes, thereby arresting its translation and suppressing the production of MOR1 protein.

INTRODUCTION

Opioid analgesics are used widely for treatment of moderate to severe pain. Their efficacy is the result of their ability to mimic endogenous opioid peptides on the opioid receptors. Currently, three major types of opioid receptors have been cloned: μ (mu), δ (delta), and κ (kappa), all of which belong to the G-protein-coupled receptor superfamily (Kieffer, 1995). Mu opioid receptor (MOR) is the major molecular target of opioid drugs, and its expression is regulated temporally and spatially (Choe et al., 1998).

Since the cloning of the mouse MOR gene in 1994 (Min et al., 1994), extensive efforts have been made to clarify the mechanism underlying its regulation. Numerous studies have identified multiple *cis*-acting elements within its promoter and 5′ untranslated region (5′UTR) (Choi et al., 2005b; Choi et al., 2007; Hwang et al., 2004; Hwang et al., 2003; Kim et al., 2006; Kim et al., 2004; Ko et al., 2003; Ko and Loh, 2001; Ko and Loh, 2005; Song et al., 2007). In contrast, current research on the role of the MOR 3′UTR has been limited. The major transcript of the mouse MOR gene, MOR1 (composed of exons 1, 2, 3, and 4) has the full capacity to produce MOR protein. We previously have identified a major transcript of MOR1 and its 3′UTR transcribed contiguously from the coding region. Northern blot and 3′RACE results revealed that the 3′UTR uses a single poly(A) sequence located 10,179 bp downstream from the stop codon (Wu et al., 2005), an observation corroborated by another group's study on the C57BL/6By mouse strain (Han et al., 2006). Human MOR1 3′UTR also uses a single poly(A) signal located 13,612–13,617 nucleotides downstream from the stop codon (Ide et al., 2005). This long 3′UTR of MOR1 is of great interest because of its potential for involvement in complex regulatory events.

The 3′UTR is the portion of the mRNA downstream from the stop codon that is not translated into protein. There is increasing evidence that this region can play a major role in regulating gene expression, especially at the post-transcriptional level. The 3′UTR has multiple functions: altering the stability of mRNA in certain genes (Balmer et al., 2001; Sachs and Wahle, 1993), influencing translational efficiency and associated signaling events (Yang et al., 1997), and controlling mRNA transport, cell growth and differentiation (Rastinejad and Blau, 1993). There is some evidence indicating that the 3′UTR functions in opioid receptor expression. Decreased levels of MOR were observed in CXBK-recombinant inbred mice, which possess an abnormally long 3′UTR (Ikeda et al., 2001). The 3′ splicing variants of the MOR gene seem to modulate the analgesic properties of the MOR (Pasternak, 2001). Similarly, both the 5′UTR and 3′UTR of the κ opioid receptor are regulated at the level of mRNA splicing/processing, and could affect mRNA transport (Bi et al., 2006; Bi et al., 2007; Hu et al., 2002; Wei et al., 2004). In the present study, we cloned the full-length MOR1 3′UTR into a luciferase reporter construct driven by the 1.3 kb MOR promoter. This construct was used to examine the function of the MOR1 3′UTR, which revealed a strongly repressive activity primarily at the level of translation.

Using a UTRscan program (http://bighost.ba.itb.cnr.it/BIG/UTRScan), we predicted the presence of two *cis*-acting elements in the 3′UTR: K Box and Brd Box. In opioid receptor genes, most of the reported *cis*-acting elements bind to proteins. In contrast, K Box and Brd Box usually bind to miRNAs and exert repression primarily at the post-transcriptional level (Lai et al., 1998; Lai et al., 2005). In *Drosophila*, K Box and Brd Box interact with miRNAs that have complementary nucleotides in their 5′ end (i.e., the “seed” region), and regulate the expression of Notch gene (Lai et al., 1998; Lai et al., 2005).

miRNAs are a large family of short, single-stranded, noncoding regulatory RNAs that control target gene expression by binding to their complementary mRNA sequences in the 3´UTRs. In the expanding miRNA family, miRNA23b has been cloned from mouse brain cortex and hippocampus (Dostie et al., 2003; Landgraf et al., 2007), and its "seed" region is conserved in various species (Lai, 2002). In P19 cells, miRNA23b targets the Hairy/enhancer of split protein (Hes1), a bHLH transcriptional repressor functioning in neuronal differentiation (Kimura et al., 2004). Microarray studies have demonstrated alteration of miRNA23b expression in diseases (Gottardo et al., 2007; Palmieri et al., 2007; Wang et al., 2007), indicating an important regulatory role.

Despite intensive efforts to elucidate the function of the promoter and 5´UTR of MOR gene, the role of its 3´UTR has not yet been revealed. The goal of our study is two-fold: to define the function of the MOR1 3´UTR, and to examine the mechanism by which the 3´UTR mediates the post-transcriptional regulation of MOR1.

MATERIALS AND METHODS

Cell culture, transient transfection and luciferase reporter assay

NS20Y mouse neuroblastoma cells and COS-1 monkey kidney cells were grown in Advanced DMEM (Invitrogen, Carlsbad, CA, USA) with 5% heat-inactivated fetal bovine serum in an atmosphere of 10% CO_2 at 37°C. NMB human neuroblastoma cells were cultured in Advanced RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum in an atmosphere of 5% CO_2.

For all transfections (except those including miRNA23b plasmid or anti-miR primer), cells were plated 24 hours prior to transfection at a density of 1.1×10^5 per well in 6-well plates and transfected using Effectene Transfection Reagent (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. One hundred ng of pCH110 plasmid (Amersham, Piscataway, NJ, USA) encoding the β-galactosidase gene were included for normalization. Transfections including a miRNA23b expression plasmid (Pmir23b or Pmir23bm) or anti-23b or anti-miR negative control primer (Ambion, Austin, TX, USA) were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were plated at a density of 0.5×10^5 cells per well in 24-well plates 24 hours prior to transfection; 0.5 ng *Renilla* luciferase plasmid pCMV-Rluc (Gift from Dr. Yan Zeng) was included for normalization.

Forty-eight hours after transfection, the firefly luciferase, *Renilla* luciferase and β-galactosidase activities were determined by luminometer (Berthold, Oak Ridge, TN, USA) using Luciferase Assay or Dual-Luciferase Reporter Assay systems (Promega, Madison, WI, USA) according to the manufacturer's protocol. Plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA) was added to equalize the total amount transfected in each sample. All reporter plasmid amounts were

adjusted according to their sizes to ensure that the same molar amount of DNA molecules was added to each transfection.

Plasmid Construction

Genomic DNA from mouse brain (C57BL/6J strain) was used as template for PCR to obtain the MOR1 3´UTR DNA region. The PCR product first was cloned into a Topo vector and subsequently into a luciferase reporter pL1.33N driven by the MOR promoter, generating the pMUTR plasmid. The MOR1 poly (A) signal (extending from 200 bp upstream of to 100 bp downstream from the hexanucleotide sequence AATAAA) was cloned into the pL1.33N plasmid, generating the pMPA plasmid. pSVUTR and pSVPA plasmids were constructed by replacing the promoter regions of pMUTR and pMPA, respectively, with SV40 promoter cleaved from pGL3promoter (Promega, Madison, WI, USA). pMUTRD1 and pMUTRD2 plasmids were produced by deleting the region between the FseI and PacI restriction enzyme sites, and PacI and PmeI sites of pMUTR, respectively.

The K Box and Brd Box mutation plasmids were generated using the PCR-based Site-directed Mutagenesis kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's protocol. The Pmir23b plasmid was generated by inserting the miRNA23b genomic sequence into the BgII and XhoI sites of psuper plasmid (generous gift of Dr. Yan Zeng). The Pmir23bm plasmid was generated by mutating the "seed" region (+1–+6) of miRNA23b and its complementary region in miRNA23b precursor's stem-loop structure. The sequences of all plasmids were confirmed by restriction enzyme digestion and DNA sequence analysis.

Genomic DNA from mouse brain (C57BL/6J strain) was used as template for PCR to obtain the MOR1 3´UTR DNA region. Twenty ng of genomic DNA was used as a template for

PCR using the Takara LA Taq Polymerase Kit (TaKaRa Bio., Madison, WI, USA). The PCR conditions were as follows: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds and 65°C for 11 minutes; and 72°C for 10 minutes. The primers were 5´-GGAAGCAGAAACTGCTCCATTGCCCTAA-3´ (sense) located at exon 4 of the mouse MOR gene, and 5´-ATGGGTGCTAGAGCTTGAACTTAGGCCCTCAT-3´ (antisense) located at intron 16. The PCR product was cloned into a Topo vector (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. All PCR reactions were performed using Taq polymerase (New England Biolabs, Ipswich, MA, USA) and the following PCR conditions, unless specified otherwise: 94°C for 2 minutes; 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute; and 72°C for 10 minutes. The sequence at the 5´ end of the MOR1 3´UTR was amplified using sense primer 5´-CTGCCCCGGGCCGGCCTGGGTCCCACGCCATCCAGA-3´ (which adds an FseI site at the 5´ end) and antisense primer 5´-AGAACGCGTCAGGGAAAATAATATACAAG-3´ (which matches the sequence flanking the MluI site in MOR1 3´UTR). This PCR product was also cloned into Topo vector, generating the TopoFseI plasmid. Both plasmids were cleaved at KpnI (from the Topo vector) and MluI (from the MOR1 3´UTR) sites. The KpnI/MluI fragment cleaved from TopoFseI was inserted into TopoUTR, generating the TopoUTRFse plasmid. Ligation was performed using the Rapid DNA Ligation Kit (Roche Applied Science, Indianapolis, IN, USA).

The pL1.33N plasmid was constructed previously in our lab. Mouse brain RNA was used as template for one-step RT-PCR (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol using the following primers: 5´-CTCCGTGTACTTCTAAGGT-3´ (sense) and 5´-CTGCTGTCCATGGGTTCTGAA-3´ (antisense). The PCR product was cloned into a Topo

vector (Invitrogen, Carlsbad, CA, USA) and cleaved between the PvuII and NcoI sites. This fragment was then inserted into a pL6 plasmid (Wu et al., 2005), generating pL1.33N. TopoUTRFse was cut between the KpnI and FseI sites. The fragment between the KpnI and FseI sites from pL1.33N (including the MOR promoter and luciferase coding region) was inserted into the TopoUTRFse vector, generating the pMUTR plasmid. The MOR1 poly(A) signal (from 200 bp upstream of to 100 bp downstream from the AATAAA hexanucleotide sequence) was generated by PCR using primers 5´-AATAGGCCGGCCGCATTAGGAGCATTGCTGAG-3´ (sense) and 5´-ACGCGTCGACCATGGGTGCTAGAGCTTGAA-3´ (antisense). The PCR product was cloned into the pL1.33N plasmid.

pSVUTR and pSVPA plasmids were constructed by replacing the promoter regions of pMUTR and pMPA, respectively, with SV40 promoter cleaved from pGL3promoter (Promega, Madison, WI, USA) between the KpnI and FseI sites. pMUTRD1 and pMUTRD2 plasmids were produced by deleting the region between the FseI and PacI sites, and the PacI and PmeI sites in pMUTR, respectively. The K Box and Brd Box mutation plasmids were generated using the PCR-based Site-directed Mutagenesis kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's protocol. The mutation primers used were as follows: K Box1: 5´-GTTTTGTGAATATCTCAGGATCAATTTTCATTAG-3´ (sense) and 5´-CTAATGAAAATTGATCCTGAGATATTCACAAAAC-3´ (antisense); K Box2: 5´-CCTGCCATCCTCTGCTCAGGATTTTCTCTGTGTG-3´ (sense) and 5´-CACACAGAGAAAATCCTGAGCAGAGGATGGCAGG-3´ (antisense); Brd Box: 5´-GAAGAGACACTAAAGATAGGGAATCTGAAGGTC-3´ (sense) and 5´-GACCTTCAGATTCCCTATCTTTAGTGTCTCTTC-3´ (antisense). The mutated nucleotides are underlined. Pfu polymerase (New England Biolabs, Ipswich, MA, USA) was used for mutation

PCRs under the following conditions: 95°C for 30 seconds; 20 cycles of 95°C for 30 seconds and 48°C for 1 minute; and 68°C for 15 minutes.

The genomic DNA sequence of miRNA23b was generated by PCR using 5´-AGTCAGATCTGTCCTTTGTCTCCCAGTCCCCTAT-3´ (sense) and 5´-CATGCTCGAGTGCACCTTGTTAGAGAGGTC-3´ (antisense) primers. Pmir23b plasmid was generated by inserting the miRNA23b genomic sequence into the BgII and XhoI sites of psuper plasmid (gift of Dr. Yan Zeng). Pmir23bm plasmid was generated by mutating the "seed" region (+1–+6) of miRNA23b and its complementary region in miRNA23b precursor's stem-loop structure using the methods described above. The PCR conditions were as follows: 95°C for 30 seconds; 16 cycles of 95°C for 30 seconds and 55°C for 1 minute; and 68°C for 3 minutes. The mutation primers for the "seed" region were 5´-GTGACTTGAGATTAAACCTGAGTTGCCAGGGATTACC-3´ (sense) and 5´-GGTAATCCCTGGCAACTCAGGTTTAATCTCAAGTCAC-3´ (antisense). The mutation primers for the nucleotides complementary to the "seed" region were 5´-CTTGGGTTCCTGGCACTCCAGGTTGTGACTTGAG-3´ (sense) and 5´-CTCAAGTCACAACCTGGAGTGCCAGGAACCC-3´ (antisense). The mutated nucleotides are underlined. The sequences of all plasmids were confirmed by restriction enzyme digestion and DNA sequence analysis.

PCR, One-step RT-PCR and Real-time qPCR and qRT-PCR

Cells were plated at a density of 1.1×10^5 cells per well in 6-well plates 24 hours before transfection. DNA and total RNA were isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). mRNA was isolated from total RNA using MicroPoly(A) Purist (Ambion,

Austin, TX, USA). Nuclear and cytoplasmic RNA were extracted as described previously (Wilkinson, 1988). RNA was treated with Turbo DNase I (2U/μg RNA) (Ambion, Austin, TX, USA) before being reverse-transcribed. Primers used in PCR, one-step RT-PCR and real-time qPCR were as follows: luciferase: 5′-GAAACTGACGGAGCCTAGG-3′ (sense) and 5′-CCACCTCGATATGTGCATCTG-3′ (antisense); mouse β-actin: 5′-TGGCCTTAGGGTGCAGGGGG-3′ (sense) and 5′-GTGGGCCGCTCTAGGCACCA-3′ (antisense); MOR1: 5′-CATCAAAGCACTGATCACGATTCC-3′ (sense) and 5′-TAGGGCAATGGAGCAGTTTCTGC-3′ (antisense). PCR conditions were as follows: 94°C for 2 minutes; 25 cycles (for luciferase) or 20 cycles (for β-actin) of 94°C for 1 minute and 55°C for 1 minute; and 72°C for 10 minutes. One-step RT-PCR conditions were: 50°C for 30 minutes; 95°C for 15 minutes, 30 cycles (for MOR1), 25 cycles (for luciferase) or 20 cycles (for β-actin) of 94°C for 1 minute and 55°C for 1 minute; and 72°C for 10 minutes. The linear range of PCR cycles for each gene was determined using relative PCR, and cycle numbers for PCR and RT-PCR were optimized according to the results. PCR and one-step RT-PCR were performed using a GeneAmp PCR System 9600 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) using Taq Polymerase (New England Biolabs, Ipswich, MA, USA) and the OneStep RT-PCR Kit (QIAGEN, Valencia, CA, USA), respectively.

Real-time qPCR and qRT-PCR for the luciferase gene were performed using a Quantitect SYBR Green RT-PCR kit (QIAGEN, Piscataway, NJ, USA). For miRNA23b, RNA was extracted using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Reverse transcription was performed using the Taqman miRNA reverse transcription Kit (Applied Biosystems, Foster City, CA USA). One-tenth of the reverse transcription mix was used for real-time qPCR using an iQ supermix kit (Bio-Rad Laboratories, Oakland, CA, USA). The *mir*Vana

qRT-PCR Primer Set for Normalization (5S) (Ambion, Austin, TX, USA) was included as an internal control. Real-time qPCR and qRT-PCR were both performed on an iCycler (Bio-Rad Laboratories, Oakland, CA, USA) and the relative expression levels were calculated as described previously (Hwang et al., 2007). β-actin and 5s RNA were included for normalization. All PCR products were electrophoresed in 1% or 2% agarose gels, quantified by ImageQuant 5.2 (Amersham Biosciences Inc., Piscataway, NJ, USA) and verified by DNA sequence analysis.

Immunoprecipitation and Western Blot

For western blots, cells were plated at a density of 1.1×10^5 cells per well in a 6-well plate 24 hours before transfection. Proteins from two wells were extracted, pooled, and lysed in RIPA buffer containing 50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. For immunoprecipitation, 5×10^5 cells per dish were plated the day before transfection. The cells from two 10-cm dishes were lysed in a buffer containing 20 mM Tris HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40 and 2 mM EDTA, supplemented with 1× Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA), as described in the Abcam protocol (Abcam, Cambridge, MA, USA). Approximately 1 mg of the clarified cell lysate was incubated overnight at 4°C with affinity-purified rabbit anti-MOR1 antibody (produced in our lab, information available upon request). Immunoprecipitates were recovered on protein G–agarose beads (Invitrogen, Carlsbad, CA, USA) and washed extensively. Proteins were separated on an 8% SDS-PAGE gel and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were incubated with anti-luciferase, anti-galactosidase, anti-β-actin (Cell Signaling Technology, Danvers, MA, USA) or anti-MOR1 antibodies (kindly provided by Dr. Lee-Yuan Liu-Chen) diluted 1:1000. Signals were detected

using a Storm 840 PhosphorImager system (Amersham Biosciences, Piscataway, NJ, USA) and quantified by ImageQuant 5.2 (Amersham Biosciences Inc., Piscataway, NJ, USA).

Polysome mRNA Extraction and Polysome Profile by Sucrose Gradient Analysis

Polysome mRNA extraction was conducted as described previously (Mukhopadhyay et al., 2003). Briefly, NS20Y cell extracts were layered onto polysomal buffer (10 mM MOPS, 250 mM NaCl, 2.5 mM MgOAc, 0.5% NP-40, 200 U/ml RNase inhibitor, and 50 μg/ml cycloheximide). The polysomes were collected by ultracentrifugation at 45,000*g* for 1 hour. Polysome mRNA was isolated from pellets with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. For sucrose gradient analysis, polysomes were isolated as described previously (Yang et al., 1997) with modifications. NS20Y cells were washed with PBS containing 0.01% cycloheximide and harvested into 1 ml of lysis buffer (20 mM Tris-HCl [pH 8], 140 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 0.5 mM dithiothreitol, 0.1 mg/ml cycloheximide, and 1 mg/ml heparin). Intact nuclei and mitochondria were removed by centrifugation, and the supernatant was loaded onto 10–50% linear sucrose gradients. The gradients were centrifuged in an SW41 rotor at 35,000 rpm for 3 hours at 4°C. Fourteen fractions (0.85 ml each) were collected from the top (fraction 1) to the bottom (fraction 14) and extracted with phenol:chloroform. The RNA was precipitated and analyzed by RT-PCR. Absorbance at 260 nm was monitored to identify fractions containing monosomes and polysomes.

Statistics

Data are presented as mean values ± SEM. Comparisons between groups were performed using the Student's *t* test. $P < 0.05$ was taken as significant.

RESULTS

MOR1 3´UTR represses reporter expression. The MOR1 3´UTR is encoded in a contiguous DNA segment extending from the end of the coding region and uses a single polyadenylation signal located 10,179 bp downstream from the MOR1 stop codon (Wu et al., 2005). To elucidate its function, we constructed the pMUTR plasmid by cloning the full length MOR1 3´UTR into a luciferase reporter (pL1.33N) driven by the 1.33 kb MOR promoter. We also constructed the pMPA plasmid, which contains the same MOR1 poly(A) signal, but lacks the 9829 bp sequence upstream of the poly(A) signal in the MOR1 3´UTR (Fig. 3-1A). In NS20Y cells, pMUTR generated significantly less luciferase activity than did pMPA (Fig. 3-1B). Considering their different sizes (17 kb for pMUTR vs. 7 kb for pMPA), the transfection amounts of both constructs were adjusted to ensure that the same molar quantities of DNA were transfected (for example, 500ng pMUTR vs. 200ng pMPA). The transfection efficiencies of the two plasmids were similar, as confirmed by their galactosidase activity and PCR experiments that amplified the luciferase DNA recovered from transfected cells (data not shown). That is, the difference in luciferase activity was not the result of different transfection efficiencies. Western blots showed that cells transfected with the pMUTR plasmid had significantly lower levels of luciferase protein than did those transfected with pMPA (Fig. 3-1C). β-actin and galactosidase protein levels were comparable in both samples, as well as in cells mock-transfected with pcDNA3 (control), confirming similar transfection efficiencies and loaded sample amounts.

MOR1 3´UTR inhibits the polysome-mRNA association. 3´UTRs affect mRNA stability, mRNA transport**,** translation efficiency, etc. (Kuersten and Goodwin, 2003). We systematically examined how MOR1 3´UTR affects cellular events, including those occurring transcriptionally and post-

transcriptionally. A comparison of pMUTR- and pMPA-transfected samples showed that the MOR1 3´UTR had no effect on the levels of the reporter mRNA produced (Fig. 3-2A), suggesting that the MOR1 3´UTR likely acts post-transcriptionally. Likewise, there was no difference in the ratio of nuclear to cytoplasmic RNA between the two transfects (Fig. 3-2B), indicating that the MOR1 3´UTR does not impair transport of reporter RNA from the nuclei.

The distribution of mRNA between ribosome-free and polysome-bound fractions has been used to determine translational control of a given mRNA. This can be done since translational control takes place, mainly at the initiation step (Sonenberg et al., 2000), and thus ribosome loading of a transcript is a robust indicator for translation efficiency (del Prete et al., 2007). One analysis measures the ratio of polysome-bound mRNA to total mRNA, which corresponds to the translation efficiency (Mukhopadhyay et al., 2003). Another approach assesses the sedimentation of mRNAs in the sucrose density gradients. The presence of mRNAs in the heavier fractions (bound with more ribosomes) indicates higher efficiency in protein synthesis (Qin et al., 2007).

