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*ACCELERATED COMMUNICATION*

# A Major Species of Mouse μ-opioid Receptor mRNA and Its Promoter-Dependent Functional Polyadenylation Signal

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**ABSTRACT**

The pharmacological effects of opioid drugs are mediated mainly by the μ-opioid receptor (MOR), which is encoded by an mRNA transcript named MOR1. Although several MOR mRNA splice variants have been reported, their biological relevance has been debated. In this study, we found that probes of regions essential for the production of functional MOR, as well as that of the 3′-downstream region of the MOR gene coding region, detected by Northern blot analyses, a major species of mature transcript MOR1 from mouse brain of ~11.5 kilobases (kb). Although 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′-untranslated region (UTR) of MOR1 is contiguously extended from the end of the coding region, and uses a single polyadenylation [poly (A)] signal (located 10,179 bp downstream of the MOR1 stop codon). The poly (A) signal (AAUAAA) is located 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 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 the MOR gene promoter and its polyadenylation signal.





Opiate drugs are widely used as pain relievers in clinic. Besides 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-, δ-, and κ-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 μ-opioid receptor (MOR). The MOR gene spans approximately 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; 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 after the initial gene structure study (Min et al., 1994) and the reports of splice variants of this gene (for review, see 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 (accession number 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), neuron-restrictive silencer factor (Kim et al., 2004), and Sp3 isoforms

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**ABBREVIATIONS:** MOR, μ-opioid receptor; kb, kilobase(s); PCR, polymerase chain reaction; bp, base pair(s); UTR, untranslated region; SV40, simian virus 40; RACE, rapid amplification of cDNA ends; RT, reverse transcription; poly (A), polyadenylation.

(Choi et al., 2005). Regulatory sequences in the 5′-UTR have also been defined by other labs, including STAT6 (Kraus et al., 2001), cAMP response element-binding protein (Lee and Lee, 2003), and neurorestrictive suppressor element (Andria and Simon, 2001). However, studies of its 3′-UTR have been very limited.

Ample evidence has shown that 3′-UTR of mRNA plays an important role in gene regulation. The average length of 3′-UTR has increased dramatically throughout the evolutionary process, from 200 nucleotides in plant and fungi to 800 and more in humans and other vertebrates. This increase suggests a potential role of longer 3′-UTR in the regulation of more complicated gene expression in higher vertebrates. It has been reported that the 3′-UTR can specifically control nuclear export, polyadenylation status, subcellular targeting, and the rate of translation and degradation of mRNA (Standart and Jackson, 1994). The *cis*-acting elements on 3′-UTR and the *trans*-acting factors that bind to them can affect the expression of genes. It has been shown that several human disease genes depend on *cis* and/or *trans* factors acting at the 3′-UTR of mRNA (Conne et al., 2000). A recent report showed that the deletion of 3′-UTR sequences of $\beta$2-adrenergic receptor resulted in a 2- to 2.5-fold increase in receptor expression and a shift of mRNA distribution toward the polysomal fraction, thereby favoring increased translation (Subramaniam et al., 2004).

