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The Potential Promoter Regions on the 5′ Flank Sequence of the mu Opioid Receptor Gene in Lymphocytes

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Abstract The human mu opioid receptor is known to mediate a variety of physiological and pharmacological effects of morphine in many tissues. However, the molecular processes that regulate the expression of the mu opioid receptor gene in immune cells are not well understood. To study regulatory elements that affect the expression of the mu opioid receptor gene in human lymphocytes (LMOR), a 2,278 bp fragment of the 5′ regulatory region of the mu opioid receptor gene was cloned and sequenced from CEM ×174 cells. The transcriptional initiation site was mapped through a primer extension assay. A series of 5′-deleted plasmids were constructed and transiently transfected into cultured CEM ×174 cells. The data indicated that morphine up-regulated the mRNA level of LMOR in a dose-dependent manner, which could be blocked by the opioid receptor antagonist naloxone. Only one transcription initiation site (TIS) about 110 bp upstream of the translation start codon was identified. The regions from −372 to −253 and −2279 to −1371 located in the 5′ regulatory sequence of the mu opioid receptor gene contained enhancer elements, while the regions from −1371 to −968 and −650 to −370 possessed repressor elements. Those promoter elements were involved in the transcriptional regulation of the mu opioid receptor gene. Collectively, this data strongly indicates that the expression of the mu opioid receptor gene in lymphocytes is subject to the regulation of *cis*-elements upstream from the TIS. J. Cell. Biochem. 95: 1204–1213, 2005.

Key words: mu opioid receptor; lymphocytes; morphine; expression; transcription initiation site; transcriptional regulation

Over the past decade, there has been a substantial increase in our understanding of the involvement of the mu opioid receptor in immune cells [Pampusch et al., 1998; McCarthy et al., 2001; Suzuki et al., 2003]. Apart from its presence in neurons, the biological and pharmacological effects of opiates, such as morphine, in lymphocytes have been well documented [Raghavendra et al., 2001; Wang et al., 2001; Hao et al., 2003; Li et al., 2004]. Opioid receptors participate in the functioning of lymphocytes, with evidence suggesting that opioids modulate both innate and acquired immune response [Suzuki et al., 2002; Szabo et al., 2003]. In addition, the ligand such as morphine may interact with its receptor to function as a mediator to downregulate immune reactions [Singhal et al., 1999; Wang et al., 2002]. The suppressive functioning of morphine on immune cells was particularly observed in the case of AIDS [Steele et al., 2003; Szabo et al., 2003]. Therefore, drug abuse was thought as a contributing factor in the progression of AIDS [Nair et al., 1997; Donahoe and Vlahov, 1998; Gurwell et al., 2001]. To date, all studies indicate that the effects of morphine on the immune system are achieved through opioid receptors though contrary data has been reported [Madden et al., 2001]. However, this conclusion was only based on the result from pharmacological and immunological research and few studies have been reported about the transcriptional regulation of the mu opioid receptor in lymphocytes (LMOR). Hence, a good

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understanding of the molecular mechanisms is still lacking.

The evidence described above indicates that the expressional behaviors and pharmacological effects of LMOR are definitely different from those of the mu opioid receptor in neurons. Therefore, it may be speculated that different regulatory mechanisms are involved in various cell sources. This presumption has been recently confirmed using molecular and pharmacological approaches [Cadet, 2004]. Recently, three human mu opioid receptors (MOR-1, MOR-2, and MOR-3) were found in the brain, gastrointestinal system, and in immunocytes respectively. In terms of pharmacological features, MOR-3 expressed in immunocytes was different from the other two. However, the expressional and regulatory properties of human LMOR have not been clearly reported.

In the present study, we examine the expression of the mu receptor gene in lymphocytes as well as analyze the 5′ flank region of the mu opioid receptor gene. The objective of this study is to clarify two questions: (1) whether the lymphocytes express the mu opioid receptor, since one negative answer has been reported [Madden et al., 2001], and (2) if it can, then elucidating the profile of the potential regulatory mechanism.