In our experiment, the translation efficiencies of pMUTR and pMPA were compared by their ratio of polysome-bound to total mRNA. Cytoplasmic lysates from transfected NS20Y cells were centrifuged and the mRNA was extracted from the pellets (polysome fraction). Although the total reporter mRNA levels were similar between two transfects (Fig. 3-2A), there was a dramatic decrease in polysome-bound luciferase mRNA in pMUTR-transfected cells, relative to that of pMPA-transfected cells (Fig. 3-2C). This was confirmed by real-time qRT-PCR (Fig. 3-2D). Deleting a 5-kb or 3-kb region of the MOR1 3´UTR partially restored the suppression of both reporter activity and polysomal mRNA levels (Fig. 3-3). Taken together, these data show that

MOR1 3´UTR represses reporter expression primarily by inhibiting mRNA association with polysomes, thereby decreasing translation efficiency.

K Box1 is a negative cis-acting element in the MOR1 3´UTR. Although 3´UTR is less conserved across species than protein-coding regions, highly conserved nucleotide blocks have been detected in the 3´UTRs of orthologous genes from different mammalian orders (Shabalina and Spiridonov, 2004). Increasingly, studies report regulatory elements in 3´UTRs, such as AU-rich elements that promote mRNA degradation (Zubiaga et al., 1995) or small motifs base-pairing with small RNAs to suppress translation (Gray and Wickens, 1998). We therefore sought to identify regulatory elements in the MOR1 3´UTR. The online program UTRscan (http://bighost.ba.itb.cnr.it/BIG/UTRScan) predicted some putative *cis*-acting elements (Fig. 3-4A): two K Boxes (K Box1 at 3805–3812 bp and K Box2 at 8276–8283 bp downstream from the stop codon) and one Brd Box (2559–2265 bp downstream from the stop codon). To validate these putative elements, we performed clustered point mutations on each (Fig. 3-4B; only pMUTRKm is shown). Reporter assays were performed comparing each mutant construct with its wild type plasmid in NS20Y cells. Only the mutation on K Box1 increased reporter activity (Fig. 3-4C; Brd Box and K Box2, data not shown). Although K Box2 shares the same "seed" sequence as K Box1, its mutation failed to affect reporter activity.

miRNA23b interacts with the MOR1 3´UTR. In *Drosophila*, K Box interacts with miRNA2 and miRNA16, both of which have "seed" sequences complementary to K Box (Lai et al., 2005). miRNA23b has a "seed" sequence homologous to those of miRNA2 and miRNA16 in *Drosophila* and has been cloned from mouse brain (Kimura et al., 2004). To determine if

miRNA23b interacts with the MOR1 3´UTR through a K Box motif, we first confirmed the expression of endogenous miRNA23b in NS20Y cells and mouse brain (Fig. 3-5A): The miRNA expression plasmid Pmir23b increased miRNA23b levels in NS20Y cells in a dose-dependent manner (Fig. 3-5B). When cotransfected with pMUTR, Pmir23b induced a sharp decrease in reporter activity (Fig. 3-6A), even at a minimal dose (10 ng) that only slightly increased miRNA23b level in NS20Y cells (Fig. 3-5B). However, the deletion of the MOR1 3´UTR in pMPA totally abolished the effect induced by Pmir23b (Fig. 3-6B). These results show that the MOR1 3´UTR is required in order for Pmir23b to suppress reporter activity.

Although K Box1 and K Box2 share an identical "seed" region, only K Box1 functions in the mutation assay. Two partial deletion plasmids lacking K Box1 and K Box2 (pMUTRD1 and pMUTRD2, respectively) act differently in response to Pmir23b. Deleting the sequence containing K Box1 abolished the Pmir23b-induced suppression of reporter activity (Fig. 3-6C). In contrast, deleting K Box2 did not alter the effect of Pmir23b (Fig. 3-6D). These data support the observation that K Box1, but not K Box2, is a functional regulatory element.

We also mutated the "seed" sequence in the Pmir23b plasmid responsible for binding to the K Box (i.e., the Pmir23bm plasmid). Mutation of the "seed" sequence attenuates the repression effect (Fig. 3-6E). At the minimal amount (i.e., 10 ng), Pmir23b downregulated reporter activity more than 50%, but Pmir23bm had no significant effect.

miRNA23b interacts with K Box1 to decrease the polysome mRNA association rate. To determine the mechanism of interaction between miRNA23b and K Box, we examined reporter RNA levels after Pmir23b transfection. Total luciferase RNA levels remained the same after the transfection (Fig. 3-7A), suggesting that miRNA23b is not likely to repress reporter expression by

inducing RNA degradation. In contrast, Pmir23b decreased luciferase polysomal mRNA levels to about half those of control transfections (Fig. 3-7B); its mutation plasmid, Pmir23bm did not induce a significant change. This suggested that miRNA23b specifically decreased the association of mRNA with polysomes in reporters containing MOR1 3´UTR. On the other hand, mutating the K Box1 (pMUTRKm) abolished the Pmir23b-induced decrease in polysome mRNA association. Pmir23b also reduced the polysomal mRNA in pMUTRD2 (Fig. 3-7C), but when the K Box1 was mutated, this downregulation was abolished (Fig. 3-7D). RT-PCR analysis of luciferase polysome mRNA levels was confirmed by real-time qRT-PCR (Fig. 3-7E).

Sucrose gradient experiments were performed to provide a detailed view of mRNA in different ribosomal fractions. Pmir23b, but not Pmir23bm, shifted the MOR1 3´UTR reporter (pMUTR) mRNA out of the heaviest polysome fraction (Fig. 3-8B, lane 7), confirming that a reduction in polysomal mRNA association was induced specifically by miRNA23b. Taken together, these data show that a specific interaction between miRNA23b and the K Box1 in the MOR1 3´UTR decreases the association of mRNA with polysomes, leading to suppressed translation.

miRNA23b regulates endogenous MOR1 protein expression. Finally, we determined if miRNA23b affects endogenous MOR1 protein production. Because NS20Y is a weak MOR-positive cell line, we used the histone deacetylase inhibitor Trichostatin A (TSA) to stimulate its global transcription, thereby enhancing endogenous MOR1 RNA levels (Fig. 3-9A). Nevertheless, MOR1 protein expression was still undetectable (Fig. 3-9C, lane 2). We confirmed that anti-23b primer decreased endogenous miRNA23b expression in NS20Y cells (Fig. 3-9B). Anti-23b was introduced into the cells pre-treated with TSA. Immunoprecipitations followed by

western blots were performed 24 hours after transfection. Knocking down miRNA23b alleviated the repression of MOR1 and significantly increased the level of MOR1 protein detected (Fig. 3-9C, lane 4). This supports the role of miRNA23b in regulating MOR1 expression *in vivo*.

DISCUSSION

Post-transcriptional regulation mediated by the 3´UTR is an important part of gene regulation. The 3´UTR is not under rigid structural constraints to accommodate transcriptional and translational machinery, rendering it highly versatile in regulating gene expression. The revolutionary expansion of 3´UTRs suggests it has substantial potential for the transcriptional and translational control in higher vertebrates (Conne et al., 2000). The 3´UTR can control various aspects of mRNA, including nuclear transport, polyadenylation status, subcellular targeting, translation efficiency and degradation rate (Sachs and Wahle, 1993). Studies on the 3´UTR of the mu opioid receptor have been limited, partly due to the technical difficulty in identifying its 3´UTR(s). The recent sequencing of the 3´UTR in mouse and human MOR genes makes it possible to begin addressing the functional role of the MOR 3´UTR (Han et al., 2006; Ide et al., 2005; Kasai et al., 2006; Wu et al., 2005).

In this study, we constructed a firefly luciferase reporter containing the full-length MOR1 3´UTR (~10 kb) including its poly (A) signal. The construct is driven by a MOR promoter, which retains any possible interaction between the MOR1 promoter, the 5´UTR and the 3´UTR. Because the 3′-end of MOR1 [i.e., the 400 bp containing the poly(A) signal] shows a distinct preference for its own promoter (Wu et al., 2005), we also examined whether the MOR promoter is involved in the function of the 3´UTR. A stable decrease in luciferase activity was observed in the reporter containing the MOR1 3´UTR; the same effect was seen when the MOR promoter was replaced with an SV40 promoter (Fig. 3-10A) or in other cell lines such as human NMB cells and monkey COS-1 cells (Fig. 3-10B and C). Overall, the results demonstrate a repressive role of MOR1 3´UTR, independent of promoter and cellular background.

The function of 3´UTRs can be interpreted largely by the interaction between the *cis*-acting elements and their binding partners (e.g., proteins or small RNAs). For example, the AU-rich element (ARE), a well-defined sequence motif in 3´UTRs, binds to a wide range of ARE-binding proteins to target mRNAs for fast degradation (Barreau et al., 2005). The first identified miRNA, lin-4, binds to a repeated sequence in the 3´UTR of lin-14 mRNA and regulates its translation (Lee et al., 1993). The function of long 3´UTRs such as the one found in MOR1, is more likely to be explained by the cooperative effect of their multiple *cis*- and *trans*- interactions.

To determine the mechanism of the MOR1 3´UTR as a whole, we carefully compared the expression of two reporter constructs with regard to their total mRNA, subcellular distribution, and association with polysomes. The MOR1 3´UTR induced a sharp decrease in polysome mRNA levels in the pMUTR construct, compared to those of the pMPA plasmid. Because no significant difference was observed between the constructs in their total mRNA, nuclear versus cytoplasmic distribution, we concluded that the MOR1 3´UTR represses reporter activity mainly by inhibiting mRNA association with polysomes. Polysome mRNA levels have been used as an indicator of translation efficiency; more mRNA molecules in the actively translated polysome fraction correlate with higher translation efficiency (Conne et al., 2000; Kuersten and Goodwin, 2003). The 3´UTR generally suppresses polysome–mRNA association because of its complex secondary structure and interactions with *trans*-acting elements that inhibit the accessibility of mRNA to polysomes (Lee et al., 2006). However, deletions within the MOR1 3´UTR only partly restored both luciferase activity and polysomal mRNA levels. Therefore, neither a specific 3´UTR region nor the general mRNA structure alone account for its function; it is likely that multiple *cis*-elements in the 3´UTR have synergistic roles affecting the function of MOR1 3´UTR.

Based on sequence homology, the UTRscan program predicted two putative *cis*-acting elements in the MOR1 3′UTR. K Box and Brd Box are 6–7-nucleotide motifs mostly seen in 3′UTRs. They are considered too small to bind to proteins but have been shown to interact with miRNAs (Lai et al., 2005). Cluster point mutations on the three predicted boxes (two K Boxes and one Brd Box) found that only the mutation of K Box1 increased reporter activity. Although K Box2 shares the identical "seed" sequence with K Box1, mutating it had no effect.

While the "seed" region is critical to miRNA:mRNA base-pairing, its sequence alone does not guarantee interaction. Plant miRNAs are easier to identify because their sequences are near-perfect complements to their targets, and their targets are mostly in the coding region. In contrast, animal miRNAs form imperfect base-pairs with their targets, which are predominantly in the 3′UTR. Identification of animal miRNAs is complicated by the fact that their sequence is small (especially the "seed" region responsible for the base-pairing); also, the interaction might be affected substantially by a protein complex, i.e., the interaction is probably not simple hybridization by optimal base-pairing (Enright et al., 2003). It is therefore possible that miRNA access to the K Box2 is blocked by nearby binding proteins, or because of the general mRNA structure in the MOR1 3′UTR.

Overexpression of miRNA23b in NS20Y cells induced by transfecting Pmir23b decreased reporter activity in a dose-dependent fashion, and this effect required the presence of the MOR1 3′UTR. Mutating the "seed" sequence of miRNA23b significantly reduced the pMUTR reporter's sensitivity. Decreased reporter activity was induced by Pmir23bm at higher doses (e.g., 25 ng), but this was likely due to the "off-target" effect of the miRNA expression plasmid. Although a nonsense mutation sequence (according to the current miRNA database) was carefully chosen, it is possible that the mutated sequence is homologous to some unknown

miRNAs. It is believed that miRNAs comprise 1–3% of the genome (Zhao and Srivastava, 2007), and it is reasonable to expect that more miRNAs will be identified that can regulate the MOR1 3′UTR in the future. Interestingly, we found that deleting the sequence containing K Box1 (pMUTRD1) abolished the effect induced by Pmir23b, but eliminating K Box2 retained the repressive effect of Pmir23b. This corroborates the mutation results showing that K Box1, not K Box2, is a functional element.

Mature miRNA molecules are partially complementary to one or more mRNAs, usually in the 3′UTR; their main function is to downregulate gene expression. When binding to 3′UTR, the RNP complex RISC associated with miRNA usually inhibits polysome–mRNA association or induces RNA degradation (Lai, 2005). The difference between the two mechanisms can be distinguished by measuring alterations in RNA levels: Inhibition of polysome–mRNA association usually does not change the equilibrium RNA amount, but repression through RNA degradation appears as decreased RNA levels (Behm-Ansmant et al., 2006).

Pmir23b does not alter the equilibrium levels of total luciferase RNA when cotransfected with pMUTR. However, Pmir23b significantly decreased the polysomal luciferase mRNA levels, and the same mutation of its “seed” region that diminished its repression of reporter activity also attenuated its suppression on polysomal mRNA levels. In sucrose gradient experiments, Pmir23b promoted a shift of mRNA from the heavy to the light polysome fraction, relative to its mutated form (i.e., Pmir23bm). These results support that a specific interaction between miRNA23b and the MOR1 3′UTR suppresses gene expression by interfering with the association of mRNA with polysomes, thereby arresting translation of the reporter.

The miRNA target, K Box1, also plays an important role in the interaction. In *Drosophila*, mutating the K Box increases accumulation of its target mRNA transcript (Lai et al.,

1998). In the MOR1 3´UTR, mutating the K Box was likewise expected to interfere with miRNA23b function. Interestingly, in the luciferase assay, K Box1 mutation failed to alter the reporter's response to miRNA23b (data not shown). However, in experiments with polysomal mRNA, pMUTRD2 (with a wild type K Box1) showed decreased polysomal mRNA levels when cotransfected with Pmir23b, consistent with the results of the luciferase assay. When the K Box1 in pMUTRD2 was mutated, Pmir23b failed to alter the association of the mRNA with the polysomes, indicating a disruption of the miRNA:mRNA interaction. The discrepancy between the results of the luciferase assay and the experiments on polysomal mRNA could be the outcome of multiple elements in MOR1 3´UTR. Although the UTRscan program identified only three putative sites in the MOR1 3´UTR, the database used is based on already-identified *cis*-acting elements. It is reasonable to speculate that there are other, presently unknown, elements in this 10-kb region. The K Box1 is therefore likely to be one among many elements, and its activity could represent just a fraction of the total effect of the MOR1 3´UTR. Hence, although the mutation disrupted the interaction, its effect could be under the limits detectable by the reporter. In contrast, measurement of polysomal mRNAs provides a more direct means of detecting the interaction between miRNA23b and K Box1, which is an event upstream of reporter activity. Also, the use of PCR in the polysome studies could have magnified the changes caused by the K Box1 mutation.

NS20Y cells express little MOR1 protein and a very low level of MOR1 RNA. Treating cells with 500 nM of the histone deacetylase inhibitor TSA stimulates general transcription activity, including that of MOR1. However, TSA treatment had no effect on MOR1 protein levels, suggesting that repression of MOR1 expression in NS20Y cells occurs at both the transcriptional and translational levels. After TSA treatment, ample MOR1 RNA accumulated in

NS20Y cells, but translation was repressed. Adding the anti-23b primer knocked down endogenous miRNA23b and released its repression of the MOR1 3´UTR, thereby stimulating the translation of MOR1 mRNA. At a moderate concentration of anti-23b (2 nM), enhanced levels of MOR1 protein were detected. The anti-miR primer (negative control) was used to equalize the transfection primer amounts; it shares the same structure as anti-23b primer but has no sequence complementary to any known miRNAs. This excludes possible nonspecific effects induced by anti-miR primers.

Taken together, our study reveals an important role for the MOR1 3´UTR in regulating expression of its gene, and reports, to our knowledge, for the first time the involvement of a specific miRNA in regulating the expression of opioid receptors. In contrast to most studies focusing on MOR1 transcriptional regulation, this study addresses its 3´UTR, a previously poorly understood regulatory region. Post-transcriptional regulation provides abundant regulation opportunities because of their distance from the rigidly constrained transcriptional machinery (Sachs and Wahle, 1993). Recent studies have found miRNAs to be potential drug targets in cancer and other diseases (Zhang et al., 2007; Zhou et al., 2007). By scanning the sequence of MOR1 3'-UTR, 24 more potential miRNA targets were predicted by RegRNA (http://regrna.mbc.nctu.edu.tw/html/prediction.html), and 43 by microRNA.org (http://www.microrna.org/microrna/getGeneForm.do). Our finding of a functional role for miRNA23b in this regard provides a novel direction for unraveling the molecular mechanisms involved in the development of opioid tolerance. It will expand the research on opioid receptors toward an area of study that largely has been ignored, and facilitate our understanding of the complex mechanisms that regulate the expression of this important drug receptor.

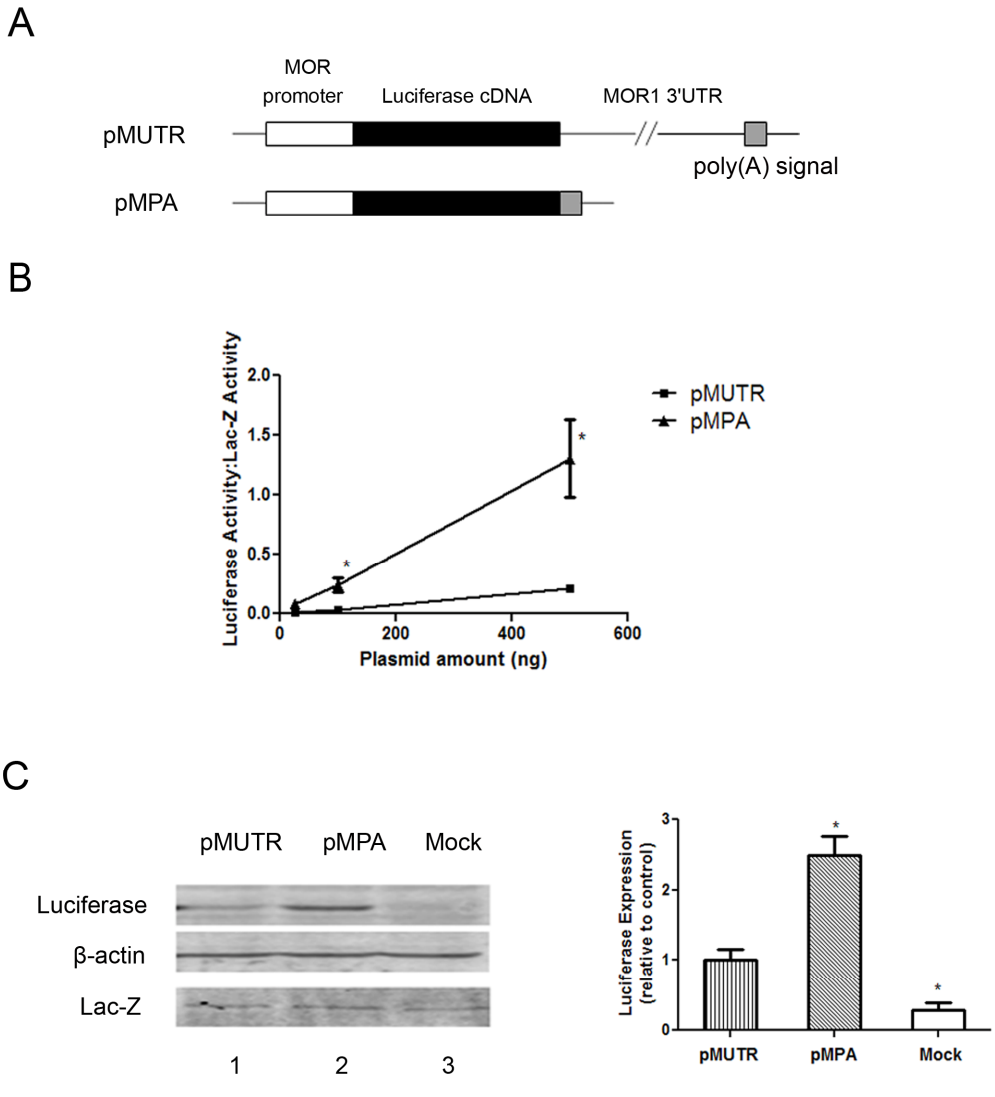

A
MOR
promoter
Luciferase cDNA
MOR1 3'UTR
pMUTR
poly(A) signal
pMPA
B
Luciferase Activity:Lac-Z Activity
2.0
1.5
1.0
0.5
0.0
0
200
400
600
Plasmid amount (ng)
pMUTR
pMPA
C
pMUTR
pMPA
Mock
Luciferase
β-actin
Lac-Z
1
2
3
Luciferase Expression
(relative to control)
3
2
1
0
pMUTR
pMPA
Mock

Figure 3-1

Figure 3-1. MOR1 3´UTR inhibits reporter expression. (A) Structure of the pMUTR and pMPA plasmids. MOR promoter (open box), luciferase cDNA (filled box), and MOR1 poly(A) signal (gray box). (B) Reporter assay performed in NS20Y cells. The amount of plasmid (shown as pMUTR's amount) transfected is plotted against the relative activity of the reporter constructs (luciferase activity/galactosidase activity). Assays were performed in triplicate. n = 3; *: $p < 0.05$. (C) Western blot of NS20Y cells transfected with pMUTR, pMPA or an equivalent amount of pcDNA3 (mock transfection). Lane 1, 500 ng pMUTR; lane 2, 200 ng pMPA; lane 3, 500 ng pcDNA3. The blot was probed with antibodies specific to luciferase, β-actin and galactosidase (lac-Z) proteins. The bar graph shows the intensity of the luciferase protein band (normalized against β-actin), relative to the intensity of the pMUTR band (control). n = 2; *: $p < 0.05$.