Despite an enormous effort focusing on MOR gene regulation by its promoter and 5′ upstream region, the identity of its 3′-UTR has not yet been revealed. The goal of our study was 1) to identify the mature MOR1 transcript that can produce functional MOR protein, 2) to obtain the complete 3′-UTR sequence of mature MOR1 mRNA; and 3) to identify biologically functional poly (A) signal(s) used 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. Furthermore, we identified a single 3′-end of the mature MOR1 mRNA and confirmed the biological function of a single poly (A) signal used by MOR gene to generate 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, Madison, WI). 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, 5′-AATAGGCCGGCCGCATTAGGAGCATTGCTGAG-3′; antisense, 5′-ACGCGTCGACCCTAACTCTGGGATGGCAAG-3′; the underlined nucleotides indicate the overhanging restriction enzyme sites for FseI and SalI, respectively). The pL6PA and pGL3pPA plasmids were constructed by subcloning this DNA fragment after digestion with FseI and SalI into pL6 and pGL3-promoter plasmid, respectively. The pL6 and pGL3-promoter plasmids also have been digested by FseI and SalI, 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 FseI and SalI 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 instructions (Molecular Research Center, Cincinnati, OH). The mRNA was isolated from total RNA using MicroPoly (A) Purist (Ambion, Austin, TX). The Northern blot analysis was performed as described in manufacturer's manual (NorthernMax Kit; Ambion). In brief, 10 $\mu$g of mRNA per lane was loaded in 1% formaldehyde agarose gel, and transferred to a Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ). 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 Applied Science, Indianapolis, IN). Probes 1, 2, and 3 are located at exon 1, 3′-UTR after exon 4, and exon 3, respectively (Fig. 1A). All probes were prepared by PCR with *Taq* DNA polymerase (Roche Applied Science) and the above cDNA template. The PCR conditions for the probes consisted of a 2-min denaturing at 94°C and 30 cycles of amplification at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min followed by a 10-min extension at 72°C. PCR products were separated in a 1.2% agarose gel. The sense primers were 5′-GAACATCAGCGACTGCTCTG-3′ for probe 1, 5′-ATTCCCTCTCACGACGTTGT-3′ for probe 2, and 5′-GATTGCACCCTCACGTTCTC-3′, for probe 3. The antisense primers were 5′-TCCAAAGAGGCCCACTACAC-3′ for probe 1, 5′-AGGTGGGAAGAAGGAACTCTCAGA-3′ for probe 2, and 5′-GTGTAACCCAAGGCAATGCAG-3′ for probe 3. The radiolabeled probes were produced by Random Labeling Kit (Amersham Biosciences) using [$\alpha$-$^{32}$P]dCTP.

**3′-RACE and DNA Sequencing.** Total RNA was extracted from mouse brain as described under *Northern Blot Analysis*. The 3′-RACE experiments were performed as described in the protocol of 3′-RACE System for rapid amplification of cDNA ends Kit (Invitrogen, Carlsbad, CA). In brief, the first-strand cDNA was synthesized by SuperScript II RT from 5 $\mu$g of mouse brain total RNA using anchored primer AP (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-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 $\mu$l of cDNA using primer A (5′-TAGCTTAACTCGGAACTGAGT-3′) and antisense primer UAP (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′, included in the kit). The second round of the nested PCR used 4 $\mu$l of PCR product from the first round amplified by sense primer B (5′-CACGCTGCCTAGCTGGATTA-3′) or primer C (5′-GCATTAGGAGCATTGCTGAG-3′) and antisense primer UAP. The PCR conditions consisted of a 2-min denaturing at 94°C and 30 cycles of amplification at 94°C for 1 min, 57°C (for primer A and B) or 60°C (for primer C) for 1 min, and 72°C for 1 min followed by a 10-min extension at 72°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 (Fig. 2C, lane 1; sense primer, primer B) as template. The primer sets used are primer C and UAP. The 700-bp PCR product was also generated (Fig. 2C, lane 2) in the third-round PCR because the PCR template from lane 1 of Fig. 2C contained sufficient 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, Valencia, CA), 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 37°C. For transfection, cells were plated 24 h before transfection at a density of $1.1 \times 10^5$ per well in six-well

plates. NS20Y cells were transfected with plasmids pL6, pL6PA, pL6N, pGL3p, pGL3pPA, and pGL3pN using the Effectene transfection reagent (QIAGEN) as described by the manufacturer's manual. One specific reporter construct (320 ng) was used for each well, and 80 ng of pCH110 plasmid (Amersham Biosciences) containing β-galactosidase gene was included for normalization. Forty-eight hours 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 Technologies, Bad Wildbad, Germany) as described in the protocol from manufacturer.