MATERIALS AND METHODS

Expression of LMOR Induced by Morphine

The CEM ×174 cell line was maintained in the RPMI (Gibco, Grand Island, NY) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2, split and subcultured every 3–4 days. Cell viability was determined to be >96% by the trypan blue dye exclusion method. To examine the effects of different concentrations of morphine on the expression of LMOR, cells (5×10^5 cells per ml) were added to 6 well plate and treated with different concentrations of morphine chloride (0.1–100 μM). After 12 h incubation, cells were pelleted and total cellular RNA was extracted from cell lines with TRIzol reagent (Promega, Madison, WI) according to the manufacturer's instructions. In the evaluation of different incubation time stimulated with morphine, morphine at the concentration of 1 μM was added into culture and harvested at different time intervals from 0 to 24 h. In the time course assay, this suboptimum dosage was used to avoid the prompt reach of maximum responsive platforms in a short period of time. In the blocking assay of naloxone, the opiate receptor antagonist, the CEM ×174 cells were preincubated with 100 μM of naloxone for 30 min and subsequently treated with 10 μM of morphine for 12 h. The mRNA extraction was performed as described above. Single strand cDNA was synthesized from 2 μg of RNA by using 1 μg of random primer and 10 units of AMV reverse transcriptase. PCR amplification was performed in a system containing the synthesized cDNA as the templet, 0.4 mM dNTP mix, 0.5 μM of forward and reverse primers, and 5 unit of *Taq* DNA polymerase (MBI Co. Lithuanian). The following oligonucleotide primers were used: 5′-GGTACTGGGAAAACCTGCTGAAGATCTGTG-3′ (forward primer) and 5′-GGTCTCTAGTGTTCTGACGAATTCGAGTGG-3′ (reverse primer). The reaction was started at 95°C for 3 min, and subsequent conditions were: 20 s at 95°C, 30 s at 63°C, 30 s at 72°C for 30 cycles, followed by a final extension for 10 min at 72°C. PCR product (441 bp) was analyzed by 2% agarose gel electrophoresis. The product size was the same as predicted from the sequences. After staining with ethidium bromide, gel was viewed under UV light, and a digital image was captured with a WDS ImageMaster VDS (Pharmacia Biotech, San Francisco, CA).

Amplification of the mu Receptor Gene 5′ Flanking Region

CEM ×174 cells (1×10^6) were removed from cell culture flasks, washed 4× in PBS pH 7.4, and incubated in a cell lysis buffer (0.2 M Tris HCl pH 8, 0.1 M Na_2 EDTA, 1% SDS, 100 μg/ml proteinase K) for 1 h at 37°C. The lysate was extracted 2× with phenol:chloroform and the DNA precipitated with 3 M sodium acetate and ethanol overnight at −20°C. The DNA was washed once with 75% ethanol, suspended in a TE buffer (pH 8) and quantified spectrophotometrically at a wavelength of 260 nm. A ~2.3 kb fragment of the LMOR gene 5′ flanking region spanning the −2,282 bp to +16 bp region was amplified from CEM ×174 cells by PCR. As the template for the PCR reaction, 0.2 μg of DNA were used in a reaction mixture consisting of 5 μl of 10× PCR buffer, 0.5 μl of 25 μM each of primer PCN1: 5′-ATTACGCGTGTGGAACTG-

GCTTGGATTGT-3′ (location −2282∼−2254, with a *Mlu I* site in 5′ terminal) and primer PCN 2: 5′-CAGCGCTGCTGTCCATGGTACTGACG-3′ (location −10∼−16, with a *Nco I* site), 8 μl of 2.5 mM dNTP's, 2.5 U LongTaq polymerase (MBI Co., Lithuanian) and H_2O to 50 μl. The reaction system was preheated in 94°C for 5 min. The PCR reaction conditions consisted of 94°C for 30 s, 59°C for 30 s, and 68°C for 2.5 min for 30 cycles to yield a DNA fragment 2,298 bp in length. Ten microliters of amplified DNA were analyzed via 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. The PCR product was cloned into pGEM-T vector (Promega) to create the pGEM-2.3 which was verified by BglII/NcoI and EcoRI digestion. The sequence and orientation of the pGEM-2.3 were confirmed by direct DNA sequencing. The identities of the PCR products were confirmed by comparing sequences with the database of the National Center for Biotechnology Information BLAST network server.