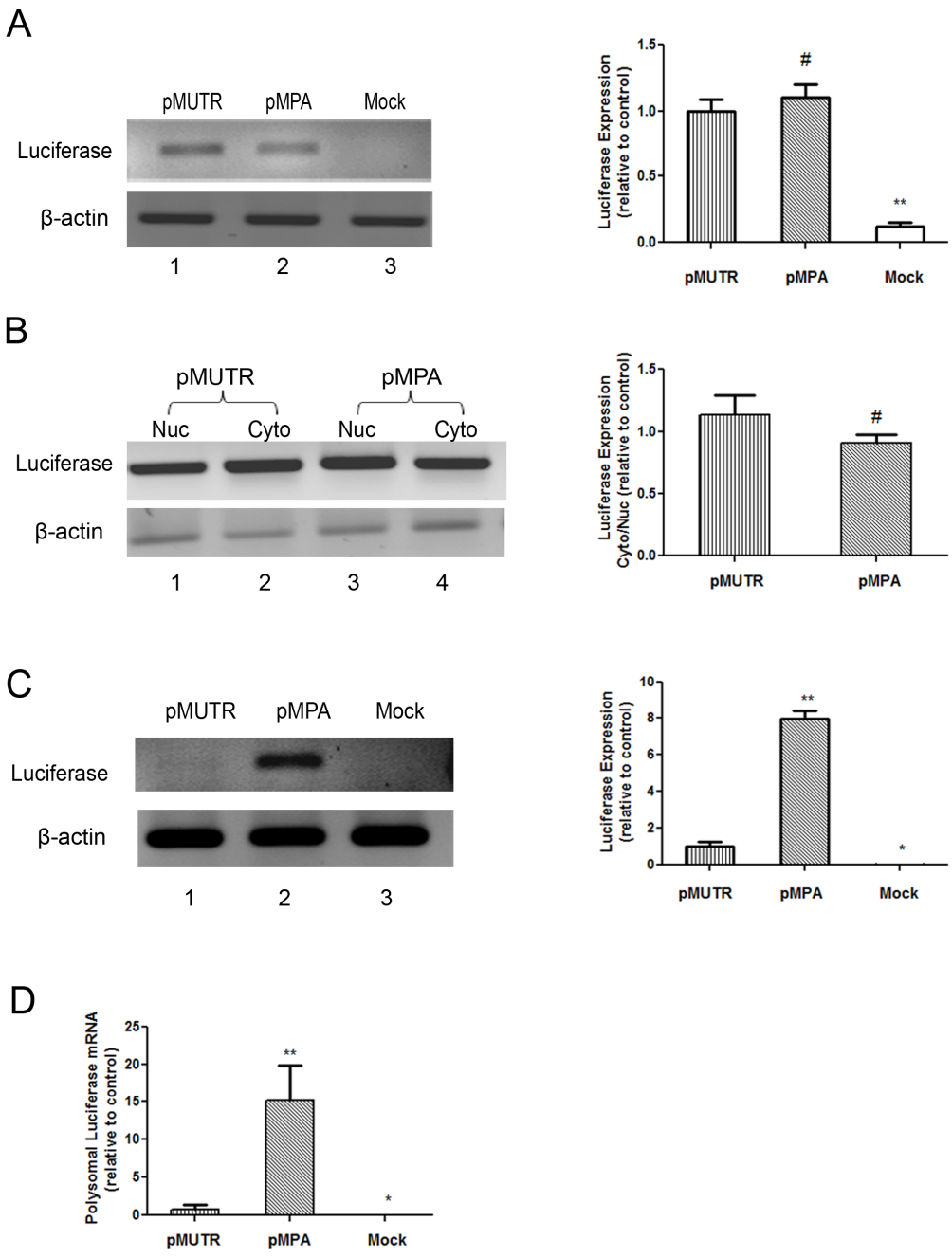

A
pMUTR
pMPA
Mock
Luciferase
β-actin
1
2
3
Luciferase Expression (relative to control)
1.5
1.0
0.5
0.0
#
**
pMUTR
pMPA
Mock
B
pMUTR
pMPA
Nuc
Cyto
Nuc
Cyto
Luciferase
β-actin
1
2
3
4
Luciferase Expression Cyto/Nuc (relative to control)
1.5
1.0
0.5
0.0
#
pMUTR
pMPA
C
pMUTR
pMPA
Mock
Luciferase
β-actin
1
2
3
Luciferase Expression (relative to control)
10
8
6
4
2
0
**
*
pMUTR
pMPA
Mock
D
Polysomal Luciferase mRNA (relative to control)
25
20
15
10
5
0
**
*
pMUTR
pMPA
Mock

Figure 3-2

Figure 3-2. MOR1 3´UTR decreases luciferase polysome mRNA levels. (A) Luciferase mRNA levels in NS20Y cells transfected with pMUTR and pMPA. NS20Y cells were transfected with 500 ng pMUTR (lane 1), 200 ng pMPA (lane 2) and 500 ng pcDNA3 (lane 3; mock transfection). Two hundred ng mRNA from each sample was used for RT-PCR. The graph shows the intensity of the normalized luciferase signal (luciferase/β-actin), relative to the intensity of the pMUTR band (control). n = 2; **: $p < 0.01$; #: $p > 0.05$. (B) Distribution of luciferase RNA between nuclear and cytoplasmic fractions from transfected NS20Y cells. One μg RNA from nuclear (Nuc) and cytoplasmic (Cyto) fractions was used for RT-PCR. The graph shows the ratio of cytoplasmic luciferase to nuclear luciferase, normalized against β-actin. n = 2; #: $p > 0.05$. (C) Polysomal mRNA levels in transfected NS20Y cells. Polysome mRNA was extracted from NS20Y cells transfected with 1 μg pMUTR, 0.4 μg pMPA or 1 μg pcDNA3 (Mock). One μg of polysomal mRNA was used for RT-PCR. The graph shows the intensity of luciferase, normalized against β-actin in each sample, relative to pMUTR (control). n = 2; *: $p < 0.05$; **: $p < 0.01$. (D) Luciferase polysomal mRNA levels analyzed by real-time qRT-PCR. One μg polysome mRNA from each sample was used as template for real-time qRT-PCR. The relative expression level of luciferase was calculated using the comparative threshold cycle method after normalization against β-actin as an internal control. The bar graph shows the expression level of each sample relative to that of pMUTR. n = 2; *: $p < 0.05$; **: $p < 0.01$.

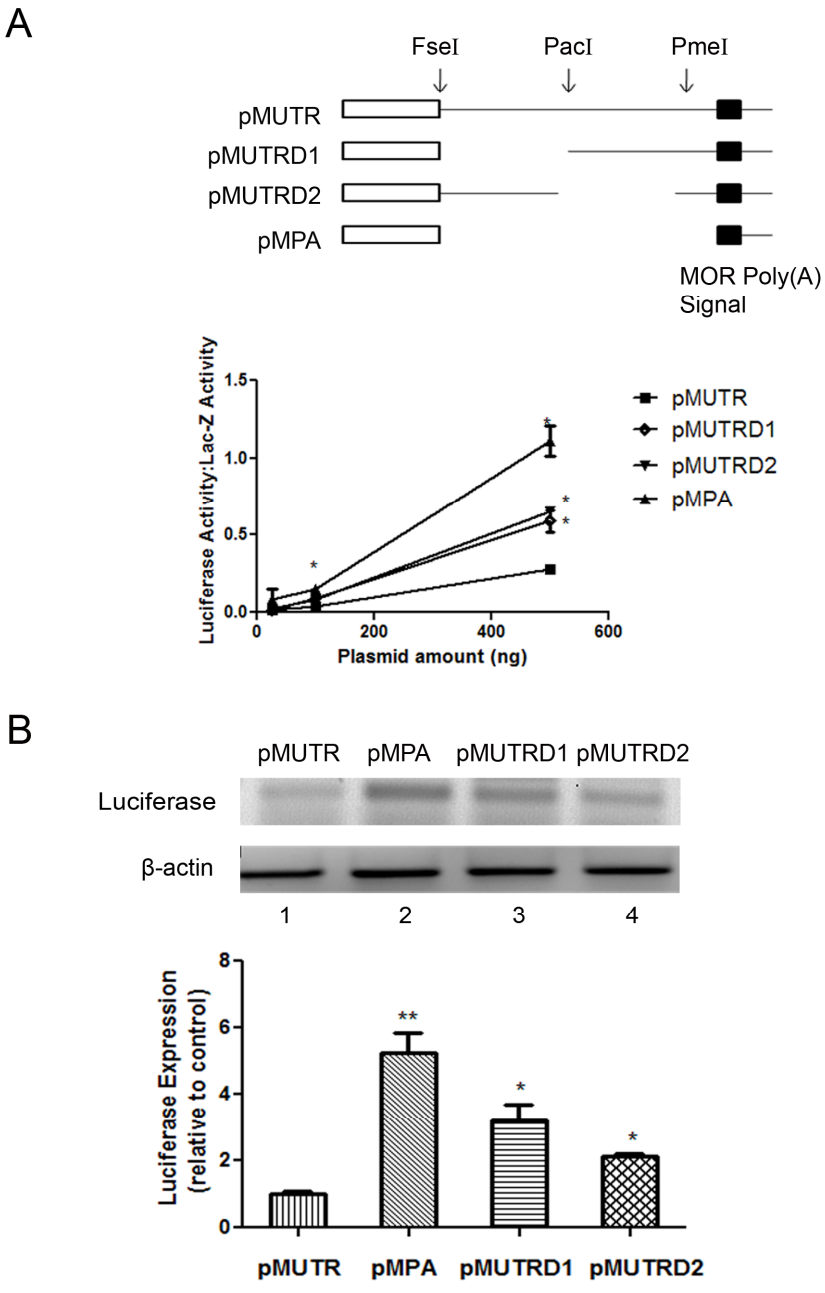

A
FseI
PacI
PmeI
pMUTR
pMUTRD1
pMUTRD2
pMPA
MOR Poly(A)
Signal
Luciferase Activity:Lac-Z Activity
1.5
1.0
0.5
0.0
0
200
400
600
Plasmid amount (ng)
pMUTR
pMUTRD1
pMUTRD2
pMPA
B
pMUTR
pMPA
pMUTRD1
pMUTRD2
Luciferase
β-actin
1
2
3
4
Luciferase Expression
(relative to control)
8
6
4
2
0
pMUTR
pMPA
pMUTRD1
pMUTRD2

Figure 3-3

Figure 3-3. Partial deletion in MOR1 3´UTR partially recovers repression. (A) Reporter assay of partial deletion constructs. Luciferase coding region (open box), MOR1 poly (A) signal (filled box). pMUTR was deleted between *Fse*I and *Pac*I restriction enzyme sites (pMUTRD1) or the *Pac*I and *Pme*I sites (pMUTRD2). The amount of pMUTR plasmid (x-axis) transfected is plotted against the activity of each reporter. The amounts of other plasmids were adjusted according to their sizes to ensure the same amount of DNA molecules was transfected into the cells. Assays were performed in triplicate. n = 3. (B) Luciferase polysome mRNA levels for each deletion construct. NS20Y cells were transfected with 1 μg pMUTR, 0.4 μg pMPA, 0.7 μg pMUTRD1, or 0.8 μg pMUTRD2. Polysome mRNA was extracted 24 hours after transfection and 1 μg polysome mRNA from each sample was used for RT-PCR. The graph shows the intensity of the luciferase signal from each sample (normalized against β-actin) relative to that of pMUTR. n = 2. *: $p < 0.05$; **: $p < 0.01$.

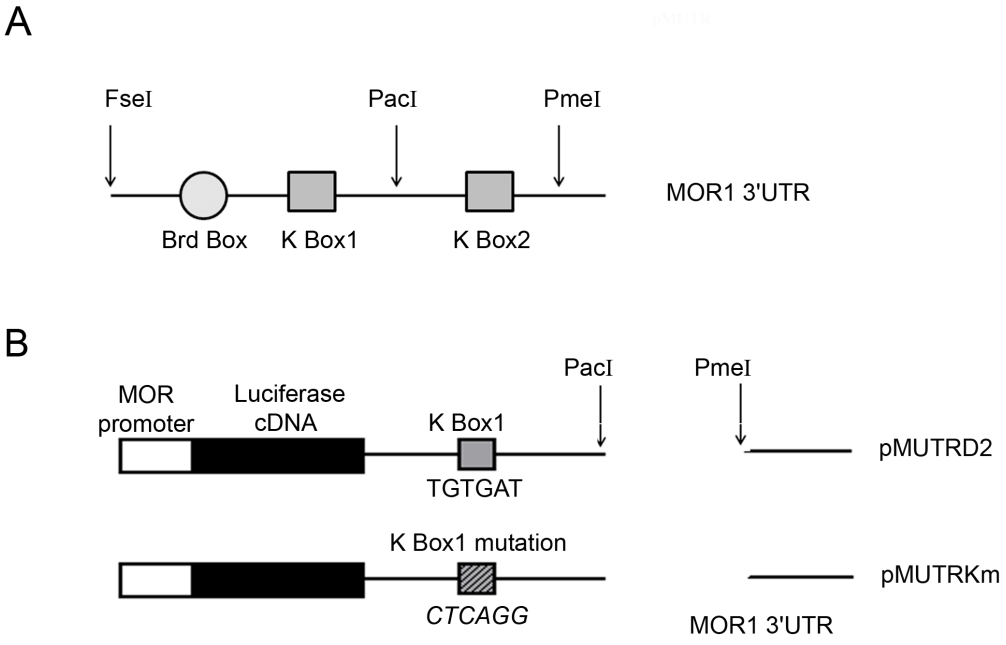
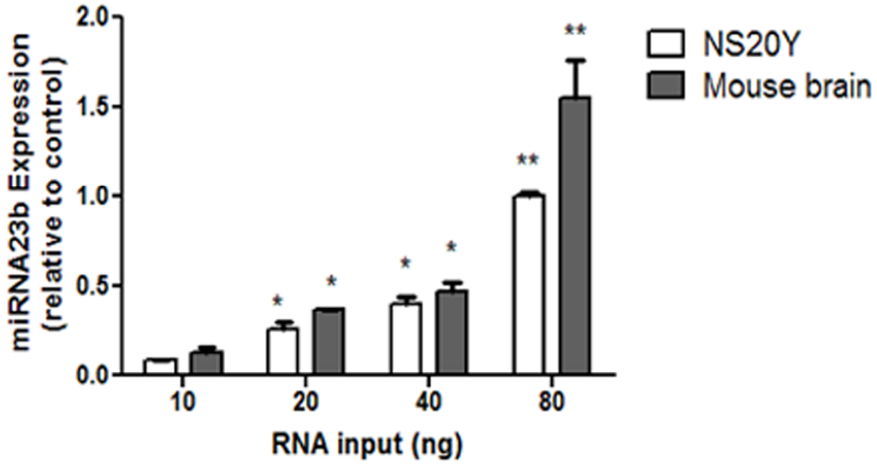

A
FseI
PacI
PmeI
Brd Box
K Box1
K Box2
MOR1 3'UTR
B
MOR
promoter
Luciferase
cDNA
K Box1
PacI
PmeI
TGTGAT
pMUTRD2
K Box1 mutation
CTCAGG
pMUTRKm
MOR1 3'UTR
C
Luciferase Activity:Lac-Z Activity
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
Plasmid amount (ng)
pMUTRD2
pMUTRKm
pMUTR
pMPA
*
*
*

Figure 3-4

Figure 3-4. K Box1 is a functional *cis*-acting element in MOR1 3´UTR. (A) The predicted *cis*-acting elements in the MOR1 3´UTR. Brd Box (light gray circle); K Boxes (gray squares). Arrows show the restriction enzyme sites in the 3´UTR. (B) Structure of the pMUTRD2 and pMUTRKm plasmids. MOR promoter (open box), luciferase cDNA (filled box), K Box1 (gray box), K Box1 mutation (striped gray box), MOR1 3´UTR (straight line). The pMUTRD2 plasmid was constructed by deleting the region between *Pac*I and *Pme*I sites of the MOR1 3´UTR in pMUTR. The pMUTRKm plasmid was constructed by mutating the six-nucleotide sequence (K Box1) TGTGAT to CTCAGG using pMUTRD2 plasmid as the template. (C) Effect of K Box1 mutation. Reporter assays were performed in NS20Y cells in triplicate. The activity of each reporter was compared relative to that of pMUTRD2. *: $p < 0.05$.

A


miRNA23b Expression
(relative to control)
2.0
1.5
1.0
0.5
0.0
10
20
40
80
RNA input (ng)
NS20Y
Mouse brain
*
**

B

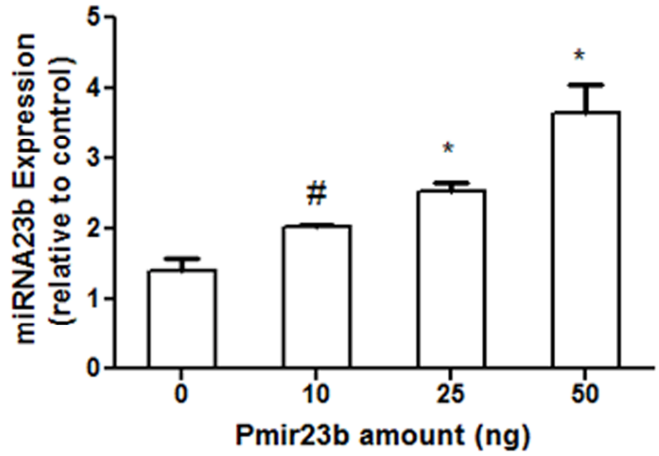

miRNA23b Expression
(relative to control)
5
4
3
2
1
0
0
10
25
50
Pmir23b amount (ng)
#
*

Figure 3-5

Figure 3-5. The expression of miRNA23b. (A) miRNA23b is expressed in NS20Y cells and mouse brain. miRNA-enriched total RNA (10, 20, 40 or 80 ng) from NS20Y cells and mouse brain was used as template for reverse transcription followed by real-time qPCR using miRNA23b-specific primers. 5s RNA primers were included as an internal control. The graph shows the amount of miRNA23b expressed, normalized against 5s RNA. n = 2. (B) The effect of Pmir23b on miRNA23b levels in NS20Y cells. Pmir23b plasmid (10, 25 or 50 ng) was transfected into NS20Y cells. Twenty-four hours after transfection, miRNA-enriched RNA was reverse-transcribed and followed by real time qPCR. 5s RNA primers were included as internal controls. Result are expressed as relative miRNA23b levels, normalized against 5s RNA. n = 2. *: $P < 0.05$; **: $P < 0.01$; #: $P > 0.05$.

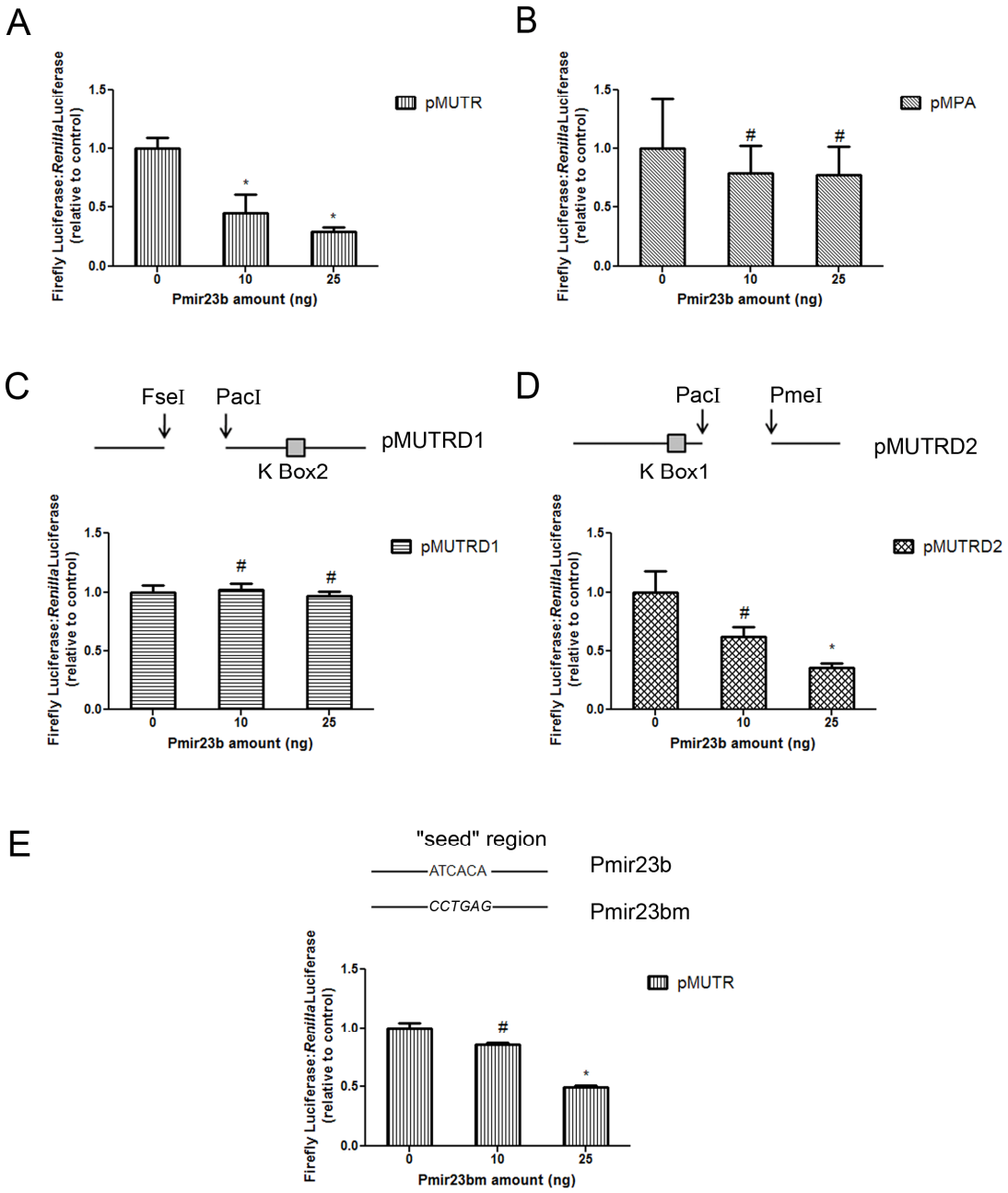

A
Firefly Luciferase:Renilla Luciferase (relative to control)
1.5
1.0
0.5
0.0
0
10
25
Pmir23b amount (ng)
pMUTR
B
Firefly Luciferase:Renilla Luciferase (relative to control)
Pmir23b amount (ng)
pMPA
C
FseI
PacI
pMUTRD1
K Box2
Firefly Luciferase:Renilla Luciferase (relative to control)
Pmir23b amount (ng)
pMUTRD1
D
PacI
PmeI
pMUTRD2
K Box1
Firefly Luciferase:Renilla Luciferase (relative to control)
Pmir23b amount (ng)
pMUTRD2
E
"seed" region
ATCACA
Pmir23b
CCTGAG
Pmir23bm
Firefly Luciferase:Renilla Luciferase (relative to control)
Pmir23bm amount (ng)
pMUTR

Figure 3-6

Figure 3-6. miRNA23b interacts with K Box1 in the MOR1 3´UTR. Pmir23b (0, 10, or 25 ng) were cotransfected into NS20Y cells with 500 ng pMUTR (A), 200 ng pMPA (B), 350 ng pMUTRD1 (C), or 400 ng pMUTRD2 (D). Assays were performed in triplicate. The intensity of the firefly luciferase signal was normalized against that of *Renilla* luciferase (internal control). n = 2; *: $p < 0.05$; #: $p > 0.05$. (E) The effect of Pmir23bm on pMUTR. The "seed" sequence in Pmir23b was mutated in Pmir23bm plasmid. Five hundred ng pMUTR were cotransfected with 0, 10 or 25 ng Pmir23bm into NS20Y cells. Reporter assays were performed as described above. n = 2; *: $p < 0.05$; #: $p > 0.05$.