## Results

**Characterization of Mature Mouse MOR1 mRNA.** 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. 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 extramembrane 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 (Fig. 1C, lanes 1 and 2). 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 the MOR1 3′-UTR, we first examined whether splicing occurred in the 3′ downstream region of the 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 Fig. 1C, lanes 3 and 4, a 350-bp probe, from 4566 bp downstream of the MOR1 stop codon, also detected a single band at 11.5 kb (Fig. 1C, lanes 3 and 4), exactly the same size as that detected by the exon 1 probe. The Northern blot results suggest that the 3′ downstream region of the MOR gene is likely to be contiguously transcribed after the end of the coding region, thus constituting the 3′-UTR of MOR1 transcript.

Because exon 3 is included in all reported MOR splice variants, we chose a 353-bp DNA fragment within exon 3 as the probe. As shown in Fig. 1C, lanes 5 and 6, the hybridization signals appeared in two sharp bands, approximately 3.7 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 because 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 investigated further. As shown in Fig. 1B, the 18 S and 28 S 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 approximately 11.5 kb and the 3′-UTR is likely to be contiguously transcribed from this gene after the end of the coding region. Taking into consideration the 5′-UTR, the coding region, and the full length of MOR1, its 3′-UTR was estimated to be around 10 kb downstream of the stop codon. We then designed three sets of primers located 8.3, 9.3, and 9.8 kb downstream of the stop codon in the 3′ RACE analyses. The predicted lengths of products would be approximately 1700, 700, and 300 bp, respectively, as shown in Fig. 2A. After the first round of 3′ RACE-PCR, a sharp band at approximately 1700 bp was detected in lane 1 of Fig. 2B. We used the PCR product from the first round (lane 1 of Fig. 2B) as the template to amplify the nested PCR products with primer B or primer C as the sense primer, and UAP

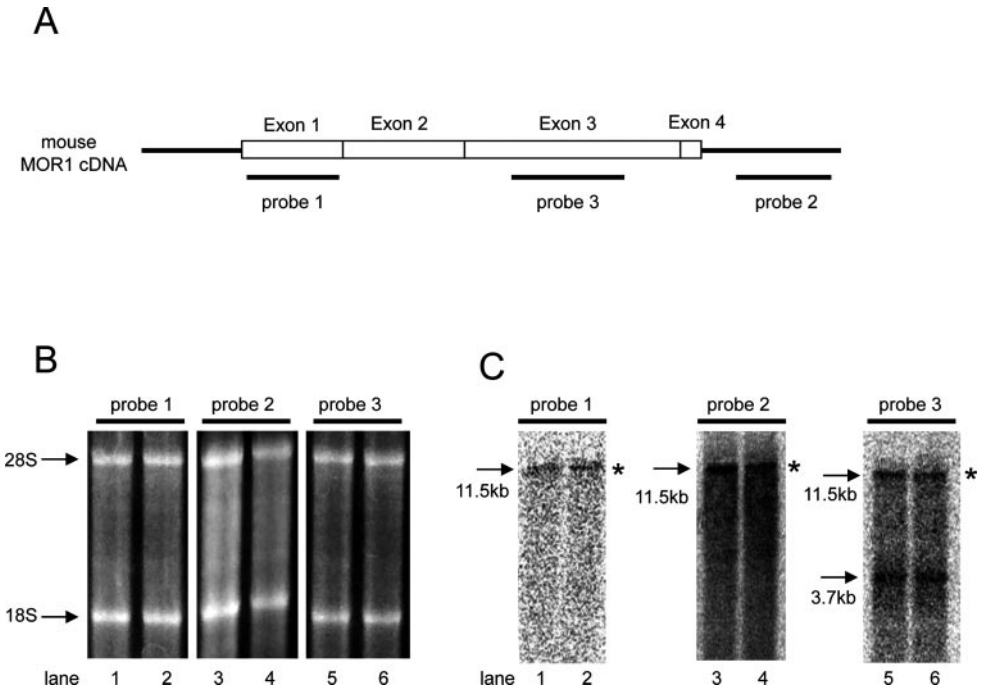


**Fig. 1.** Northern blot results of mouse brain mRNA using different probes. A, the structure of the MOR1 cDNA and the positions of probes. B, the denaturing RNA gel stained with ethidium bromide, showing the integrity of mRNA samples. The same amount (10 μg) of mRNA was loaded in two wells for duplicating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