Analysis of the Transcription Start Site by a Primer Extension Assay

Primer extension was carried out using Primer Extension System (Promega) as described in the protocol of the manufacturer. Briefly, an antisense oligonucleotide (5′-GCAATTGCTGGCGTTCGTGGGGG-3′) complementary to the +39 to +17 of the LMOR gene was end labeled with (^{32}P)-ATP (Furui Bio Co. China) [Wendel and Hoehe, 1998]. About 2 pM of the labeled oligonucleotide and 20 ng of RNA were mixed in 5 μl of the reverse transcription buffer (pH 8.3, containing 2 mM dNTPs) and incubated for 20 min at 58°C and 10 min at room temperature. Hybridization between the mRNA and labeled oligonucleotide was accomplished for 30 min at 42°C. Reverse transcription was initiated by adding 1 U of avian myeloblastosis virus reverse transcriptase (Promega) to the mRNA-oligonucleotide mixture in a total volume of 8 μl and the reaction carried out at 42°C for 30 min. The primer extended product was separated on 8% urea polyacrylamide gels and visualized by autoradiography at −70°C overnight.

Verification of Transcription Start Site by RT-PCR

RT-PCR was performed to verify the fragment produced by primer extension from mRNA of CEM ×174 cells. The extraction of total RNA and the synthesis of cDNA were described in "Expression of LMOR." PCR amplification consisted of 30 cycles (95°C, 20 s; 63°C, 30 s; 72°C, 30 s) to yield a 146 bp fragment with primer 1: 5′-AGGAGCTGTGGCAGCGGCGAAAG-3′ and primer 2: 5′-GCAATTGCTGGCGTTCGTGGGGG-3′. Ten microliters of amplified DNA product was analyzed via 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Plasmid Construction

Subsequently serial 5′ deleted mu-Luc fusion constructs (pLuc 1-6) were created using a deletional and PCR strategy. *MluI*/*NcoI* fragment (2,278 bp) of pGEM-2.3 was cloned into pGL3-basic vector containing the firefly luciferase reporter gene, resulting in pLuc-1. pLuc-1 was verified by *HindIII*/*Sac I* and *BglII*/*Pst I* digestion. *BglII*/*MluI* and *NdeI*/*MluI* fragments of pLuc-1 were deleted from pLuc-1 in order to obtain the pLuc-2 (containing inserter 1,370 bp) and pLuc-3 (containing inserter 983 bp). The pLuc-2 and pLuc-3 were verified by *HindIII*/*SacI* and *HindIII*/*KpnI* digestion respectively. The pLuc-4 was constructed by inserting pGL3-basic vector an oligonucleotide (651 bp) derived from *XhoI*/*HindIII* digestion on a fragment from PCR assay (primer Pr699U: 5′CACTCGAGATGTTGGCAACGGAG 3′ and primer PrD: 5′ACAGTACCGGAATGCCAAGC 3′) on pLuc-1. Inserting into the pGL3-basic vector an oligonucleotide (371 bp) obtained by digestion with *XhoI*/*HindIII* on a fragment from PCR assay (primer Pr419U: ACCTCGAGAAGAGTGCCCAGTGAAG 3′ and primer PrD) on pLuc-1 to finally obtain the pLuc-5. The PCR products constructing pLuc 4 and pLuc-5 were confirmed by sequencing. The pLuc-4 and pLuc-5 were also verified by *XhoI*/*PstI* digestion. *PstI*/*HindIII* fragment (278 bp) of pLuc-1 was subcloned into pBluescript II SK(±), and then a fragment (252 bp) digested by *SmaI*/*HindIII* on the constructed pBluescript II SK(±) was inserted into pGl3-basic to create pLuc-6. The pLuc-6 was verified by *SacI*/*HindIII* digestion.

Transient Transfection and Reporter Expression Assays

The large-scale purification of expression vectors was conducted using the Large-scale Purification Kit (Vigorous, China), according to the protocol of the manufacturer. Transient transfection was carried out using cationic lipid

DMRIE-C Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 1.6 μg of each pLuc-fusion construct was incubated at room temperature with 200 μl of a serum-free RPMI-1640 medium. The medium was gently added to 4.8 μl of DMRIE-C Reagent and was allowed to stand for 20 min at room temperature. 0.016 μg of pRL-SV 40 promoter driven Renilla luciferase (Promega) was co-transfected with each individual transfectant to standardize the results for transfection efficiency. The luciferase reporter activity was assayed by using the Dual-Luciferase assay system (Promega). Briefly, after 36 h post-transfection, the cells were washed with PBS and lysed using 50 μl of lysis buffer followed by a freeze and thaw method. The cell lysate (20 μl) was mixed with 100 μl of LAR II reagent and the firefly luciferase activities were measured as light output (10 s) in a Centro LB 960 luminometer (Berthold, Germany). Subsequently the Renilla luciferase activity was estimated after the addition of 100 μl of the Stop and Glo reagent, and the light output (10 s) measured separately. The corrected pLuc promoter driven luciferase activity was expressed as the ratio of pLuc promoter driven luciferase activity to the renilla luciferase activity. The promoterless luciferase reporter vector, pLuc-basic, served as the negative control.