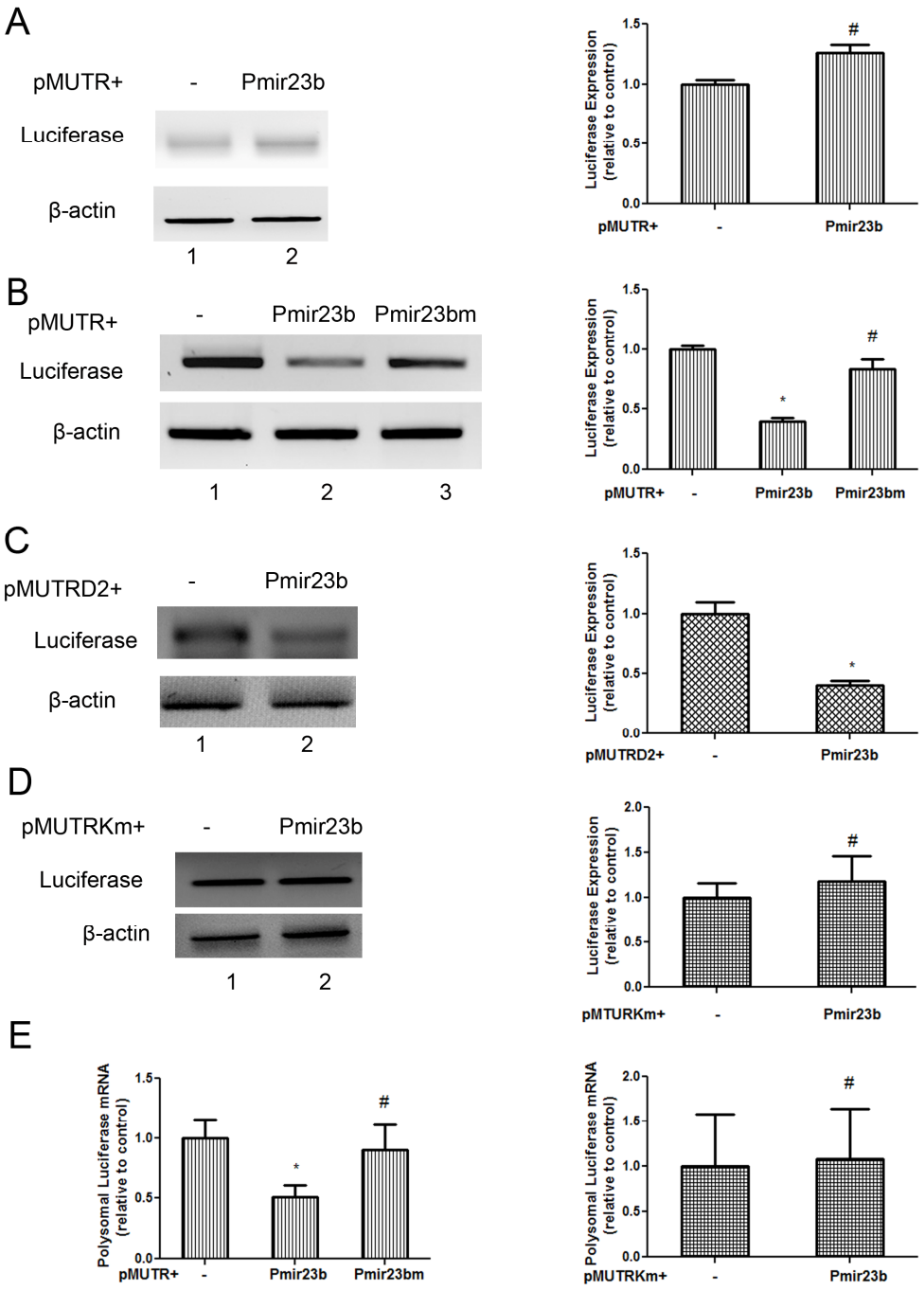

A
pMUTR+
-
Pmir23b
Luciferase
β-actin
1
2
Luciferase Expression (relative to control)
pMUTR+
-
Pmir23b
B
pMUTR+
-
Pmir23b
Pmir23bm
Luciferase
β-actin
1
2
3
Luciferase Expression (relative to control)
pMUTR+
-
Pmir23b
Pmir23bm
C
pMUTRD2+
-
Pmir23b
Luciferase
β-actin
1
2
Luciferase Expression (relative to control)
pMUTRD2+
-
Pmir23b
D
pMUTRKm+
-
Pmir23b
Luciferase
β-actin
1
2
Luciferase Expression (relative to control)
pMTURKm+
-
Pmir23b
E
Polysomal Luciferase mRNA (relative to control)
pMUTR+
-
Pmir23b
Pmir23bm
Polysomal Luciferase mRNA (relative to control)
pMUTRKm+
-
Pmir23b

Figure 3-7

Figure 3-7. miRNA23b decreases polysomal reporter mRNA levels in NS20Y cells. (A) Pmir23b does not affect the total luciferase RNA levels. One μg pMUTR was transfected alone (–) or cotransfected with 100 ng Pmir23b into NS20Y cells. One μg of RNA from each sample was used for RT-PCR. The graph shows the intensity of the luciferase signal (normalized against β-actin) relative to the pMUTR signal. n = 2; #: $p > 0.05$. (B) Pmir23b decreases the level of luciferase polysome mRNA of pMUTR. Two μg pMUTR were transfected alone (–), or cotransfected with 100 ng Pmir23b or 100 ng Pmir23bm into NS20Y cells. Two μg polysome mRNA from each sample were used for RT-PCR. The graph shows the intensity of the luciferase signal (normalized against β-actin) relative to the pMUTR signal. n = 2; *: $p < 0.05$; #: $p > 0.05$. (C) Pmir23b decreases the luciferase mRNA levels in pMUTRD2. pMUTRD2 (1.6 μg) was transfected alone (–) or cotransfected with 100 ng Pmir23b into NS20Y cells. n = 2; *: $p < 0.05$. (D) K Box mutation abolishes the effect of Pmir23b. pMUTRKm (1.6 μg) was transfected alone (–) or cotransfected with 100 ng Pmir23b into NS20Y cells. n = 2; #: $p > 0.05$. (E) Polysomal mRNA levels analyzed by real-time qRT-PCR. Polysome mRNA from (B) (left panel) and from (D) (right panel) were used for real-time qRT-PCR. One μg polysome mRNA from each sample was used as template. Luciferase expression levels of each sample are shown relative to the sample transfected with only pMUTR (left panel) or pMUTRKm (right panel). n = 2; *: $p < 0.05$; #: $p > 0.05$.

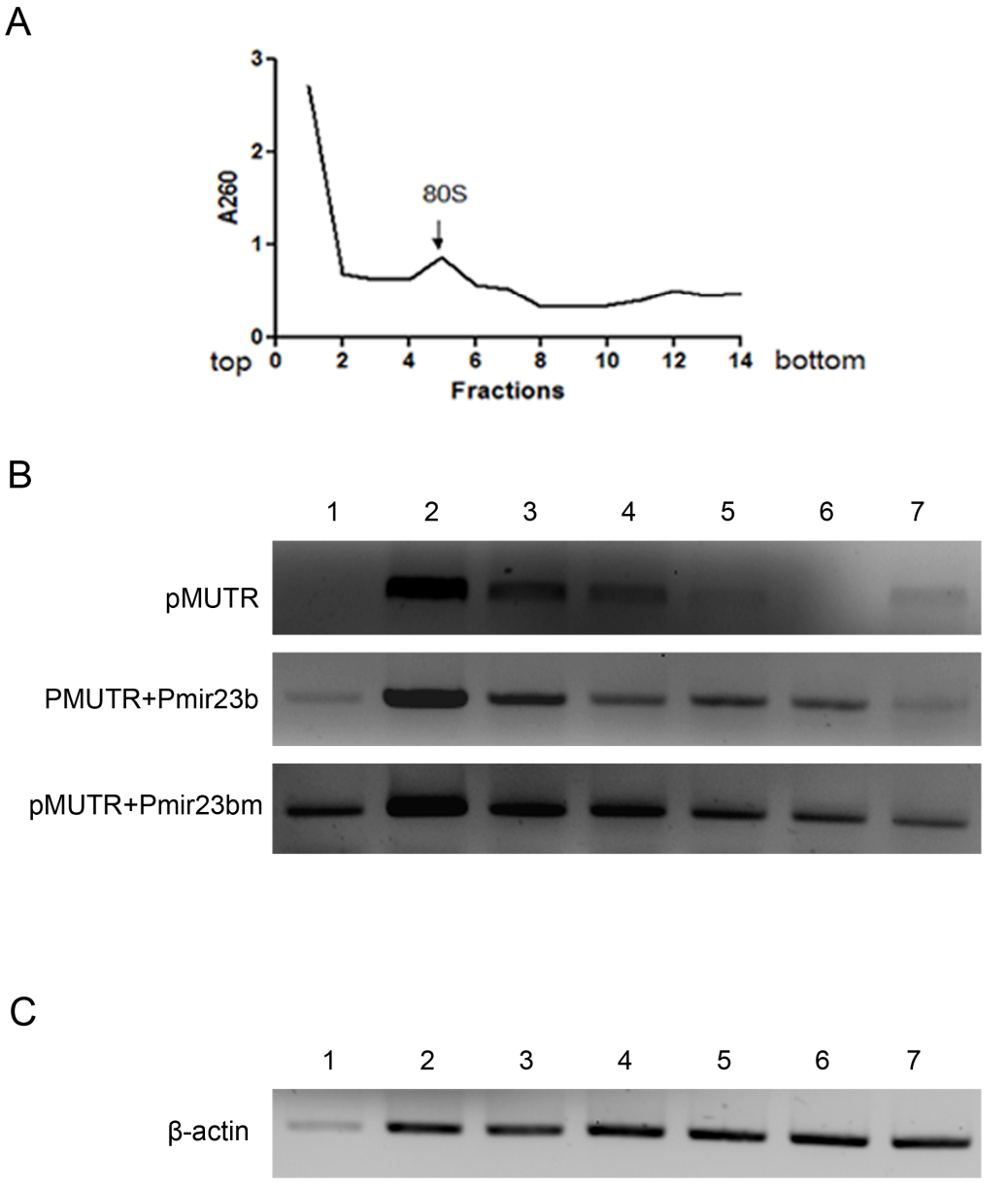

A
A260
80S
top
bottom
Fractions
B
1 2 3 4 5 6 7
pMUTR
PMUTR+Pmir23b
pMUTR+Pmir23bm
C
1 2 3 4 5 6 7
β-actin

Figure 3-8

Figure 3-8. Polysome profile of luciferase mRNA in sucrose gradients. (A) Representative profile of the UV absorption of sucrose gradient fractions. The first two fractions were devoid of ribosomes; fractions 3–10 contained monosomes; polysomes were present in fractions 11–14. (B) RT-PCR analysis of luciferase mRNA. Four μg pMUTR were transfected alone or cotransfected with 200 ng Pmir23b or Pmir23bm into NS20Y cells. Cells were lysed and layered onto 10–50% sucrose gradients. Fourteen fractions were collected from top to the bottom, and two successive fractions were pooled to obtain enough mRNA for RT-PCR using luciferase primers. The experiment was repeated twice with similar results. (C) RT-PCR analysis of β-actin mRNA (internal control). Results were similar among three transfects. A representative gel is shown.

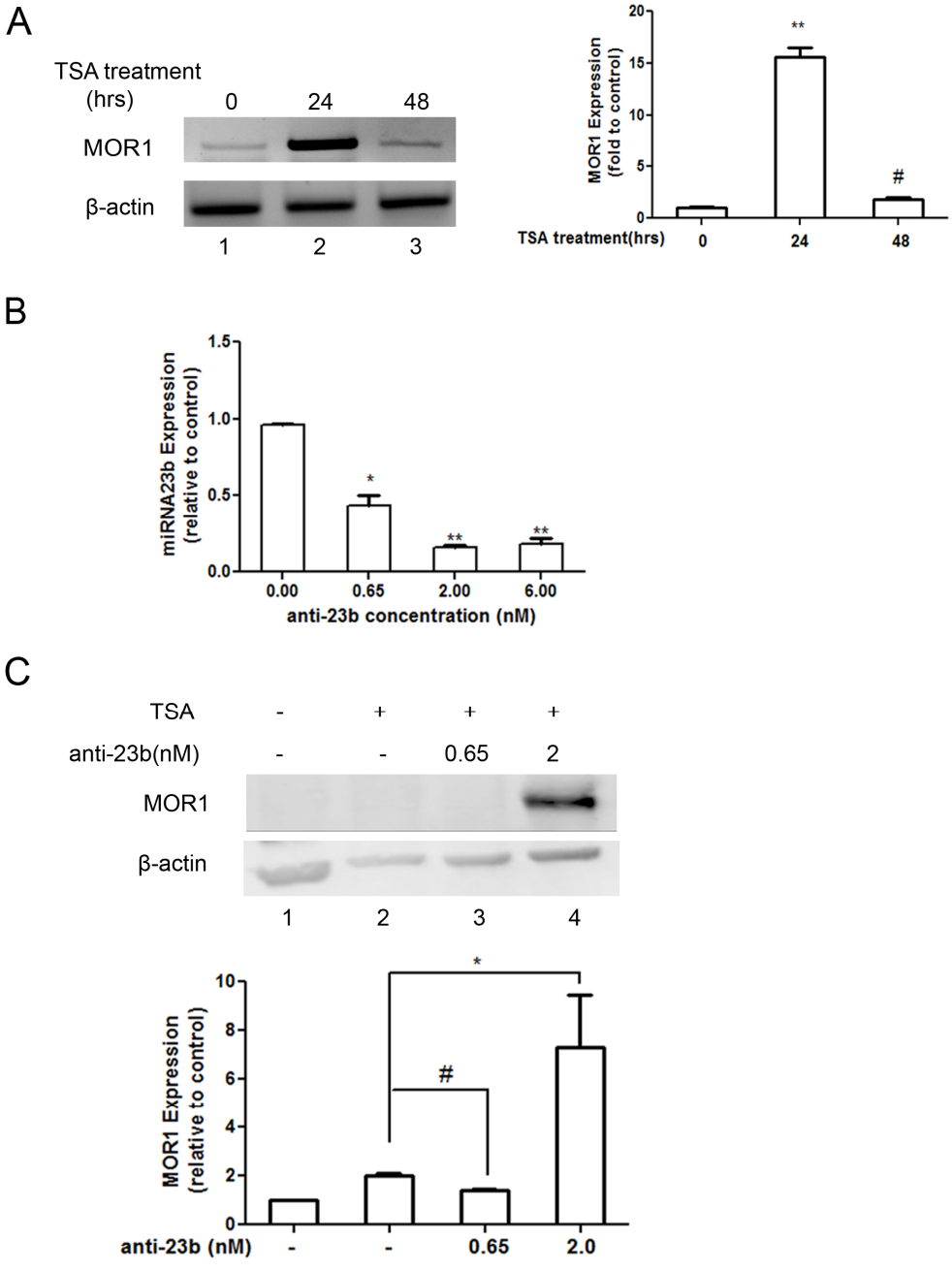
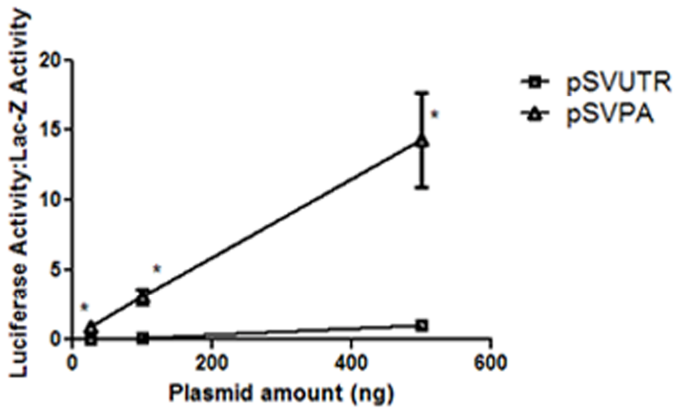

A
TSA treatment
(hrs)
0
24
48
MOR1
β-actin
1
2
3
MOR1 Expression
(fold to control)
20
15
10
5
0
**
#
TSA treatment(hrs)
0
24
48
B
miRNA23b Expression
(relative to control)
1.5
1.0
0.5
0.0
*
**
**
0.00
0.65
2.00
6.00
anti-23b concentration (nM)
C
TSA
-
+
+
+
anti-23b(nM)
-
-
0.65
2
MOR1
β-actin
1
2
3
4
MOR1 Expression
(relative to control)
10
8
6
4
2
0
*
#
anti-23b (nM)
-
-
0.65
2.0

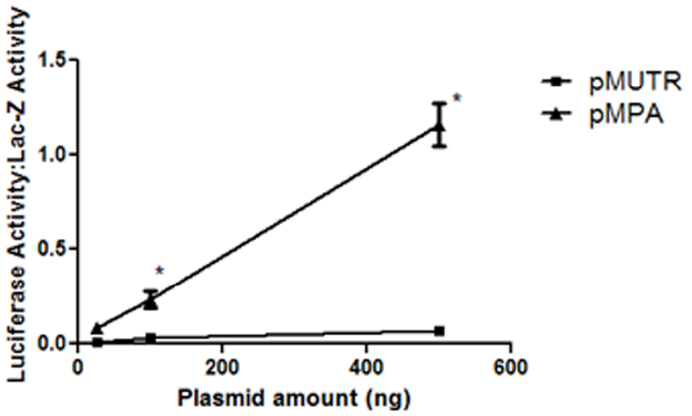
Figure 3-9

Figure 3-9. miRNA23b inhibits endogenous MOR1 protein expression in NS20Y cells. (A) TSA treatment increases MOR1 RNA levels in NS20Y cells. TSA (500 nM) was added to NS20Y cells four hours after plating. RNA was extracted 0, 24 or 48 hours after TSA treatment. One μg RNA was used for RT-PCR. The graph shows MOR1 expression levels in each sample relative to lane 1. n = 2; *: $p < 0.05$; #: $p > 0.05$. (B) Anti-23b primer knocks down the endogenous miRNA23b expression in NS20Y cells. Anti-23b primer (0, 0.65, 2, or 6 nM) was transfected into NS20Y cells. miRNA-enriched RNA was extracted 24 hours after transfection. Forty ng RNA were used for reverse transcription using miRNA23b and 5s RNA primers, followed by real-time qPCR. The graph shows miRNA23b levels, normalized to 5s RNA. n = 2; *: $p < 0.05$; **: $p < 0.01$. (C) Anti-23b increases endogenous MOR1 protein expression in NS20Y cells. NS20Y cells were pre-treated with TSA for 24 hours before transfection with anti-23b primer. Lane 1, no TSA treatment; lane 2, TSA treatment without anti-23b transfection; lane 3, TSA treatment with 0.65 nM anti-23b; lane 4, TSA treatment with 2 nM anti-23b. Anti-miR (negative control) was added to transfectants in lanes 2 and 3 (2 nM and 1.35 nM, respectively) to equalize the total transfection concentration to 2 nM. Western blots of immunoprecipitates were probed with antibodies specific to MOR1 and β-actin. The graph shows the normalized level of MOR1 protein (MOR1/β-actin) relative to the levels in lane 2. n = 2; *: $p < 0.05$; #: $p > 0.05$.

A

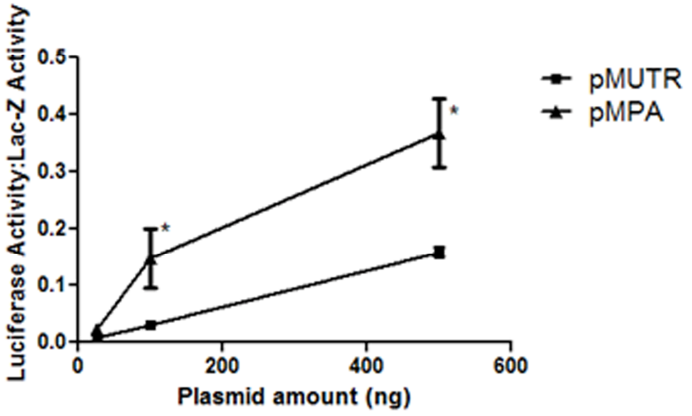


B



NMB cells

C



COS-1 cells

Figure 3-10

Figure 3-10. MOR1 3'-UTR represses reporter expression independent of promoter and cell type. (A) Reporter assays of pSVUTR in NS20Y cells. The amounts of pSVUTR plasmid (x-axis) transfected are plotted against the relative activity of the reporter constructs (luciferase activity/galactosidase activity). The amounts of other plasmids were adjusted according to their sizes to ensure the same amount of DNA molecules was transfected into the cells. Assays were performed in triplicate. n = 3. Reporter assay of pMUTR in NMB cells (B) and COS-1 cells (C). Figure legends are the same as in (A). Assays were performed in triplicate. n = 3. *: $P < 0.05$.

Chapter IV

Chronic Morphine Treatment Decreases the Association of Mu-opioid Receptor (MOR1) mRNA with Polysomes through miRNA23b

Abstract

Mu-opioid receptor (MOR) mediates most of the pharmacological effects of opioid drugs. The expression of MOR is temporarily and spatially regulated at both the transcriptional and post-transcriptional levels. Chronic morphine treatment that induces tolerance does not alter MOR mRNA expression, suggesting no direct link between agonist treatment and MOR gene transcription. We previously identified the 3′-untranslated region (3′-UTR) of the major transcript of mu-opioid receptor (MOR1) and revealed a novel *trans*-acting factor, miRNA23b, that binds to the K Box motif in the 3′-UTR. The interaction between miRNA23b with the MOR1 3′-UTR suppressed receptor translation by inhibiting polysome–mRNA association. In this report, we demonstrate that chronic morphine treatment increases miRNA23b expression in a dose- and time-dependent manner, and represses the association of MOR1 mRNA with polysomes through the MOR1 3′-UTR. The translational luciferase reporter assay shows a suppression effect of morphine on reporter activity that requires the MOR1 3′-UTR. This suggests a potential link between MOR expression and morphine treatment at the post-transcriptional level in which a specific miRNA, miRNA23b, is involved.