(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 Fig. 2C, lanes 1 and 3. The PCR product from Fig. 2C, lane 1, was further amplified by the addition of primer C, and, as predicted, two bands were detected (Fig. 2C, lane 2). 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. 2D). The result agreed with the genomic DNA information on the mouse MOR1 gene contained in GenBank, 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. 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 after the coding region. Based upon sequence comparison, a single poly (A) signal is located 10,153 bp downstream of the stop codon, which is likely to be used to generate the mature 11.5-kb MOR1 transcript. This information is summarized in Fig. 4A.

**Biological Activity of MOR1 Poly (A) Signal.** To determine whether the poly (A) signal of MOR1 is biologically

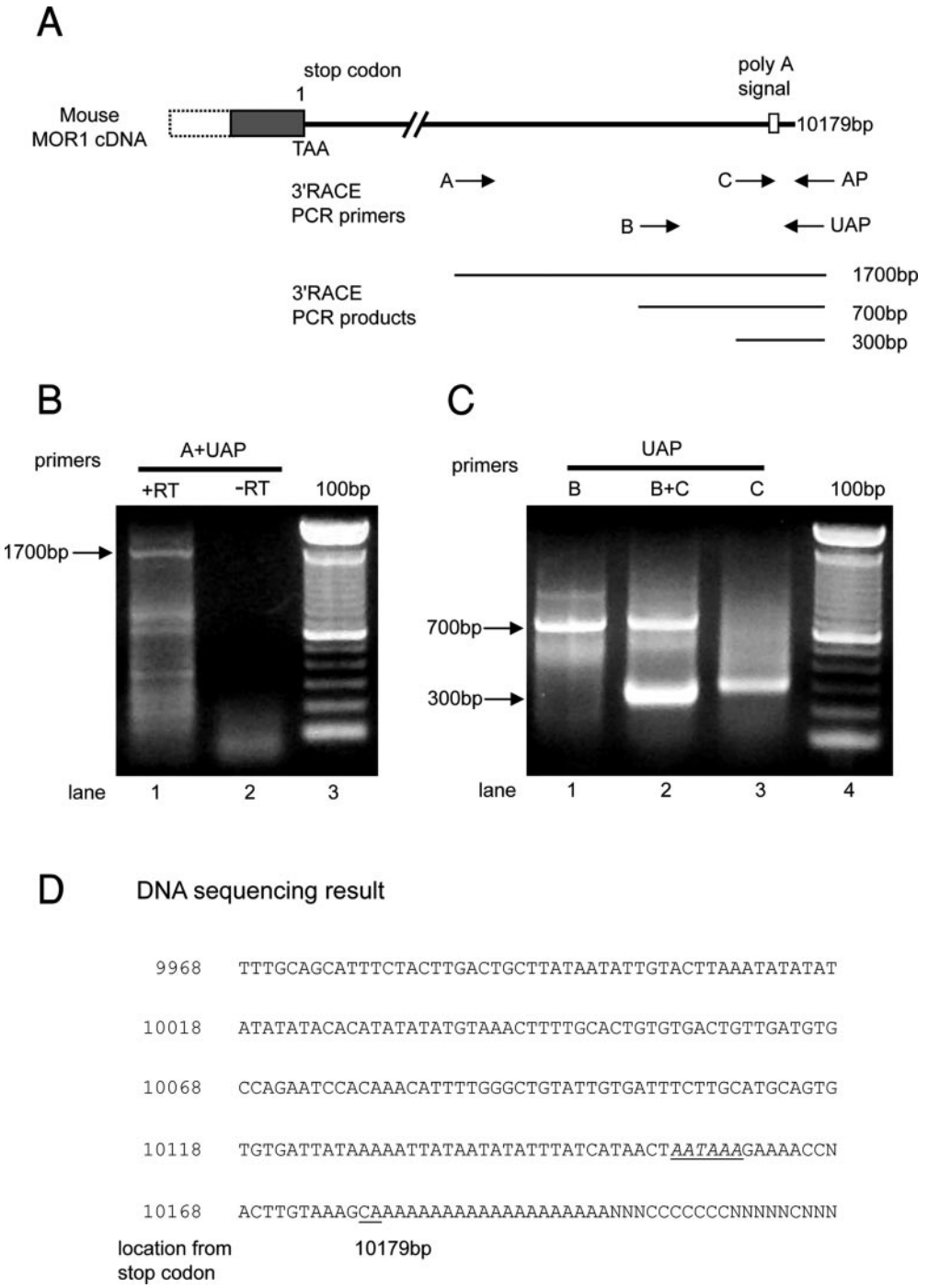


**Fig. 2.** 3′ RACE, the nested PCR and DNA sequencing result. A, locations of 3′ RACE-PCR primers are shown corresponding to MOR1 3′-UTR. The sense primers used in PCR reactions are primers A, B, and C. The antisense primers (AP and UAP) are provided in the kit (Invitrogen). Expected sizes for 3′ RACE 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 that was reverse-transcribed (+RT) using primer AP from 5 $\mu$g of 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. Lanes 1 and 3, second-round PCR product. First-round PCR product (Fig. 2B, lane 1) was used as template, and sense primers for lanes 1 and 3 are primer B and C, respectively. Lane 2, the third round PCR product. Second-round PCR product from C, lane 1, was used as the template, and sense primer is primer C. PCR (700 bp) 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. 