Data Analysis

All data were depicted as mean ± SD. Differences between the two groups were validated by the Student *t*-test with the software SPSS 11.5.

RESULTS

Certification for Expression of LMOR in lymphocytes

Our approach to identify the expression of the LMOR gene was to amplify the mRNA of LMOR in the presence of different treated dosages of morphine or at different treatment times by a RT-PCR assay. In these assays, different concentrations of morphine (0, 0.1, 1.0, 10, and 100 μM) were administrated into cultured CEM ×174 cells to stimulate the expression of the LMOR gene. The data showed that the expression of LMOR was low in the sample from normal cells (non-treated) and markedly elevated after treatment with morphine (Fig. 1a). A dose-dependent increase was observed at the concentration range of morphine from 0.1 to 10 μM (Fig. 1b). The density ratios of the mu receptor to actin at different dosages were 0.53, 0.75, 0.82, 1.19, and 1.12 respectively. As shown in Figure 1c, the expression of the LMOR gene was triggered at the stimulated time 8 h and reached the maximum at 12 h. The density ratios of the mu receptor to actin at different time points were 1.04, 0.96, 0.99, 1.4, 2.7, 1.65, and 0.42 respectively. Interestingly, After 12 h treatment with morphine, the expression of the mu receptor declined. Naloxone (100 μM) could reverse the induced effect of morphine (10 μM) on the expression of the LMOR gene, indicating the involvement of a mu opioid receptor mechanism in lymphocytes (Fig. 1d). All data were reproducible in at least three independent experiments.

Cloning and Analysis of the 5′-Region of the LMOR Gene in Lymphocytes

The 5′-flanking region of the LMOR gene was amplified from CEM ×174 cells. The size of the PCR product as compared with a marker on an agarose gel was approximately 2.3 kb as shown in Figure 2a, which was confirmed by sequencing (Fig. 2b). The sequence of this fragment was identical to that of the human genomic nucleotide sequence in the neuron with overall homology of 99.8%, except for a T instead of a G at position −1653, missing a G at position −448, a C at position −2027, and 2A at position −2174. Further analysis revealed several putative *cis*-elements corresponding to binding sites for transcription factors, including a glucocorticoid responsive element (GRE), activator proteins 1 (AP-1), Yin Yang-1 (YY1) boxes, Oct-1, and cAMP response elements (CRE). The 5′ regulatory sequence did not contain a classical TATA box. To study the promoter sequence of the LMOR gene, the PCR product was cloned into pGEM-T to create the pGEM-2.3 fusion construct. As shown in Figure 3, the expected fragments with EcoRI or BglII/NcoI digestion could be visualized.

Identification of the Transcriptional Initiation Site (TIS) of the LMOR Gene in CEM ×174 Cells

Primer extension experiments were carried out to identify the TIS of the LMOR gene. The LMOR specific primer-2 complementary from +39 to +17 of the LMOR gene was employed for this assay. Only one cDNA product was identified that extended about 150 bp from the 5′-end