Introduction

Opioid drugs are widely used clinically to treat moderate to severe pain. Three major opioid receptors, μ (mu), δ (delta), and κ (kappa), belong to the G-protein-coupled receptor superfamily (Kieffer, 1995). Mu-opioid receptor (MOR) mediates most of the pharmacological effects of opiates; its regulation is of great importance to unravel the molecular mechanisms underlying the physical responses to opioid treatment, such as tolerance and dependence. In addition to the multiple *cis*-acting elements that regulate the transcription of MOR (Choi et al., 2007; Hwang et al., 2004; Kim et al., 2006; Song et al., 2007), a recent study on the 3′-UTR of the major mu-opioid receptor mRNA (MOR1) has started to address the regulation of MOR at the post-transcriptional level (Wu et al., 2008).

The phenomenon of MOR down-regulation has been observed in various cell lines and neurons caused by chronic agonist treatment (Tao et al., 1998; Yabaluri and Medzihradsky, 1997; Yamamoto et al., 2008). Down-regulation results primarily by sequestration of membrane receptors to the cytosol via clathrin-coated pits and dynamin following receptor phosphorylation. The internalized endosomes merge with intracellular lysosomes and the receptors are degraded proteolytically, resulting in a decrease of the total number of receptors in the cell (Binyaminy et al., 2008). In addition to this classic model, accumulating evidence shows the involvement of many other factors in the processes, e.g., protein kinase C (Kramer and Simon, 1999), mitogen-activated protein kinase (Schmidt et al., 2000), and Ca^{2+}/calmodulin-dependent protein kinase (Koch et al., 1997). However, whether a decreased receptor biosynthesis is involved is still under debate. One study measured the receptor turn-over rate in mouse neuronal N2A cells expressing a cloned mu-opioid receptor (N2A-MOR) and found that the down-regulation of MOR caused by

agonist stimulation is the sum of both accelerated receptor degradation and decreased receptor biosynthesis (Afify, 2002).

The expression of MOR can be regulated at both the transcriptional and post-transcriptional levels. It is widely accepted that chronic morphine treatment does not alter MOR mRNA levels (Brodsky et al., 1995a), indicating that morphine has no significant effect on the transcription of MOR gene. Nonetheless, it is not known if morphine can regulate MOR mRNA at the post-transcriptional level, possibly through interactions between *trans*-acting factors and its 3′-UTR. Although morphine can induce discrete and fluctuating expression of important factors related to MOR function (Ammon-Treiber and Hollt, 2005), whether these factors are involved in the post-transcriptional regulation of MOR is still unknown. Our recent study identified miRNA23b as a *trans*-acting factor that represses MOR translation efficiency through interaction with the 3′-UTR of MOR1 (Wu et al., 2008).

Little is known about the mechanisms responsible for regulating miRNA expression. In some cells, the production of miRNAs appears to be actively regulated (Boyd, 2008; Woods et al., 2007). It would be of interest to investigate if activating a membrane receptor can control the expression of a miRNA, thereby regulating expression of the receptor gene. In this report, we investigated if morphine treatment could change miRNA23b expression and consequently regulate the translation of MOR mRNA.

miRNA23b interacts with the MOR1 3′-UTR through binding to a K Box motif (Wu et al., 2008). The MOR1 3′-UTR is absent from the plasmid DNA sequences in all cell lines that express cloned MOR, such as N2A-MOR and HEK-MOR (Chakrabarti et al., 1995; Wu and Wong, 2005). N2A cells are not known to express any opioid receptors (Im et al., 2001); N2A-MOR cells were established by stably transfecting MOR1 into N2A cells. MOR1, a 1.4-kb insert,

was subcloned into the expression vector pRC/CMV; the cDNA sequence contains the entire coding region of the MOR, together with 200 base pairs of 5′ non-coding region and only 30 base pairs of 3′ non-coding region (i.e., the 3′-UTR). The cloned MOR (without the major part of MOR1 3′-UTR sequence) behaves like native receptor in terms of its desensitization and down-regulation effects (Chakrabarti et al., 1995). However, it has not yet been determined if the absence of MOR1 3′-UTR influences the regulation of the MOR gene. In this report, we investigated the effect of morphine on miRNA23b expression in N2A-MOR cells and the resulting changes in MOR1 mRNA translation efficiency. In addition, we revealed the critical roles of MOR receptor and MOR1 3′-UTR in this pathway.

Materials and Methods

Cell Culture, Transfection and Luciferase Reporter Assay

Mouse neuronal cells N2A, N2A-MOR (Chakrabarti et al., 1995) and human neuronal cells NMB, SHSY-5Y were maintained in Advanced DMEM or RPMI (for NMB) (Invitrogen, Carlsbad, CA, USA) with 5% heat-inactivated fetal bovine serum in an atmosphere of 10% (for N2A and N2A-MOR) or 5% (for NMB and SHSY-5Y) CO_2 at 37°C. The medium for N2A-MOR was supplemented with 0.2% Geneticin (G418). Transfections of anti-23b or anti-miR negative control primer (Ambion, Austin, TX, USA) were performed using Lipofectamine 2000 (Invitrogen) as described before (Wu et al., 2008).

For the luciferase reporter assay, cells were plated at a density of 0.5×10^5 cells per well in 24-well plates 24 hours prior to transfection; 2 ng *Renilla* luciferase plasmid pCMV-Rluc (Gift from Dr. Yan Zeng) was included for normalization. Morphine was added 3 hrs before transfecting pSVUTR or pSVPA plasmids (Wu et al., 2008). Twenty-four hours after transfection, the firefly and *Renilla* luciferase activities were determined by a luminometer (Berthold, Oak Ridge, TN, USA) using Dual-Luciferase Reporter Assay systems (Promega, Madison, WI, USA) according to the manufacturer's protocol.

RT-PCR, real-time qPCR, and qRT-PCR

RNA was isolated from cells using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and treated with Turbo DNase I (2U/μg RNA) (Ambion) before being reverse-transcribed. One-step RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen, Valencia, CA, USA) and the following primers: Mouse MOR1, 5′-CTGCTCGAATCCGTCAAAACA-3′ (sense) and 5′-AGCAACCTGATTCCAAGTAGA-3′ (antisense); HA-MOR1, 5′-

CTGCTCGAATCCGTCAAAACA-3′ (sense) and 5′-GGCAACTAGAAGGCACAGTC-3′ (antisense); and mouse β-actin, 5′-TGGCCTTAGGGTGCAGGGGG-3′ (sense) and 5′-GTGGGCCGCTCTAGGCACCA-3′ (antisense). For MOR1 RNA, the product from the one-step RT-PCR was re-amplified for a second round using Taq polymerase (New England Biolabs, Ipswich, MA, USA) and primers: 5′-CTGCTCGAATCCGTCAAAACA-3′ (sense) and 5′-GTAGATGGCAGCCTCTAA-3′ (antisense) primers. PCR was performed on a GeneAmp PCR System 9600 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) using 30 cycles (for MOR1 and HA-MOR1) or 20 cycles (for β-actin) of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by 72°C for 10 min. The linear range of PCR cycles for each gene had been pre-determined using relative PCR, and cycle numbers for PCR and RT-PCR were optimized according to the results. PCR products were electrophoresed in 1% or 2% agarose gels, quantified by ImageQuant 5.2 (Amersham Biosciences Inc., Piscataway, NJ, USA) and verified by DNA sequence analysis.

miRNA-enriched RNA was extracted and reverse transcribed followed by qPCR as described before (Wu et al., 2008). One-tenth of the reverse transcription mix was used for real-time qPCR. The miRNA primer sets hsa-miR23b and snoRNA234 (as an internal control) (Applied Biosystems) were used for reverse transcription, and qPCR was performed according to the manufacturer's protocol.

Real-time qPCR and qRT-PCR were performed on an iCycler (Bio-Rad Laboratories, Oakland, CA, USA) using either an iQ Supermix Kit (Bio-Rad) for miRNA23b and snoRNA234 or a Quantitect SYBR Green RT-PCR kit (Qiagen) for MOR1, HA-MOR1 and β-actin. The relative expression levels of miRNA23b were calculated using the Gene Expression Macro

(http://www.bio-rad.com/) normalized to those of snoRNA234; and the levels of MOR1 and HA-MOR1 were calculated against those of β-actin.

Polysome mRNA Extraction

Polysome mRNA extraction was conducted as described previously (Wu et al., 2008). Polysomal mRNA was isolated from pellets using TRI reagent (Molecular Research Center) following the protocol.

Statistics

Data are presented as mean values ± SD. Comparisons between groups were performed using the Student's *t*-test. $P < 0.05$ was taken as significant.

Results

Chronic morphine treatment increases miRNA23b levels. N2A-MOR cells expressing HA-tagged MOR1 receptor were treated with morphine (10^{-8} to 10^{-5} M) for 24 hours. miRNA23b levels were determined by reverse transcription followed by real-time qPCR. There was a dose-dependent increase in miRNA23b levels, with the maximum effect reached under a treatment of 10^{-6} M morphine (Fig. 4-1A). Human and mouse miRNA23b sequences have 100% homology (http://microrna.sanger.ac.uk/). In human neuronal cell lines NMB and SHSY-5Y (both express MOR endogenously), morphine stimulated a similar dose-dependent increase of miRNA23b levels (Fig. 4-1B). N2A-MOR cells were treated with 10^{-6} M morphine for 1, 3, 6, or 24 hrs (Fig. 4-1C). The up-regulation of miRNA23b was only observed after treatments longer than 6 hours, indicating that prolonged exposure to morphine was required to alter the expression of miRNA23b.

Wild-type N2A cells are devoid of opioid receptors (Im et al., 2001). To determine the role of MOR receptor, the dose-response and time-course of miRNA23b in wild-type N2A cells was assessed. An increasing dose of morphine (10^{-8} to 10^{-5} M) did not change the miRNA23b levels in N2A cells (Fig. 4-1D). Also the same dose (10^{-6} M) that stimulated a three-fold increase of miRNA23b expression in N2A-MOR cells did not induce any significant change in N2A cells over the course of a 24-hour treatment period (Fig. 4-1E), confirming the role of MOR receptor in the morphine-induced up-regulation of miRNA23b.

Morphine inhibits the association of MOR1 mRNA with polysomes through an interaction between miRNA23b and the MOR1 3′-UTR. We reported previously that miRNA23b interacts with a K Box motif in the MOR1 3′-UTR and suppresses the translation of MOR1 mRNA (Wu et

al., 2008). Because morphine enhances miRNA23b expression in N2A-MOR cells, it is possible that these elevated levels of miRNA23b could repress the polysome association of MOR1 mRNA. In N2A-MOR cells, the pRC/CMV plasmid encoding the HA-tagged MOR1 protein lacks the MOR1 3′-UTR region. Presumably, RNA transcribed from the pRC/CMV plasmid (i.e., HA-MOR1) will not be affected by miRNA23b. In contrast, RNA transcribed from the native MOR DNA (i.e., MOR1) includes the 3′-UTR and should be regulated by miRNA23b via its interaction with the K Box.

RT-PCR was used to distinguish the HA-MOR1 RNA from MOR1 RNA, by using two different antisense primers specific to pRC/CMV plasmid or the MOR1 3′-UTR (Fig. 4-2A). N2A-MOR cells express little native MOR1 RNA (Fig. 4-2B, lane 1). In order to detect changes in MOR1 transcript levels, the histone deacetylase inhibitor, sodium butyric acid (SBA), was used to stimulate general transcription in N2A-MOR cells. Cells treated with 200 nM SBA for 24 hours showed increased levels of both MOR1 and HA-MOR1 RNA by RT-PCR (Fig. 4-2B, lane 2). SBA treatment had no effect on the morphine-induced changes in miRNA23b expression (data not shown). Cells expressing these transcripts (i.e., HA-MOR1, without the MOR1 3′-UTR and MOR1, with the MOR1 3′-UTR) were used to examine the role of MOR1 3′-UTR in terms of their ability to associate with polysomes.

In NS20Y cells, when endogenous miRNA23b was knocked down using an anti-23b primer, the repression of miRNA23b on MOR1 translation was released, as shown by increased MOR protein levels (Wu et al., 2008). In N2A-MOR cells, anti-23b primer can also effectively knock down endogenous miRNA23b expression (Fig. 4-2C). miRNA23b inhibits the MOR1 expression mainly by repressing its association with polysomes rather than by inducing RNA degradation (Wu et al., 2008). N2A-MOR cells were treated with 200 nM SBA for 24 hours

before transfection with anti-23b primer. Total RNA was extracted and analyzed by one-step RT-PCR. Both MOR1 and HA-MOR1 RNA levels remained unchanged by anti-23b primer transfection alone, treatment with 10^{-6} M morphine alone, or by morphine treatment of anti-23b-transfected cells (Fig. 4-3A). This is consistent with the previous observation suggesting that miRNA23b does not induce significant RNA degradation (Wu et al., 2008).

Polysomal mRNA levels are a robust indicator for translation efficiency (del Prete et al., 2007). In N2A-MOR cells, the MOR1 polysomal mRNA levels increased significantly after transfection with anti-23b primer (Fig. 4-3B, lane 2). Treatment with 10^{-6} M morphine (i.e., a dose that increases miRNA23b expression) reversed the effect of anti-23b primer (Fig. 4-3B, lane 3). In contrast, when the 3′-UTR was truncated (e.g., in the HA-MOR1 transcript), neither anti-23b transfection nor morphine treatment affected the mRNA's ability to associate with polysomes (Fig. 4-3B), confirming that the MOR1 3′-UTR is required for the interaction with miRNA23b. This result was further confirmed by real-time qRT-PCR (Fig. 4-3C). In summary, chronic morphine treatment increases miRNA23b expression, thereby inhibiting the polysome-mRNA association of MOR1 via interactions with the MOR1 3′-UTR.

Morphine inhibits the translational reporter activity through MOR1 3′-UTR pSVUTR and pSVPA plasmids were constructed by inserting the complete MOR1 3′-UTR or only MOR1 poly (A) (400bp sequence flanking the poly (A) signal) into a translational luciferase reporter construct, respectively (Wu et al., 2008). N2A-MOR cells were pretreated with morphine (10^{-8} to 10^{-5} M) before transfection. In cells transfected with pSVUTR (with the MOR1 3′-UTR), the reporter activity was repressed by morphine in a dose-dependent manner (Fig. 4-4A); however, no significant change was observed in cells transfected with pSVPA (without the MOR1 3′-UTR)

(Fig. 4-4B). It shows that morphine treatment inhibits the translational reporter expression through the MOR1 3′-UTR.

Discussion

Morphine tolerance after chronic treatment includes a series of profound changes. There are three phenomena that have been studied extensively: desensitization, which happens when the activated receptor is phosphorylated by a G-protein-receptor-coupled kinase and associates with β-arrestin that uncouples the receptor-G-protein complex; internalization, which refers to the sequestration of the receptor from the membrane to the cytosol via clathrin-coated pits and dynamin; and down-regulation, which is shown by a general decrease in receptor numbers (Binyaminy et al., 2008).

For acute treatment, it is generally agreed that morphine does not induce receptor down-regulation (Castelli et al., 1997). However, there are equivocal reports as to whether chronic morphine exposure can result in receptor down-regulation. Binding studies of MOR following chronic morphine treatment have shown significant mu-opioid receptor down-regulation in mouse (Yoburn et al., 1993), rat (Bhargava and Gulati, 1990), and cells in culture (Zadina et al., 1993). The decrease of mu-receptor protein quantity was also observed by western blot in the mouse brainstem following chronic morphine administration (Bernstein and Welch, 1998). In contrast, some studies failed to show any change in MOR binding site numbers after chronic morphine treatment (Hitzemann et al., 1974). Several reports suggested that down-regulation of opioid receptors is readily observed after chronic exposure to high-intrinsic-efficacy agonists (e.g. etorphine), but not following low-intrinsic-efficacy agonists (e. g., morphine) (Duttaroy and Yoburn, 1995; Shen et al., 2000; Whistler et al., 1999; Yabaluri and Medzihradsky, 1997; Zaki et al., 2000).

The down-regulation of MOR can be seen as the sum of increased receptor degradation and decreased receptor synthesis (Afify, 2002). It is generally known that MOR mRNA levels do

not change following morphine treatment (Brodsky et al., 1995a), indicating no major alteration involved at the transcriptional level. However, whether morphine can affect MOR mRNA at the post-transcriptional level is still not clear.

Previously, we identified and cloned the MOR1 3′-UTR and demonstrated its ability to suppress the translation efficiency of receptor mRNA. A *trans*-acting factor, miRNA23b interacts with the *cis*-acting element K Box in the MOR1 3′-UTR, inhibiting the association of MOR1 mRNA with polysomes, thereby arresting its translation. In this study, we employed the mouse neuronal N2A-MOR cell line to determine if through miRNA23b, morphine can affect the polysome-mRNA association of MOR1, a critical step in translation control. These cells stably express exogenous MOR encoded from a plasmid containing only MOR1 cDNA (i.e., excluding the major part of the MOR1 3′-UTR). The cell line produces a homogenous population of MOR proteins, and imitates the native receptor in response to opioid agonists at the signal transduction level (Chakrabarti et al., 1995).

Morphine induced a dose-dependent increase of miRNA23b in N2A-MOR cells. A prolonged morphine treatment was required for the up-regulation of miRNA23b. This delayed response could result from the time it takes to alter the miRNA maturation pathway. In wild-type N2A cells, which do not express MOR protein, morphine did not change the expression level of miRNA23b, suggesting an indispensable role for MOR. In addition, the upregulation of miRNA23b by morphine was confirmed in NMB and SHSY-5Y cells, which endogenously express MOR.

The expression of miRNA23b seems to be under complicated regulation. An opioid antagonist, naloxone also increases the miRNA23b levels, but not in a dose-dependent manner (Fig. 4-5A), and this effect was also seen in the MOR-negative cell line N2A (Fig. 4-5B). The

result suggests that naloxone regulates miRNA23b expression, but likely through pathways other than that of the MOR receptor. In N2A-MOR cells pre-treated with naloxone before adding morphine, the miRNA23b levels were not significantly different from those seen with morphine treatment alone (Fig. 4-5C). This is likely the result of combined effect of naloxone's up-regulating miRNA23b and blocking the MOR receptor.

miRNAs are small non-coding RNAs that participate in the spatiotemporal regulation of mRNA and protein synthesis. Aberrant miRNA expression can lead to developmental abnormalities and diseases, but the stimuli and processes regulating miRNA biogenesis are largely unknown (Davis et al., 2008).

The maturation of miRNAs includes multiple-step processes from the transcription of pri-miRNAs to the association of mature miRNAs with the RISC complex targeting mRNAs (Boyd, 2008). miRNAs can be regulated exquisitely at each step through their maturation cascade, by controlling the transcription of pri-miRNAs, altering the processing of pri- and pre-miRNAs, changing the miRNA turnover, or modulating the regulators involved in the biosysthesis of miRNAs, etc. (Ding et al., 2008).

miRNA23b was the first miRNA identified to regulate MOR. It suppresses the polysome–mRNA association of MOR1 through its interaction with a K Box in the MOR1 3′-UTR (Wu et al., 2008). An important feature of this *trans*-acting element is that it responds to morphine treatment and can act as a "messenger" to regulate MOR gene expression at the post-transcriptional level. Morphine increases the miRNA23b level and leads to a decrease of the polysome association of MOR1 mRNA. This effect was only observed in native MOR1 mRNA (i.e., those with the MOR1 3′-UTR), but not in HA-MOR1 mRNA (lacking the MOR1 3′-UTR). It confirms that the MOR1 3′-UTR with the K Box motif is required for the repression by

miRNA23b. In the reporter assay, morphine treatment induced a dose-dependent decrease of luciferase activity in the plasmid with the MOR1 3′-UTR (pSVUTR), but not in the one without MOR1 3′-UTR (pSVPA). Due to technical difficulties, we couldn't show the change of MOR protein level by western blot. However, using the reporter assay, we confirmed the suppression of morphine on the reporter expression which requires the MOR1 3′-UTR. Taken together, our data support a post-transcriptional pathway for MOR gene that is induced by morphine through increasing miRNA23b expression.

When studying the regulation of MOR gene, a long-existing dilemma is that the transcription level of MOR does not reflect the agonist activation. The current advance in studying the 3′- UTR of MOR has made it possible to inspect its post-transcriptional regulation, which has the potential to answer this critical question. i.e. how the treatment by a MOR-agonist that induces tolerance regulates the gene expression of the receptor. Our study serves a preliminary investigation in such purpose. miRNA23b not only inhibits the MOR translation efficiency, but also is up-regulated by chronic morphine treatment. It also comes to our attention that miRNA23b appears to be under profound regulation i.e., its level can be affected by agonists like morphine as well as antagonists like naloxone. The signaling pathways that regulate miRNAs are starting to unfold. It is of great interest to systemically examine the cellular events that transduce the morphine-activated signal to the synthesis or functioning of miRNAs. On the other hand, it must be noted that the 3′-UTR of MOR1 is absent from all plasmids that encode cloned MOR proteins in stably transfected cell lines. Caution should therefore be taken when studying the regulation of MOR1 that involves its 3′-UTR in these cell lines.

As the major molecular target of opiates, understanding the regulation of MOR, transcriptionally and post-transcriptionally, is important for unraveling the molecular mechanism

of tolerance development. However, a direct link between agonist treatment and MOR gene transcription is lacking. From the aspect of post-transcriptional regulation, this report presents evidence for a new pathway that transduces membrane receptor signals to regulate the intracellular MOR expression in which a novel regulator miRNA is involved.