2C have been analyzed by DNA sequencing. The identity of DNA fragments was checked by comparison with the mouse genomic sequence. The nucleotides near the poly (A) signal site are shown here. AATAAA represents the poly (A) signal site; *CA* is identified as cleavage site where the poly (A) tail is added. Numberings are indicated as the locations from MOR1 stop codon.

functional, a 430-bp genomic DNA fragment was generated by PCR. This fragment contained the highly conserved AAUAAA sequence, the poly (A) site, and the U-rich downstream element (Fig. 4B). The 430-bp fragment was used to replace the SV40 poly (A) signal in a reporter construct pL6, which uses the mouse MOR promoter to drive the luciferase reporter (Fig. 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 NS20Y neuroblastoma cells, together with a galactosidase reporter (pCH110 plasmid; Amersham Biosciences) as the internal control. Forty-eight 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 construct 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. 3A, pL6PA, the construct containing MOR1 poly (A) signal, activated the luciferase reporter activity by 12- and 3-fold compared with pL6N and pL6, respectively. The result of the 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) compared with 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 the SV40 promoter, generating pGL3pPA plasmid (Fig. 3B). It seems that the MOR1 poly (A) signal was only half as potent as that SV40 poly (A) signal 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 MOR1 as the major mature MOR gene transcript that has the capacity to produce functional MOR protein. This mature transcript is approximately 11.5 kb long. Many splice variants of the MOR gene have been reported (for review, see 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. 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. Therefore, exons encoding the amino-terminal extracellular domain, the seven transmembrane domain, 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 the 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

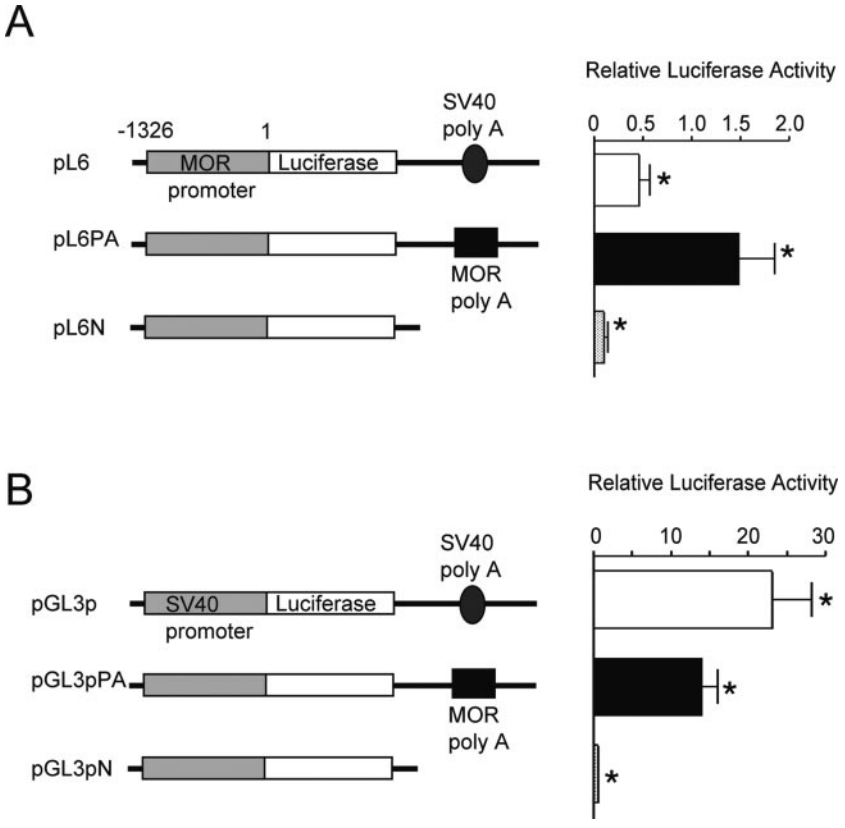


**Fig. 3.** Transient transfection assay demonstrating the biological activity of the poly (A) signal. A, analysis of homologous MOR promoter reporters, including pL6 and its derivatives. Left side shows the map of reporter constructs used in these transient transfection experiments. Gray box, MOR promoter; blank box, luciferase reporter; filled black box, MOR1 poly (A) signal; filled oval box, SV40 poly (A) signal. Right-hand shows 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 (S.E.M.). *, 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 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 ± S.E.M. and analyzed by one-way ANOVA test. Significance was set at $P < 0.05$.

mRNAs. Studies of RNA splice variants of the 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. Furthermore, it remains to be verified whether all these variants contain poly (A), an essential feature of functional mRNAs in a physiological context.

From a technical standpoint, 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 as 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, although more sensitive, cannot 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 required 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. 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