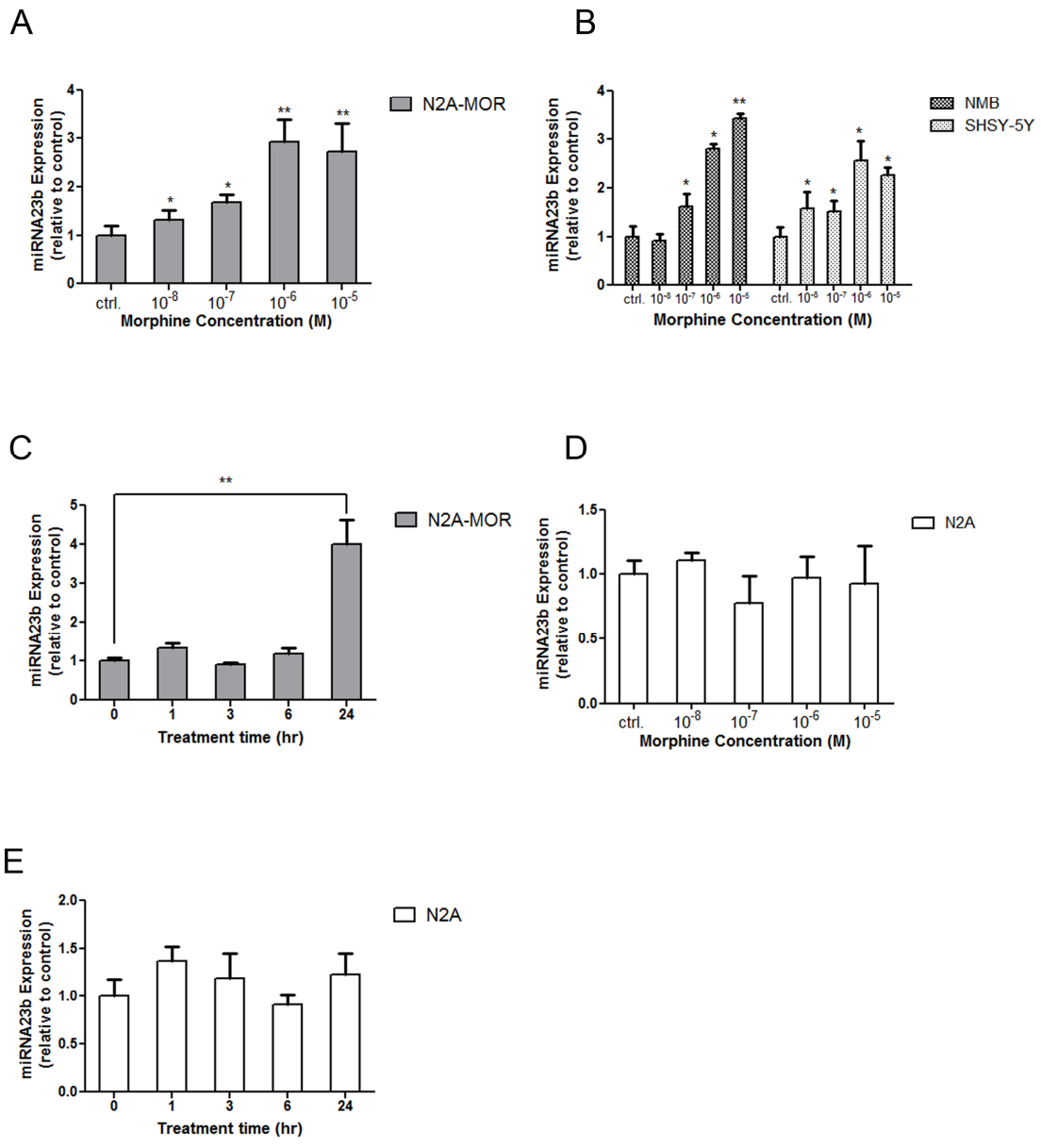

A
B
C
D
E
N2A-MOR
NMB
SHSY-5Y
N2A
miRNA23b Expression
(relative to control)
Morphine Concentration (M)
Treatment time (hr)
ctrl.
0
1
3
6
24

Figure 4-1

Figure 4-1. Morphine increases miRNA23b expression. (A) miRNA-enriched RNA was extracted from N2A-MOR cells 24 hrs after morphine treatment. Twenty ng RNA were used for reverse transcription using miRNA23b and snoRNA234 (control) primers, followed by real-time qPCR. The y-axis represents the levels of miRNA23b expression normalized relative to control (i.e., without treatment; miRNA23b/snoRNA234); x-axis represents different concentrations of morphine (10^{-8} to 10^{-5} M). Student *t*-test was performed by comparing each sample to the control sample. The graph shows a single experiment performed in duplicate; the experiment was repeated three times with similar results. n=3; *: $p < 0.05$; **: $p < 0.01$. (B) miRNA was extracted from NMB and SHSY-5Y cells treated with morphine for 24 hrs. Student *t*-test was performed by comparing each sample to the control sample in each cell line. The legends are the same as in (A). n=3; *: $p < 0.05$; **: $p < 0.01$. (C) miRNA was extracted from N2A-MOR cells treated with 10^{-6} M morphine for different lengths of time. X-axis represents the different time points (1 to 24 hrs). Student *t*-test was performed by comparing each sample to the control (0 hr treatment). n=3; **: $p < 0.01$. (D) miRNA was extracted from N2A cells 24 hrs after morphine treatment. The legends are the same as in (A); n=3. (E) miRNA was extracted from N2A cells treated with 10^{-6} M morphine for different lengths of time. The legends are the same as in (C). n=3.

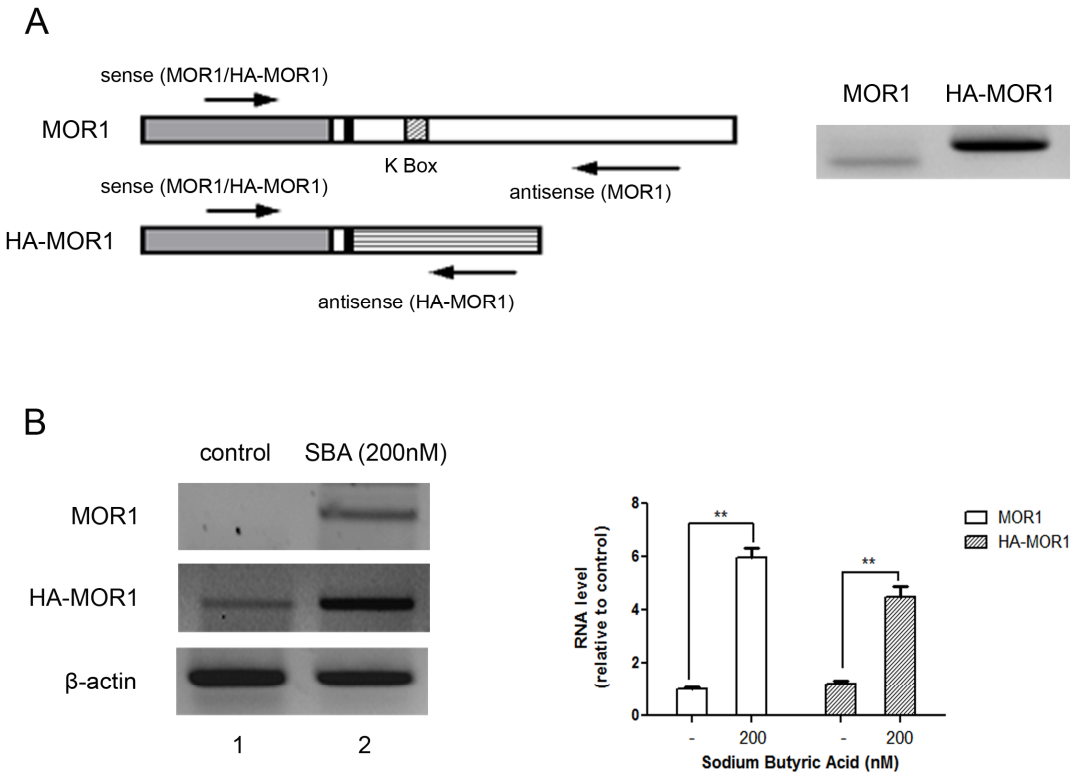

A
sense (MOR1/HA-MOR1)
MOR1
K Box
antisense (MOR1)
sense (MOR1/HA-MOR1)
HA-MOR1
antisense (HA-MOR1)
MOR1
HA-MOR1
B
control
SBA (200nM)
MOR1
HA-MOR1
β-actin
1
2
RNA level (relative to control)
MOR1
HA-MOR1
Sodium Butyric Acid (nM)
C
miRNA23b Expression (relative to control)
anti-23b concentration (nM)
0.00
0.65
2.00
6.00

Figure 4-2

Figure 4-2. MOR1 and HA-MOR1 RNAs in N2A-MOR cells. (A) Structure of MOR1 and HA-MOR1 RNAs and the positions of RT-PCR primers. Left panel: MOR1 cDNA (gray box), MOR1 3′-UTR (large blank box), sequence in pRc/CMV plasmid downstream from the MOR1 cDNA (large striped box); K Box (small striped box); 30-bp MOR1 3′-UTR that is included in both mRNAs (small blank box). The arrows represent the approximate positions for the sense and antisense RT-PCR primers. Right panel: N2A-MOR cells were treated with 200 nM sodium butyric acid (SBA) for 24 hrs, 1 μg RNA was amplified by RT-PCR using MOR1 (left lane) or HA-MOR1 (right lane) primers. (B) N2A-MOR cells were treated with 200 nM SBA for 24 hrs. RNA was extracted and 1 μg RNA (or 500 ng for β-actin) was analyzed by one-step RT-PCR. Lane 1, control (mock treatment); lane 2, treated with 200 nM SBA. The graph shows the intensity of MOR1 or HA-MOR1 signals normalized to that of β-actin; data are expressed relative to the intensity of the control sample (lane 1). The experiments were repeated three times; the Student *t*-test was performed by comparing each sample to the control sample. n=3; **: $p < 0.01$. (C) Anti-23b primer (0, 0.65, 2, or 6 nM) was transfected into N2A-MOR cells and miRNA-enriched RNA was extracted 24 hrs later. Twenty ng RNA were used for reverse transcription using miRNA23b and snoRNA234 primers, followed by real-time qPCR. The graph shows the miRNA23b levels, normalized to snoRNA234 from a single experiment performed in duplicate. The experiment was repeated three times with similar results. Student *t*-test was performed by comparing each sample to the control sample (0.00 nM anti-23b). n=3; **: $p < 0.01$; ***: $p < 0.001$.

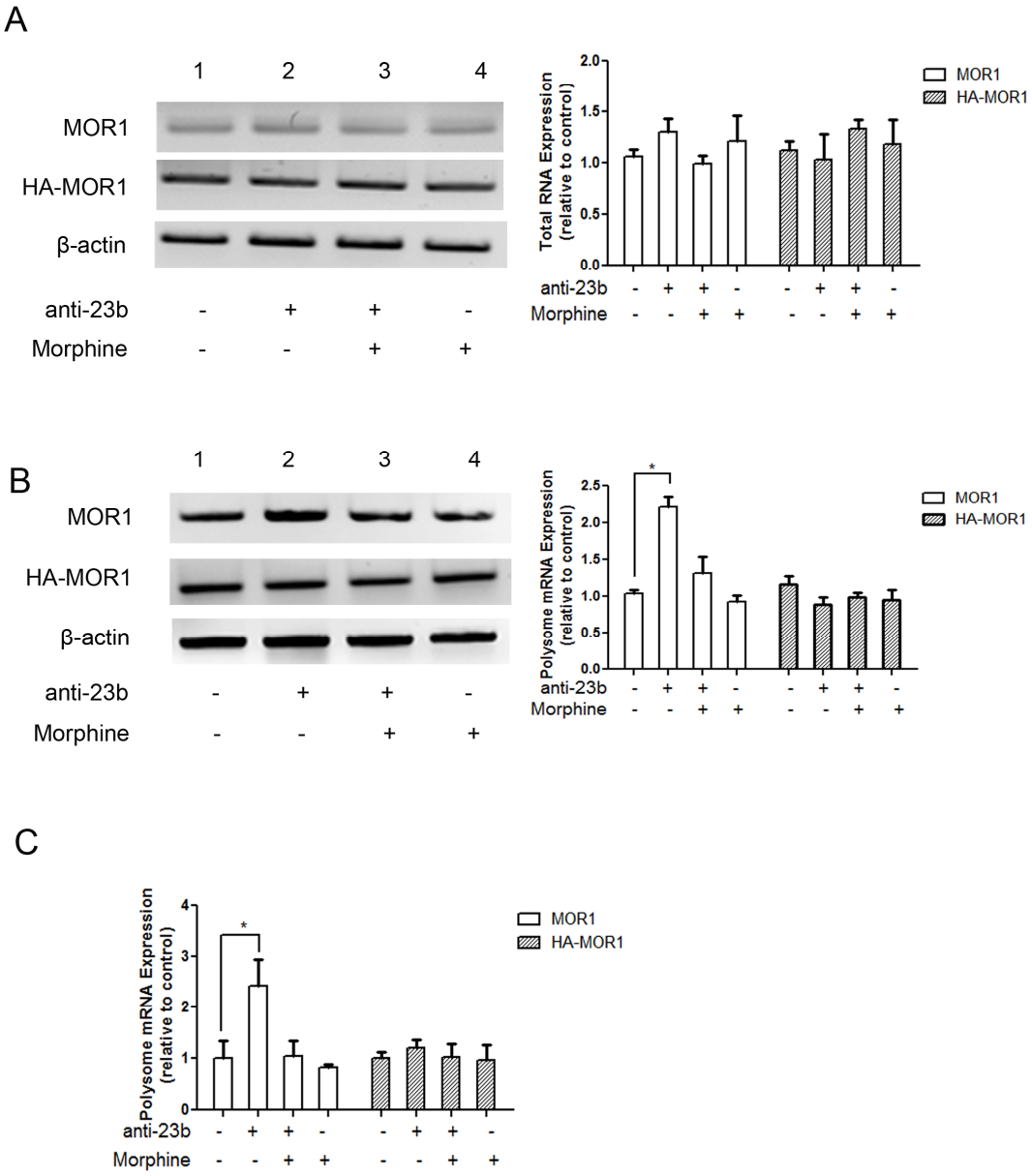
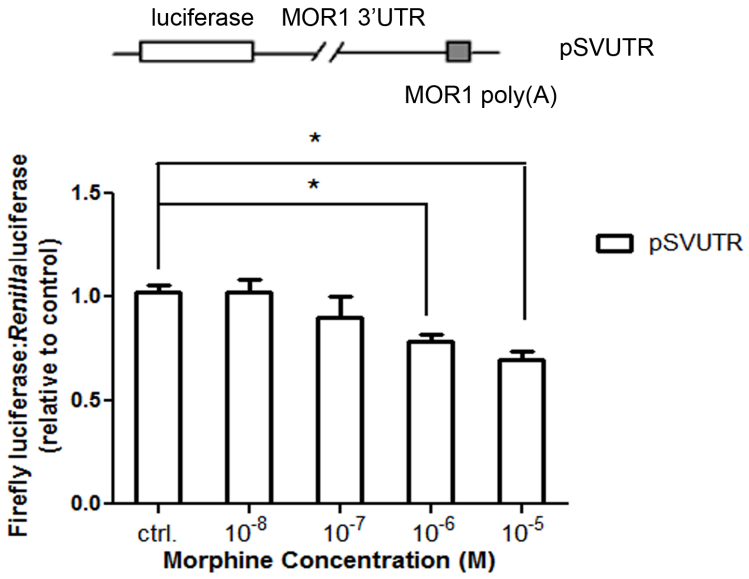

A
1
2
3
4
MOR1
HA-MOR1
β-actin
anti-23b - + + - - + + -
Morphine - - + + - - + +
Total RNA Expression (relative to control)
2.0
1.5
1.0
0.5
0.0
MOR1
HA-MOR1
anti-23b - + + - - + + -
Morphine - - + + - - + +
B
1
2
3
4
MOR1
HA-MOR1
β-actin
anti-23b - + + -
Morphine - - + +
Polysome mRNA Expression (relative to control)
*
2.5
2.0
1.5
1.0
0.5
0.0
MOR1
HA-MOR1
anti-23b - + + - - + + -
Morphine - - + + - - + +
C
Polysome mRNA Expression (relative to control)
*
4
3
2
1
0
MOR1
HA-MOR1
anti-23b - + + - - + + -
Morphine - - + + - - + +

Figure 4-3

Figure 4-3. Morphine inhibits the polysome association of MOR1 mRNA through the interaction between miRNA23b and MOR1 3′-UTR. (A) N2A-MOR cells were pre-treated with 200 nM SBA for 4 hrs before transfection with 2 nM anti-miR negative control primer (lanes 1 and 4) or 2 nM anti-23b primer (lanes 2 and 3). At the transfection, experimental and control cells were treated with 10^{-6} M morphine (lanes 3 and 4). Twenty-four hours after transfection, RNA was extracted and 1 μg (or 500 ng for β-actin) RNA was analyzed by one-step RT-PCR using primers specific for MOR1, HA-MOR1, and β-actin. The graph shows the intensity of the MOR1 and HA-MOR1 signals normalized to that of β-actin; data are expressed relative to the intensity of control sample (lane 1). Each experiment was repeated three times. Student *t*-test was performed by comparing each sample to the control sample; n=3. (B) N2A-MOR cells were prepared as described in (A). Twenty-four hours after transfection, polysomal mRNA was extracted and 2 μg polysomal mRNA (or 500 ng for β-actin) was analyzed by one-step RT-PCR. The legends are the same as in (B). Each experiment was repeated three times; n=3; *: $p < 0.05$. (C) One μg of polysomal mRNA (or 500 ng for β-actin) was analyzed by real-time qRT-PCR. The graph shows the MOR1 and HA-MOR1 polysomal mRNA levels normalized to that of β-actin; data are expressed relative to the expression of control sample (i.e., w/o anti-23b transfection or morphine treatment). The experiment was repeated three times; Student *t*-test was performed by comparing each sample to the control; n=3; *: $p < 0.05$.

A


luciferase
MOR1 3'UTR
pSVUTR
MOR1 poly(A)
*
*
pSVUTR
Firefly luciferase:Renilla luciferase (relative to control)
1.5
1.0
0.5
0.0
ctrl.
10-8
10-7
10-6
10-5
Morphine Concentration (M)

B

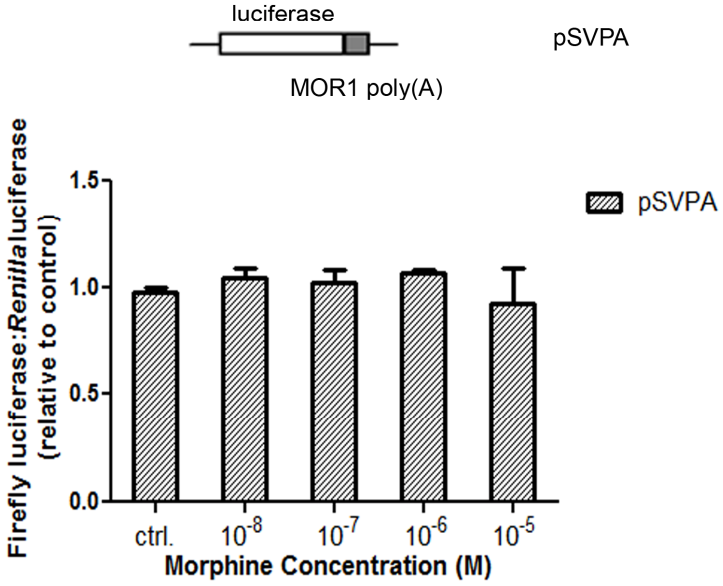

luciferase
pSVPA
MOR1 poly(A)
pSVPA
Firefly luciferase:Renilla luciferase (relative to control)
1.5
1.0
0.5
0.0
ctrl.
10-8
10-7
10-6
10-5
Morphine Concentration (M)

Figure 4-4

Figure 4-4. Morphine suppresses reporter activity through MOR1 3′-UTR. (A) pSVUTR reporter assay. Luciferase coding region (blank box); MOR1 poly (A) signal (gray box); MOR1 3′-UTR (broken line). N2A-MOR cells were treated with morphine (10^{-8} to 10^{-5} M) 3 hrs before transfected with 500 ng pSVUTR plasmid; 2 ng pCMV-Rluc was cotransfected for normalization. The morphine concentration is plotted against the normalized luciferase activity of reporter constructs (firefly luciferase /*Renilla* luciferase) relative to control. The graph shows a single experiment performed in duplicate. The experiment was repeated three times with similar results. Student *t*-test was performed by comparing each sample to the control. n=3; *: $p < 0.05$. (B) pSVPA reporter assay. Legends are the same as in (A), except for 200 ng pSVPA used in the transfection. The experiment was repeated three times with similar results. Student *t*-test was performed by comparing each sample to the control; n=3.

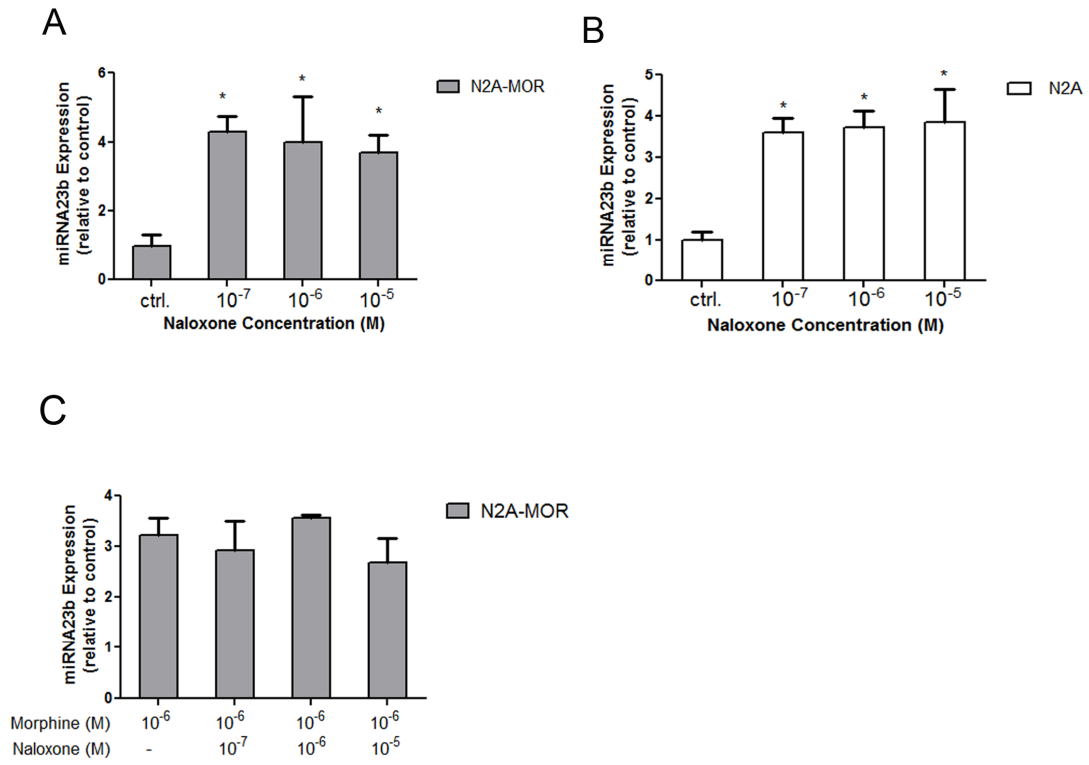

A
B
C
N2A-MOR
N2A
miRNA23b Expression (relative to control)
Naloxone Concentration (M)
ctrl.
10^{-7}
10^{-6}
10^{-5}
Morphine (M)
Naloxone (M)
-

Figure 4-5

Figure 4-5. Naloxone treatment increases miRNA23b expression independent of mu-opioid receptor. (A) miRNA-enriched RNA was extracted from N2A-MOR cells 24 hrs after naloxone treatment. Twenty ng RNA were used for reverse transcription using miRNA23b and snoRNA234 RNA primers, followed by real-time qPCR. The x-axis represents the naloxone concentration (10^{-7} to 10^{-5} M), y-axis represents the normalized expression levels of miRNA23b (miRNA23b/snoRNA234), relative to control (without treatment). The graph shows a single experiment performed in duplicate; the experiment was repeated three times with similar results. Student *t*-test was conducted by comparing each sample to the control. n=3, *: $p<0.05$. (B) miRNA was extracted from N2A cells 24 hrs after treated with naloxone. The legends are the same as in (A). n=3, *: $p<0.05$. (C) N2A-MOR cells were pre-incubated with different concentrations of naloxone (10^{-7} to 10^{-5} M) for one hour. 10^{-6} M morphine was then added and miRNA was extracted 24 hrs later. Y-axis represents the normalized expression levels of miRNA23b (miRNA23b/snoRNA234), relative to control (w/o morphine or naloxone treatment, not shown in the graph). Student *t*-test was conducted by comparing each sample to the one with only morphine treatment (the first sample); n=3.

Chapter V

Conclusions and Questions to be Answered

The cloning of μ-, δ-, κ-opioid receptors in the early 1990s has allowed the genetic regulation of each receptor to be studied. Among three opioid receptors, μ-opioid receptor (MOR) mediates most of the pharmacological effects of opioids, therefore understanding the regulation of the mu-opioid receptor is central to unraveling the molecular mechanisms that underlie tolerance, addiction, etc. .

Opioid receptor function appears to be dependent on receptor concentration at the cell surface. In addition to covalent modification, such as phosphorylation, the receptor density is controlled by the expression level of opioid receptor genes (Law and Loh, 1999). A gene can be modulated both transcriptionally and post-transcriptionally. This study focused on elucidating the post-transcriptional regulation of MOR mediated by its 3′-UTR.

We chose to study the mouse μ-opioid receptor gene which has long been a classical model for opioid research. To focus our effort and to avoid the complexity of MOR splice variants, we used a high-stringency northern blot to detect its major transcript, i.e. MOR1, which includes four exons separated by three introns and encodes the functional MOR receptor protein. The mature mRNA isolated from mouse brain was used in the northern blot to ensure the detection of natural MOR transcript and avoid the artificial splice variants observed in the cell lines.

The aim was to identify mature MOR transcripts, including their contiguous MOR1 3′-UTR. Using three probes that bind to exon 1, exon 3, and 3′-UTR, respectively, only the transcript detected by all three probes produces mature MOR1 protein and was selected for subsequent study. A single band of 11.5kb was detected in all three blots, which is also consistent with results from other groups (Pan et al., 2000).

This result should not be directly compared to the studies that focused on identifying novel exons and MOR splice variants. For strategic and technical consideration, a high stringency but low sensitivity method, northern blot, was used to identify the transcript. The mRNA species revealed by such method is the unambiguous dominant transcript of MOR (MOR-1), but it does not rule out the existence of other lower-abundance species. In contrast, other studies used highly sensitive methods, such as 3′ RACE combined with nested PCR; therefore were able to reveal the lower-abundance species of MOR transcript.

The poly (A) signal is responsible for binding to the cleavage/polyadenylation complex. Like the promoter, it can have relatively independent function to direct the termination events of transcription. After sequencing the 3′-end of MOR1 3′-UTR, we found a single MOR1 poly (A) signal located at 10153 bp downstream of the stop codon. The 400bp genomic sequence flanking the poly (A) signal was cloned into luciferase constructs driven by a MOR or SV40 promoters. The MOR1 poly (A) signal showed significantly higher activity than SV40 poly (A) signal. Interestingly, this is a promoter-dependent effect; changing to a SV40 promoter totally abolished the increase in reporter activity. The connection between MOR promoter and poly (A) signal provides evidence in favor of the emerging proposition that despite the traditional perceived distance between mRNA “head” and “end”, these two components can be spatially close in the transcription machinery. Together with the transcription initiation and cleavage/polyadenylation machinery, the transcription of DNA could be synchronized between the 5′-UTR/promoter and the poly (A) signal from the very start. In the future, it would be interesting to investigate such interaction in detail.

Cloning of the full-length MOR1 3′-UTR offered a basic tool for the following study. When transfected into the mouse neuronal cell lines, NS20Y, the MOR1 3′-UTR manifested a

robust repression on reporter activity, compared to the construct that contains only the poly (A) signal. This is consistent with the report that the 3′-UTR is repressive because it binds proteins that interfere with either the function of the translation initiation complex or the assembly of the ribosome (Mazumder et al., 2003).

A series of direct and indirect assays were performed to determine the mechanism for the repression from MOR1 3′UTR. We substituted the MOR promoter with the SV40 promoter; the inhibitory effect was preserved, indicating that it is not likely at the transcriptional level. Analysis of the cytoplamsic/nuclear RNA ratio showed no difference between the put (with MOR1 3′-UTR) and papa (without MOR1 3′-UTR), confirming the hypothesis that MOR1 3′-UTR does not affect the reporter transcription and also excluded the possibility of a lower transportation efficiency of the reporter RNA.

We chose to measure polysome mRNA levels, as an indicator for the translation efficiency. The method takes advantage of the fact that mRNA bound to polysomes (polyribosome) is actively being translated status, i.e., more mRNA in the polysome fraction corresponds to higher translation efficiency. Because the polysome mRNA is directly separated from cells, the *in vivo* interactions in the translation complex can be retained. The polysome mRNA level from pMUTR was significantly lower than that of the pMPA plasmid, confirming that the MOR1 3′-UTR represses the translation of reporter mRNA by inhibiting its association with polysomes.

To explore the *cis*- and *trans*- interactions in MOR1 3′-UTR, we used software UTRscan to predict putative elements. The reason for choosing such approach instead of performing sequential deletions is due to the extensive size of MOR1 3′-UTR, which posed major difficulty

for narrowing down the location of functional elements. To our surprise, the UTRscan predicted two types of elements that bind to miRNAs instead of proteins, i.e., K Box and Brd Box.

Although miRNAs have been shown to regulate various genes through inhibiting the translation or inducing RNA degradation, no study had reported its role in opioid receptors before. By mutating the three predicted motifs (two K Box and one Brd Box), we identified the only functional motif, K Box 1 (3805-3812 bp downstream of the stop codon). Sequence analysis showed that miRNA23b, which is a homolog to miRNA2 and miRNA16 in *Drosophila*, has the complementary "seed" sequence to the K Box. We confirmed its expression in NS20Y cells as well as the mouse brain. When miRNA23b was overexpressed in cells, the luciferase activity was repressed in the plasmid with MOR1 3′-UTR (pMUTR); mutilation of the K Box diminished the effect of miRNA23b, confirming a specific interaction between miRNA23b and K Box. The miRNA23b represses the association of reporter mRNA with polysomes, therefore inhibiting the translational efficiency of the repoter gene. Evidence on endogenous MOR was obtained in NS20Y cells that had a TSA-stimulated high level of transcription, although the translation of the MOR gene was still repressed. When miRNA23b was knocked down by an anti-sense primer, its inhibitory effect on MOR mRNA was repressed, and a significant increase of MOR protein was observed.

Considering the 10 kb size of MOR1 3′-UTR, the K Box is probably one of the many elements whose synergistic effect accounts for the function of 3′-UTR. This hypothesis is also supported by the fact that deletion of a 5 kb and 3 kb sequences in the MOR1 3′-UTR partly restored the luciferase activity and also the polysome mRNA levels.

We proved that miRNA23b regulates the MOR gene expression at the post-transcriptional level. However, whether miRNA23b can respond to morphine treatment became a

more intriguing question, as the study on the transcriptional factors of MOR gene had not provided a link to the morphine treatment.

miRNA23b levels showed a dose- and time- dependent increase in cells treated with morphine. Subsequent experiments confirmed a "morphine-miRNA23b-MOR translation" pathway that requires the MOR receptor and MOR1 3′-UTR. Details still await further investigation. This provides the exciting new evidence indicating that the MOR gene expression can be actively regulated by agonist treatment, which points to a feedback loop from the trigger of tolerance (chronic agonist treatment) to one of consequences of tolerance (receptor downregulation). The MOR receptor downregulation had been traditionally perceived to be mediated only at protein level i.e., increased receptor degradation. One study showed indirect evidence that the downregulation is the sum of accelerated degradation and repressed receptor synthesis (Afify, 2002). Our results suggest a new type of regulator that has the potential to act as an intermediate in tolerance development: transducing the molecular signals between the membrane receptor and intracellular receptor gene regulation (Fig. 5-1).

Generally speaking, future studies on the post-transcriptional regulation of MOR gene can be interesting in the following aspects. First, the MOR1 3′-UTR spans more than 10 kb, which can harbor more *cis*-acting elements like miRNA23b (Fig. 5-2). The advance in the miRNA prediction technology has enabled an improved analysis on the MOR1 3′-UTR (regRNA.com). In addition to miRNA23b, 24 more miRNAs are predicted to bind to the MOR1 3′-UTR (Table 5-1). As for other types of *cis*-acting elements, a report showed more than 40 putative ARE (AU rich element) sites in the human MOR1 3′-UTR (Ide et al., 2005). The prediction of AREs in the mouse MOR1 3′-UTR is shown in Table 5-2 (Han et al., 2004) . Understanding the function and mechanism of these individual elements would help put together

a comprehensive picture of the MOR1 3′-UTR and its post-transcriptional regulation. Second, the function and mechanisms of miRNAs have been appreciated in more and more genes. However, the investigation on how these "regulators" are regulated is still in an early stage. The highly-regulated miRNA maturation pathway has posed major difficulties but also brought intriguing perspectives to the miRNA study. It would be interesting to discover the signaling cascade that transduces the signal from MOR receptor to the synthesis or functioning of miRNAs and how this subsequently affects the MOR expression. This pathway can be part of the molecular mechanisms for opioid tolerance. A comprehensive miRNA array can be helpful in this regard. Third, this study serves as an exploratory effort for studying the post-transcriptional regulation of MOR. The post-transcriptional regulation refers to various events after a gene is transcribed, such as mRNA processing, nucleo-cytoplasmic export, mRNA localization, mRNA stabilization and translational regulation (Audic and Hartley, 2004). The cloning of full-length MOR1 3′-UTR provides a necessary tool to conduct investigation on these events, in addition to the translation.

As the major drug target for opioid analgesics, the control of μ-opioid receptor expression is pivotal to understanding the pharmacological effects of opioids at the molecular level. The identification of MOR1 3′-UTR and the finding of a "morphine-miRNA23b-MOR translation" pathway shed light on the importance of its post-transcriptional regulation. Unfolding of the story could open a new field for the gene regulation study of μ-opioid receptor, and bring novel perspectives to the understanding of molecular mechanisms of opioid tolerance.

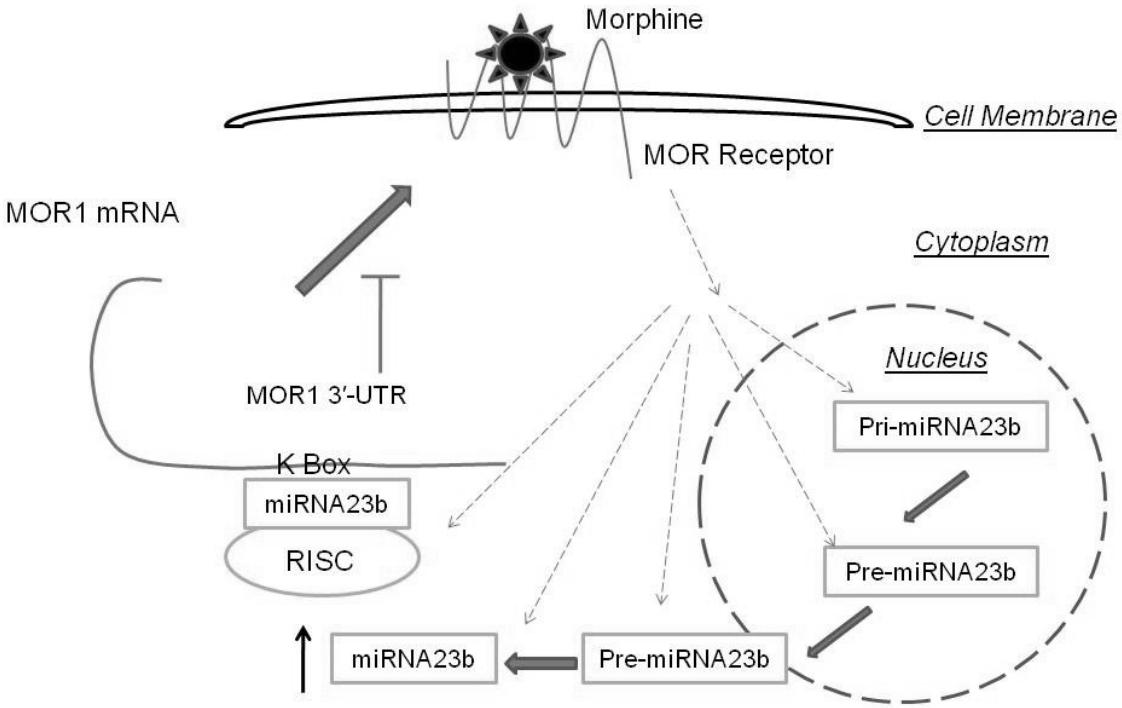

Morphine
Cell Membrane
MOR Receptor
MOR1 mRNA
Cytoplasm
Nucleus
MOR1 3′-UTR
Pri-miRNA23b
K Box
miRNA23b
RISC
Pre-miRNA23b
miRNA23b
Pre-miRNA23b

Figure 5-1

Figure 5-1. Working model of the miRNA23b-mediated translational repression of MOR1 mRNA. The chronic treatment of morphine activates the μ-opioid receptor on the cell membrane and transduces the signal into cytoplasm or nucleus. Through unknown mechanism, the miRNA23b is upregulated. miRNA23b, together with the RISC (RNA-induced Silencing Complex) binds to the K Box in the MOR1 3′-UTR and inhibits the translation of MOR mRNA. Objects are for demonstration and not in ratio. Dashed lines indicate unknown pathways.

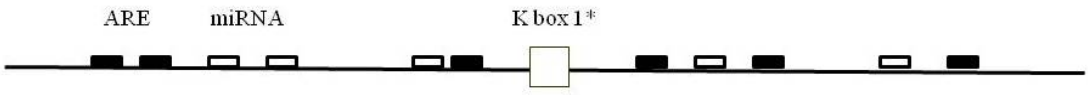


Figure 5-2

Figure 5-2. Putative *cis*-acting elements in the MOR1 3′-UTR. Blank square: K Box1; filled rectangle: ARE (AU-rich element); blank rectangle: miRNA; *: confirmed element. The depiction of ARE and miRNA is for illustration only. The detailed information on ARE and miRNA are described in Table 5-1 and 5-2.

Table 5-1. Putative miRNAs that bind to the MOR1 3′-UTR

miRNA ID	Location (bp distance from the stop codon)
134	434-459
140	271-297
141	8987-9012
149	5398-5422
17-3p	637-656
181-a	5626-5648
181-c	5626-5648
184	5861-5882
185	9121-9138
200-a	8989-9012
224	6538-6560
24	2985-3009
296	3686-3708
298	9647-9669
329	3807-3828
331	4112-4135, 535-556
345	9295-9325
34c	9479-9500
370	4721-4742
483	1259-1288
486	5369-5391
503	6108-6131
540	423-445
546	755-772

Table 5-2. Putative AREs (AU-rich element) in the MOR1 3′-UTR

ARE core sequence	Location (bp distance from the stop codon)
AUUUA	600, 1038, 1487, 1698, 2081, 3945, 4177, 4360, 4508, 4847, 5694, 5979, 6345, 6386, 6819, 6877, 6932, 8341, 8493, 8613, 8779, 8879, 10143

References

Goodman & Gilman's the pharmacological basis of therapeutics, in *McGraw-Hill's AccessMedicine Clinical library* pp 1 electronic text (xxiii, 2021 p.), McGraw-Hill, New York.

Afify EA (2002) Turnover of mu-opioid receptors in neuroblastoma cells. *Brain Res Mol Brain Res* **106**(1-2):83-87.

Ammon-Treiber S and Hollt V (2005) Morphine-induced changes of gene expression in the brain. *Addict Biol* **10**(1):81-89.

Andria ML and Simon EJ (2001) Identification of a neurorestrictive suppressor element (NRSE) in the human mu-opioid receptor gene. *Brain Res Mol Brain Res* **91**(1-2):73-80.

Audic Y and Hartley RS (2004) Post-transcriptional regulation in cancer. *Biol Cell* **96**(7):479-498.

Bagga S (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* **122**:553-563.

Balmer LA, Beveridge DJ, Jazayeri JA, Thomson AM, Walker CE and Leedman PJ (2001) Identification of a novel AU-Rich element in the 3' untranslated region of epidermal growth factor receptor mRNA that is the target for regulated RNA-binding proteins. *Mol Cell Biol* **21**(6):2070-2084.

Bao N, Lye KW and Barton MK (2004) MicroRNA binding sites in Arabidopsis class III HD-ZIP mRNAs are required for methylation of the template chromosome. *Dev Cell* **7**:653-662.

Barreau C, Paillard L and Osborne HB (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res* **33**(22):7138-7150.

Behm-Ansmant I (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* **20**:1885-1898.

Behm-Ansmant I, Rehwinkel J and Izaurralde E (2006) MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. *Cold Spring Harb Symp Quant Biol* **71**:523-530.

Bernstein MA and Welch SP (1998) mu-Opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. *Brain Res Mol Brain Res* **55**(2):237-242.

Bhargava HN and Gulati A (1990) Down-regulation of brain and spinal cord mu-opiate receptors in morphine tolerant-dependent rats. *Eur J Pharmacol* **190**(3):305-311.

Bi J, Tsai NP, Lin YP, Loh HH and Wei LN (2006) Axonal mRNA transport and localized translational regulation of kappa-opioid receptor in primary neurons of dorsal root ganglia. *Proc Natl Acad Sci U S A* **103**(52):19919-19924.

Bi J, Tsai NP, Lu HY, Loh HH and Wei LN (2007) Copb1-facilitated axonal transport and translation of kappa opioid-receptor mRNA. *Proc Natl Acad Sci U S A* **104**(34):13810-13815.

Binyaminy B, Gafni M, Shapira M and Sarne Y (2008) Agonist-specific down regulation of mu-opioid receptors: Different cellular pathways are activated by different opioid agonists. *Life Sci* **82**(15-16):831-839.

Boyd SD (2008) Everything you wanted to know about small RNA but were afraid to ask. *Lab Invest* **88**(6):569-578.

Brennecke J, Stark A, Russell RB and Cohen SM (2005) Principles of microRNA-target recognition. *PLoS Biol* **3**:404-418.

Brewer G (1991) An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. *Mol Cell Biol* **11**(5):2460-2466.

Briata P, Ilengo C, Corte G, Moroni C, Rosenfeld MG, Chen CY and Gherzi R (2003) The Wnt/beta-catenin-->Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs. *Mol Cell* **12**(5):1201-1211.

Brodsky M, Elliott K, Hynansky A and Inturrisi CE (1995a) CNS levels of mu opioid receptor (MOR-1) mRNA during chronic treatment with morphine or naltrexone. *Brain Res Bull* **38**(2):135-141.

Brodsky M, Elliott K, Hynansky A, Jenab S and Inturrisi CE (1995b) Quantitation of mu-opioid receptor (MOR-1) mRNA in selected regions of the rat CNS. *Neuroreport* **6**(5):725-729.

Bushati N and Cohen SM (2007) microRNA functions. *Annu Rev Cell Dev Biol* **23**:175-205.

Bussing I, Slack FJ and Grosshans H (2008) let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med* **14**(9):400-409.

Castelli MP, Melis M, Mameli M, Fadda P, Diaz G and Gessa GL (1997) Chronic morphine and naltrexone fail to modify mu-opioid receptor mRNA levels in the rat brain. *Brain Res Mol Brain Res* **45**(1):149-153.

Chakrabarti S, Law PY and Loh HH (1995) Neuroblastoma Neuro2A cells stably expressing a cloned mu-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. *Brain Res Mol Brain Res* **30**(2):269-278.

Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV, Thomas-Tikhonenko A and Mendell JT (2008) Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* **40**(1):43-50.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M and Karin M (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**(4):451-464.

Chen CY and Shyu AB (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* **20**(11):465-470.

Chendrimada TP (2007) microRNA silencing through RISC recruitment of eIF6. *Nature* **447**:823-828.

Choe C, Im HJ, Ko JL and Loh HH (1998) Mouse mu opioid receptor gene expression. A 34-base pair cis-acting element inhibits transcription of the mu opioid receptor gene from the distal promoter. *J Biol Chem* **273**(52):34926-34932.

Choi HS, Hwang CK, Kim CS, Song KY, Law PY, Wei LL and Loh HH (2005a) Transcriptional Regulation of Mouse {micro} Opioid Receptor Gene: Sp3 Isoforms (M1, M2) Function as Repressors in Neuronal Cells to Regulate the mu Opioid Receptor Gene. *Mol Pharmacol*.

Choi HS, Hwang CK, Kim CS, Song KY, Law PY, Wei LN and Loh HH (2005b) 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* **67**(5):1674-1683.

Choi HS, Kim CS, Hwang CK, Song KY, Law PY, Wei LN and Loh HH (2007) Novel function of the poly(C)-binding protein alpha CP3 as a transcriptional repressor of the mu opioid receptor gene. *FASEB J* **21**(14):3963-3973.

Conne B, Stutz A and Vassalli JD (2000) The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? *Nat Med* **6**(6):637-641.

Dantonel JC, Murthy KG, Manley JL and Tora L (1997) Transcription factor TFIID recruits factor CPSF for formation of 3' end of mRNA. *Nature* **389**(6649):399-402.

Davis BN, Hilyard AC, Lagna G and Hata A (2008) SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454**(7200):56-61.

del Prete MJ, Vernal R, Dolznig H, Mullner EW and Garcia-Sanz JA (2007) Isolation of polysome-bound mRNA from solid tissues amenable for RT-PCR and profiling experiments. *RNA* **13**(3):414-421.

Diederichs S and Haber DA (2007) Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* **131**(6):1097-1108.

Ding XC, Weiler J and Grosshans H (2008) Regulating the regulators: mechanisms controlling the maturation of microRNAs. *Trends Biotechnol*.