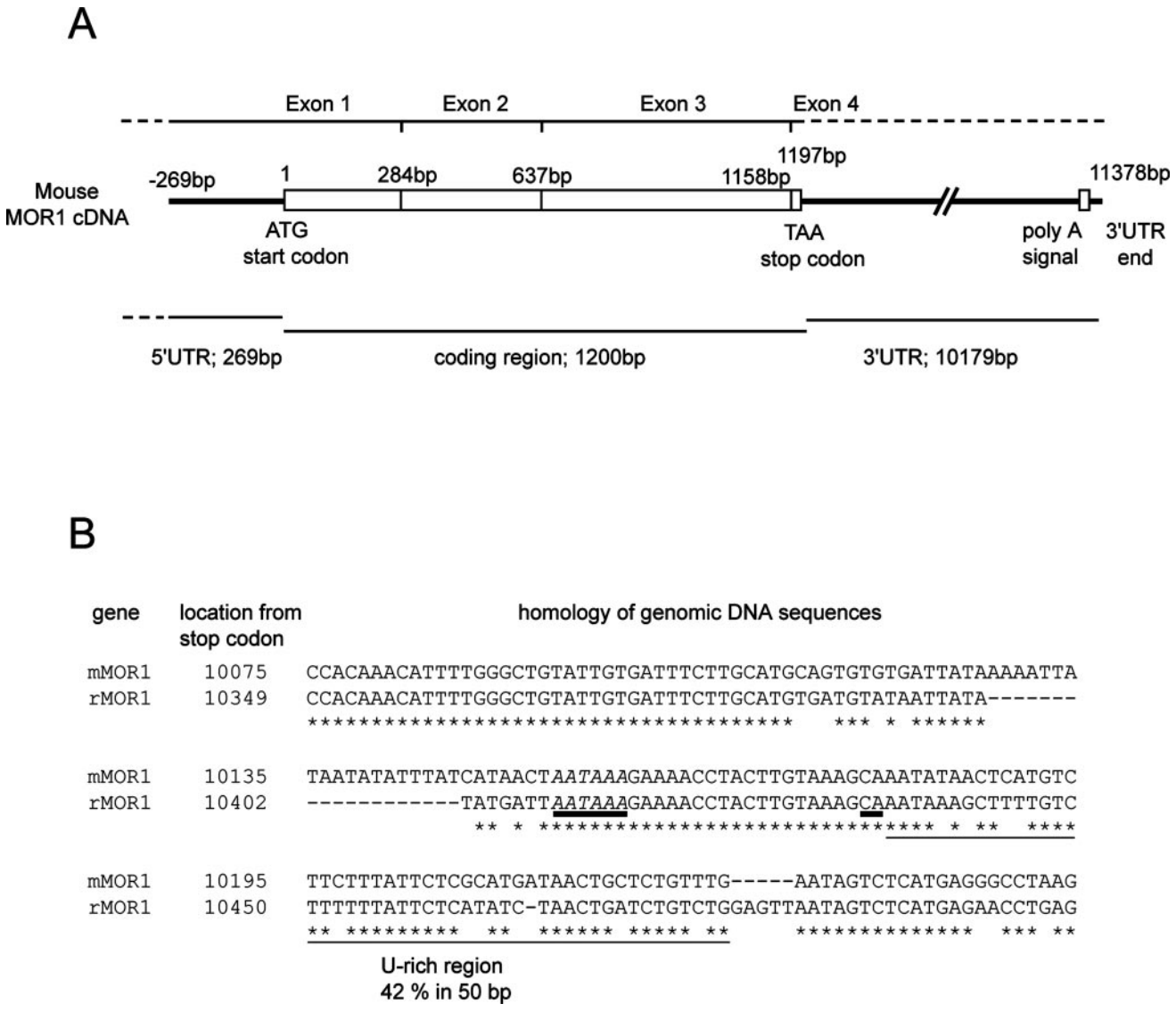


**Fig. 4.** The entire mRNA structure of mouse MOR1 gene. A, the cDNA structure of mouse MOR1. Translational start site was designated as 1. Open boxes are coding exons, except small open box at 3′ end indicates poly (A) signal. B, homology analysis of genomic DNA sequences near MOR1 poly (A) signal between mouse (mMOR1) and rat (rMOR1) species. Genomic sequences of mouse MOR1 and rat MOR1 are from GenBank accession numbers NC000076 (*Mus musculus*) and accession number NW047550.1 (*Rattus norvegicus*), 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 (Fukuda et al., 1993; Brodsky et al., 1995).

(Fig. 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. In all probability, this region can interact with the MOR promoter region, possibly through RNA polymerase II (Stutz et al., 1998; Conne et al., 2000). 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 have 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, it has been reported that the 5′- and 3′-UTRs are subjected to regulation at the level of mRNA splicing/processing, stability, translation, and κ-opioid receptor mRNA transport in neuronal cells (Wei et al., 2000; Hu et al., 2002). In this study, we demonstrated that the 3′ downstream region of MOR gene is contiguously transcribed after the end of the coding region, and it uses a single poly (A) signal at 10,153 bp downstream of stop codon. The discovery of uninterrupted 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 involve 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, because the majority of the 3′-UTR (approximately 10 kb) is missing in our construct, which 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 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 the carboxyl-terminal domain of RNA polymerase II (Wahle and Ruegsegger, 1999). Possible interaction between MOR 5′- and 3′-UTR has been reported for the human MOR gene (Zöllner et al., 2000). Therefore, it would be interesting to examine the 3′-UTR, including secondary and tertiary structures of the 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.

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