Doench JG and Sharp PA (2004) Specificity of microRNA target selection in translational repression. *Genes Dev* **18**:504-511.

Dostie J, Mourelatos Z, Yang M, Sharma A and Dreyfuss G (2003) Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA* **9**(2):180-186.

Doyle GA, Rebecca Sheng X, Lin SS, Press DM, Grice DE, Buono RJ, Ferraro TN and Berrettini WH (2007a) Identification of three mouse mu-opioid receptor (MOR) gene (Oprm1) splice variants containing a newly identified alternatively spliced exon. *Gene* **388**(1-2):135-147.

Doyle GA, Sheng XR, Lin SS, Press DM, Grice DE, Buono RJ, Ferraro TN and Berrettini WH (2007b) Identification of five mouse mu-opioid receptor (MOR) gene (Oprm1) splice variants containing a newly identified alternatively spliced exon. *Gene* **395**(1-2):98-107.

Du T and Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. *Development* **132**:4645-4652.

DuPen A, Shen D and Ersek M (2007) Mechanisms of Opioid-Induced Tolerance and Hyperalgesia. *Pain Management Nursing* **8**(3):113-121.

Duttaroy A and Yoburn BC (1995) The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology* **82**(5):1226-1236.

Easow G, Teleman AA and Cohen SM (2007) Isolation of microRNA targets by miRNP immunopurification. *RNA* **13**:1198-1204.

Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E and Dahlberg JE (2005) Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* **102**(10):3627-3632.

Enright AJ, John B, Gaul U, Tuschl T, Sander C and Marks DS (2003) MicroRNA targets in Drosophila. *Genome Biol* **5**(1):R1.

Eulalio A (2007) Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. *Genes Dev* **21**:2558-2570.

Eulalio A, Huntzinger E and Izaurralde E (2008) Getting to the root of miRNA-mediated gene silencing. *Cell* **132**(1):9-14.

Fabbri M (2008) MicroRNAs and cancer epigenetics. *Curr Opin Investig Drugs* **9**(6):583-590.

Filipowicz W, Bhattacharyya SN and Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**(2):102-114.

Filipowicz W, Jaskiewicz L, Kolb FA and Pillai RS (2005) Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr Opin Struct Biol* **15**:331-341.

Fukuda K, Kato S, Mori K, Nishi M and Takeshima H (1993) Primary structures and expression from cDNAs of rat opioid receptor delta- and mu-subtypes. *FEBS Lett* **327**(3):311-314.

Fukuda T, Yamagata K, Fujiyama S, Matsumoto T, Koshida I, Yoshimura K, Mihara M, Naitou M, Endoh H, Nakamura T, Akimoto C, Yamamoto Y, Katagiri T, Foulds C, Takezawa S, Kitagawa H, Takeyama K, O'Malley BW and Kato S (2007) DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nat Cell Biol* **9**(5):604-611.

Gherzi R, Lee KY, Briata P, Wegmuller D, Moroni C, Karin M and Chen CY (2004) A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. *Mol Cell* **14**(5):571-583.

Giraldez AJ (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**:75-79.

Gottardo F, Liu CG, Ferracin M, Calin GA, Fassan M, Bassi P, Sevignani C, Byrne D, Negrini M, Pagano F, Gomella LG, Croce CM and Baffa R (2007) Micro-RNA profiling in kidney and bladder cancers. *Urol Oncol* **25**(5):387-392.

Gray NK and Wickens M (1998) Control of translation initiation in animals. *Annu Rev Cell Dev Biol* **14**:399-458.

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N and Shiekhattar R (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**(7014):235-240.

Grimson A (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* **27**:91-105.

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G and Mello CC (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* **106**(1):23-34.

Han J, Lee Y, Yeom KH, Kim YK, Jin H and Kim VN (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18**(24):3016-3027.

Han W, Kasai S, Hata H, Takahashi T, Takamatsu Y, Yamamoto H, Uhl GR, Sora I and Ikeda K (2006) Intracisternal A-particle element in the 3' noncoding region of the mu-opioid receptor gene

in CXBK mice: a new genetic mechanism underlying differences in opioid sensitivity. *Pharmacogenet Genomics* **16**(6):451-460.

Hitzemann RJ, Hitzemann BA and Loh HH (1974) Binding of 3H-naloxone in the mouse brain: effect of ions and tolerance development. *Life Sci* **14**(12):2393-2404.

Hu X, Bi J, Loh HH and Wei LN (2002) Regulation of mouse kappa opioid receptor gene expression by different 3'-untranslated regions and the effect of retinoic acid. *Mol Pharmacol* **62**(4):881-887.

Hwang CK, Kim CS, Choi HS, McKercher SR and Loh HH (2004) Transcriptional regulation of mouse mu opioid receptor gene by PU.1. *J Biol Chem* **279**(19):19764-19774.

Hwang CK, Song KY, Kim CS, Choi HS, Guo XH, Law PY, Wei LN and Loh HH (2007) Evidence of endogenous mu opioid receptor regulation by epigenetic control of the promoters. *Mol Cell Biol* **27**(13):4720-4736.

Hwang CK, Wu X, Wang G, Kim CS and Loh HH (2003) Mouse mu opioid receptor distal promoter transcriptional regulation by SOX proteins. *J Biol Chem* **278**(6):3742-3750.

Ide S, Han W, Kasai S, Hata H, Sora I and Ikeda K (2005) Characterization of the 3' untranslated region of the human mu-opioid receptor (MOR-1) mRNA. *Gene* **364**:139-145.

Ikeda K, Kobayashi T, Ichikawa T, Kumanishi T, Niki H and Yano R (2001) The untranslated region of (mu)-opioid receptor mRNA contributes to reduced opioid sensitivity in CXBK mice. *J Neurosci* **21**(4):1334-1339.

Im HJ, Smirnov D, Yuhi T, Raghavan S, Olsson JE, Muscat GE, Koopman P and Loh HH (2001) Transcriptional modulation of mouse mu-opioid receptor distal promoter activity by Sox18. *Mol Pharmacol* **59**(6):1486-1496.

Jackson RJ (1993) Cytoplasmic regulation of mRNA function: the importance of the 3' untranslated region. *Cell* **74**(1):9-14.

Jaskiewicz L and Filipowicz W (2008) Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol Immunol* **320**:77-97.

Jones-Rhoades MW, Bartel DP and Bartel B (2006) MicroRNAS and their regulatory roles in plants. *Annu Rev Plant Biol* **57**:19-53.

Kasai S, Han W, Ide S, Hata H, Takamatsu Y, Yamamoto H, Uhl GR, Sora I and Ikeda K (2006) Involvement of the 3' non-coding region of the mu opioid receptor gene in morphine-induced analgesia. *Psychiatry Clin Neurosci* **60 Suppl 1**:S11-17.

Keene JD (1999) Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. *Proc Natl Acad Sci U S A* **96**(1):5-7.

Kieffer BL (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell Mol Neurobiol* **15**(6):615-635.

Kim CS, Choi HS, Hwang CK, Song KY, Lee BK, Law PY, Wei LN and Loh HH (2006) Evidence of the neuron-restrictive silencer factor (NRSF) interaction with Sp3 and its synergic repression to the mu opioid receptor (MOR) gene. *Nucleic Acids Res* **34**(22):6392-6403.

Kim CS, Hwang CK, Choi HS, Song KY, Law PY, Wei LN and Loh HH (2004) Neuron-restrictive silencer factor (NRSF) functions as a repressor in neuronal cells to regulate the mu opioid receptor gene. *J Biol Chem* **279**(45):46464-46473.

Kim CS, Hwang CK, Song KY, Choi HS, Kim do K, Law PY, Wei LN and Loh HH (2008) Novel function of neuron-restrictive silencer factor (NRSF) for posttranscriptional regulation. *Biochim Biophys Acta* **1783**(10):1835-1846.

Kim VN and Nam JW (2006) Genomics of microRNA. *Trends Genet* **22**:165-173.

Kimura H, Kawasaki H and Taira K (2004) Mouse microRNA-23b regulates expression of Hes1 gene in P19 cells. *Nucleic Acids Symp Ser (Oxf)*(48):213-214.

Ko JL, Liu HC and Loh HH (2003) Role of an AP-2-like element in transcriptional regulation of mouse micro-opioid receptor gene. *Brain Res Mol Brain Res* **112**(1-2):153-162.

Ko JL, Liu HC, Minnerath SR and Loh HH (1998) Transcriptional regulation of mouse mu-opioid receptor gene. *J Biol Chem* **273**(42):27678-27685.

Ko JL and Loh HH (2001) Single-stranded DNA-binding complex involved in transcriptional regulation of mouse mu-opioid receptor gene. *J Biol Chem* **276**(1):788-795.

Ko JL and Loh HH (2005) Poly C binding protein, a single-stranded DNA binding protein, regulates mouse mu-opioid receptor gene expression. *J Neurochem* **93**(3):749-761.

Koch T, Kroslak T, Mayer P, Raulf E and Hollt V (1997) Site mutation in the rat mu-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization. *J Neurochem* **69**(4):1767-1770.

Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, Kahl E and Hollt V (2001) C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. *J Biol Chem* **276**(33):31408-31414.

Kramer HK and Simon EJ (1999) Role of protein kinase C (PKC) in agonist-induced mu-opioid receptor down-regulation: II. Activation and involvement of the alpha, epsilon, and zeta isoforms of PKC. *J Neurochem* **72**(2):594-604.

Kraus J, Borner C, Giannini E, Hickfang K, Braun H, Mayer P, Hoehe MR, Ambrosch A, Konig W and Hollt V (2001) Regulation of mu-opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. *J Biol Chem* **276**(47):43901-43908.

Kraus J, Borner C, Giannini E and Hollt V (2003) The role of nuclear factor kappaB in tumor necrosis factor-regulated transcription of the human mu-opioid receptor gene. *Mol Pharmacol* **64**(4):876-884.

Kuersten S and Goodwin EB (2003) The power of the 3' UTR: translational control and development. *Nat Rev Genet* **4**(8):626-637.

Lai EC (2002) Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* **30**(4):363-364.

Lai EC (2005) miRNAs: whys and wherefores of miRNA-mediated regulation. *Curr Biol* **15**(12):R458-460.

Lai EC, Burks C and Posakony JW (1998) The K box, a conserved 3' UTR sequence motif, negatively regulates accumulation of enhancer of split complex transcripts. *Development* **125**(20):4077-4088.

Lai EC, Tam B and Rubin GM (2005) Pervasive regulation of Drosophila Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. *Genes Dev* **19**(9):1067-1080.

Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M and Tuschl T (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**(7):1401-1414.

Lau NC, Lim LP, Weinstein EG and Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **294**(5543):858-862.

Law PY and Loh HH (1999) Regulation of opioid receptor activities. *J Pharmacol Exp Ther* **289**(2):607-624.

Le Cam A and Legraverend C (1995) Transcriptional repression, a novel function for 3' untranslated regions. *Eur J Biochem* **231**(3):620-627.

Lee EJ, Baek M, Gusev Y, Brackett DJ, Nuovo GJ and Schmittgen TD (2008) Systematic evaluation of microRNA processing patterns in tissues, cell lines, and tumors. *RNA* **14**(1):35-42.

Lee KJ, Choi MK, Lee WH and Rajkumar M (2006) Molecular analysis of Korean isolate of barley yellow mosaic virus. *Virus Genes* **32**(2):171-176.

Lee PW and Lee YM (2003) Transcriptional regulation of mu opioid receptor gene by cAMP pathway. *Mol Pharmacol* **64**(6):1410-1418.

Lee RC, Feinbaum RL and Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**(5):843-854.

Liang Y and Carr LG (1996) Identification of an octamer-1 transcription factor binding site in the promoter of the mouse mu-opioid receptor gene. *J Neurochem* **67**(4):1352-1359.

Mazumder B, Seshadri V and Fox PL (2003) Translational control by the 3'-UTR: the ends specify the means. *Trends Biochem Sci* **28**(2):91-98.

Min BH, Augustin LB, Felsheim RF, Fuchs JA and Loh HH (1994) Genomic structure analysis of promoter sequence of a mouse mu opioid receptor gene. *Proc Natl Acad Sci U S A* **91**(19):9081-9085.

Molnar A, Schwach F, Studholme DJ, Thuenemann EC and Baulcombe DC (2007) miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. *Nature* **447**:1126-1129.

Mukhopadhyay D, Houchen CW, Kennedy S, Dieckgraefe BK and Anant S (2003) Coupled mRNA stabilization and translational silencing of cyclooxygenase-2 by a novel RNA binding protein, CUGBP2. *Mol Cell* **11**(1):113-126.

Neumuller RA, Betschinger J, Fischer A, Bushati N, Poernbacher I, Mechtler K, Cohen SM and Knoblich JA (2008) Mei-P26 regulates microRNAs and cell growth in the Drosophila ovarian stem cell lineage. *Nature* **454**(7201):241-245.

Nielsen CB (2007) Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. *RNA* **13**:1894-1910.

O'Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, Miska EA and Tarakhovsky A (2007) A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev* **21**(16):1999-2004.

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV and Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**(7043):839-843.

Obernosterer G, Leuschner PJ, Alenius M and Martinez J (2006) Post-transcriptional regulation of microRNA expression. *RNA* **12**(7):1161-1167.

Palmieri A, Brunelli G, Guerzoni L, Lo Muzio L, Scarano A, Rubini C, Scapoli L, Martinelli M, Pezzetti F and Carinci F (2007) Comparison between titanium and anatase miRNAs regulation. *Nanomedicine* **3**(2):138-143.

Pan YX, Xu J, Bolan E, Chang A, Mahurter L, Rossi G and Pasternak GW (2000) Isolation and expression of a novel alternatively spliced mu opioid receptor isoform, MOR-1F. *FEBS Lett* **466**(2-3):337-340.

Pan YX, Xu J, Mahurter L, Bolan E, Xu M and Pasternak GW (2001) Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. *Proc Natl Acad Sci U S A* **98**(24):14084-14089.

Pasternak GW (2001) Insights into mu opioid pharmacology the role of mu opioid receptor subtypes. *Life Sci* **68**(19-20):2213-2219.

Peters L and Meister G (2007) Argonaute proteins: mediators of RNA silencing. *Mol Cell* **26**:611-623.

Qin X, Ahn S, Speed TP and Rubin GM (2007) Global analyses of mRNA translational control during early Drosophila embryogenesis. *Genome Biol* **8**(4):R63.

Rana TM (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nature Rev Mol Cell Biol* **8**:23-36.

Rastinejad F and Blau HM (1993) Genetic complementation reveals a novel regulatory role for 3' untranslated regions in growth and differentiation. *Cell* **72**(6):903-917.

Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R and Wulczyn FG (2008) A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat Cell Biol* **10**(8):987-993.

Sachs A and Wahle E (1993) Poly(A) tail metabolism and function in eucaryotes. *J Biol Chem* **268**(31):22955-22958.

Schmidt H, Schulz S, Klutzny M, Koch T, Handel M and Hollt V (2000) Involvement of mitogen-activated protein kinase in agonist-induced phosphorylation of the mu-opioid receptor in HEK 293 cells. *J Neurochem* **74**(1):414-422.

Seggerson K, Tang L and Moss EG (2002) Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. *Dev Biol* **243**(2):215-225.

Shabalina SA and Spiridonov NA (2004) The mammalian transcriptome and the function of non-coding DNA sequences. *Genome Biol* **5**(4):105.

Shaw G and Kamen R (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**(5):659-667.

Shen J, Benedict Gomes A, Gallagher A, Stafford K and Yoburn BC (2000) Role of cAMP-dependent protein kinase (PKA) in opioid agonist-induced mu-opioid receptor downregulation and tolerance in mice. *Synapse* **38**(3):322-327.

Simantov R (1989) Glutamate neurotoxicity in culture depends on the presence of glutamine: implications for the role of glial cells in normal and pathological brain development. *J Neurochem* **52**(6):1694-1699.

Simantov R and Levy R (1986) Plasticity in the phenotypic expression of brain opioid receptors: differential response of forebrain and hindbrain cultures to chemical depolarization. *Brain Res* **391**(2):301-304.

Sonenberg N, Hershey JWB and Mathews M (2000) *Translational control of gene expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Song KY, Hwang CK, Kim CS, Choi HS, Law PY, Wei LN and Loh HH (2007) Translational repression of mouse mu opioid receptor expression via leaky scanning. *Nucleic Acids Res* **35**(5):1501-1513.

Sontheimer EJ (2005) Assembly and function of RNA silencing complexes. *Nature Rev Mol Cell Biol* **6**:127-138.

Stutz A, Conne B, Huarte J, Gubler P, Volkel V, Flandin P and Vassalli JD (1998) Masking, unmasking, and regulated polyadenylation cooperate in the translational control of a dormant mRNA in mouse oocytes. *Genes Dev* **12**(16):2535-2548.

Subramaniam K, Chen K, Joseph K, Raymond JR and Tholanikunnel BG (2004) The 3'-untranslated region of the beta2-adrenergic receptor mRNA regulates receptor synthesis. *J Biol Chem* **279**(26):27108-27115.

Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, Cha KY, Chung HM, Yoon HS, Moon SY, Kim VN and Kim KS (2004) Human embryonic stem cells express a unique set of microRNAs. *Dev Biol* **270**(2):488-498.

Tao PL, Han KF, Wang SD, Lue WM, Elde R, Law PY and Loh HH (1998) Immunohistochemical evidence of down-regulation of mu-opioid receptor after chronic PL-017 in rats. *Eur J Pharmacol* **344**(2-3):137-142.

Wahle E and Ruegsegger U (1999) 3'-End processing of pre-mRNA in eukaryotes. *FEMS Microbiol Rev* **23**(3):277-295.

Wahle S and Stoffel W (1998) Cotranslational integration of myelin proteolipid protein (PLP) into the membrane of endoplasmic reticulum: analysis of topology by glycosylation scanning and protease domain protection assay. *Glia* **24**(2):226-235.

Wang T, Zhang X, Obijuru L, Laser J, Aris V, Lee P, Mittal K, Soteropoulos P and Wei JJ (2007) A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. *Genes Chromosomes Cancer* **46**(4):336-347.

Wei LN, Hu X, Bi J and Loh H (2000) Post-transcriptional regulation of mouse kappa-opioid receptor expression. *Mol Pharmacol* **57**(2):401-408.

Wei LN, Law PY and Loh HH (2004) Post-transcriptional regulation of opioid receptors in the nervous system. *Front Biosci* **9**:1665-1679.

Wei LN and Loh HH (2002) Regulation of opioid receptor expression. *Curr Opin Pharmacol* **2**(1):69-75.

Whistler JL, Chuang HH, Chu P, Jan LY and von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23**(4):737-746.

Wilkinson M (1988) A rapid and convenient method for isolation of nuclear, cytoplasmic and total cellular RNA. *Nucleic Acids Res* **16**(22):10934.

Woods K, Thomson JM and Hammond SM (2007) Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. *J Biol Chem* **282**(4):2130-2134.

Wu EH and Wong YH (2005) Activation of delta-, kappa-, and mu-opioid receptors induces phosphorylation of tuberin in transfected HEK 293 cells and native cells. *Biochem Biophys Res Commun* **334**(3):838-844.

Wu L and Belasco JG (2005) Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. *Mol Cell Biol* **25**:9198-9208.

Wu L, Fan J and Belasco JG (2006) microRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci USA* **103**:4034-4039.

Wu Q, Hwang CK, Yao S, Law PY, Loh HH and Wei LN (2005) A major species of mouse mu-opioid receptor mRNA and its promoter-dependent functional polyadenylation signal. *Mol Pharmacol* **68**(2):279-285.

Wu Q, Law PY, Wei LN and Loh HH (2008) Post-transcriptional regulation of mouse {micro} opioid receptor (MOR1) via its 3' untranslated region: a role for microRNA23b. *FASEB J*.

Xu Y and Carr LG (2001) Functional characterization of the promoter region of the human mu opioid receptor (hMOR) gene: identification of activating and inhibitory regions. *Cell Mol Biol (Noisy-le-grand)* **47 Online Pub**:OL29-38.

Yabaluri N and Medzihradsky F (1997) Down-regulation of mu-opioid receptor by full but not partial agonists is independent of G protein coupling. *Mol Pharmacol* **52**(5):896-902.

Yamamoto J, Kawamata T, Niiyama Y, Omote K and Namiki A (2008) Down-regulation of mu opioid receptor expression within distinct subpopulations of dorsal root ganglion neurons in a murine model of bone cancer pain. *Neuroscience* **151**(3):843-853.

Yang Q, McDermott PJ, Duzic E, Pleij CW, Sherlock JD and Lanier SM (1997) The 3'-untranslated region of the alpha2C-adrenergic receptor mRNA impedes translation of the receptor message. *J Biol Chem* **272**(24):15466-15473.

Yoburn BC, Billings B and Duttaroy A (1993) Opioid receptor regulation in mice. *J Pharmacol Exp Ther* **265**(1):314-320.

Yu Z and Hecht NB (2008) The DNA/RNA-binding protein, translin, binds microRNA122a and increases its in vivo stability. *J Androl* **29**(5):572-579.

Zadina JE, Chang SL, Ge LJ and Kastin AJ (1993) Mu opiate receptor down-regulation by morphine and up-regulation by naloxone in SH-SY5Y human neuroblastoma cells. *J Pharmacol Exp Ther* **265**(1):254-262.

Zaki PA, Keith DE, Jr., Brine GA, Carroll FI and Evans CJ (2000) Ligand-induced changes in surface mu-opioid receptor number: relationship to G protein activation? *J Pharmacol Exp Ther* **292**(3):1127-1134.

Zhang W, Dahlberg JE and Tam W (2007) MicroRNAs in tumorigenesis: a primer. *Am J Pathol* **171**(3):728-738.

Zhao J, Hyman L and Moore C (1999) Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol Mol Biol Rev* **63**(2):405-445.

Zhao T (2007) A complex system of small RNAs in the unicellular green alga Chlamydomonas reinhardtii. *Genes Dev* **21**:1190-1203.

Zhao Y and Srivastava D (2007) A developmental view of microRNA function. *Trends Biochem Sci* **32**(4):189-197.

Zhou GK, Kubo M, Zhong R, Demura T and Ye ZH (2007) Overexpression of miR165 affects apical meristem formation, organ polarity establishment and vascular development in Arabidopsis. *Plant Cell Physiol* **48**(3):391-404.

Zollner C, Johnson PS, Bei Wang J, Roy AJ, Jr., Layton KM, Min Wu J and Surratt CK (2000) Control of mu opioid receptor expression by modification of cDNA 5'- and 3'-noncoding regions. *Brain Res Mol Brain Res* **79**(1-2):159-162.

Zubiaga AM, Belasco JG and Greenberg ME (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol Cell Biol* **15**(4):2219-2230.