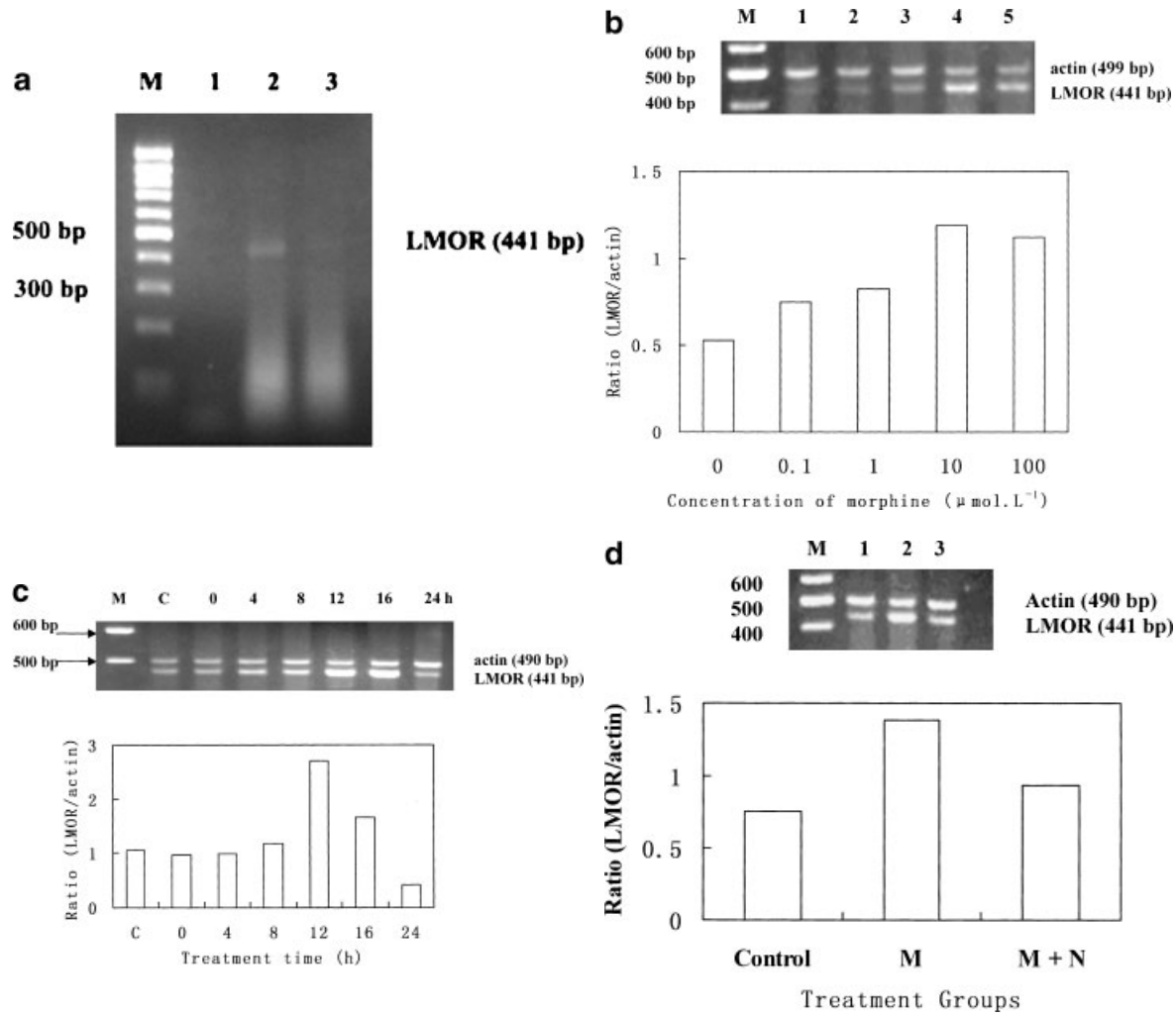

Fig. 1. Expression of LMOR in CEM ×174 cells. **a**: The effect of morphine on the expression of LMOR. M: 100 bp DNA marker; **lane 1**: negative control (without reverse transcriptase). **Lane 2**: morphine (1 μM) treated group; **lane 3**: normal cells without treatment with morphine. **b**: The effects of different concentration of morphine on the expression of LMOR. M: 100 bp DNA marker; **lane 1**: control (normal cells without treatment with morphine); **lanes 2–5**: morphine (0.1–100 μM) treated groups. **c**: The expression of LMOR in CEM ×174 cells treated with morphine at the different incubation time intervals (0, 4, 8, 12, 16, and 24 h). M: 100 bp DNA marker; **lane 1**: control (normal cells without treatment with morphine); **lanes 2–5**: groups at the different treated time intervals. **d**: The blocking effect of naloxone on the morphine-induced expression of LMOR in CEM ×174 cells. M: 100 bp DNA marker; **lane 1**: control (normal cells without the treatment of morphine); **lane 2**: morphine treated cells; **lane 3**: morphine and naloxone cotreated cells. These data above were representative of three independent experiments.

of the primer-2 (Fig. 4a). The extension product was validated by PCR amplification with primer-1 and 2, which generated a 146 bp fragment (Fig. 4b). The 5′-end of the primer-2 was at 39 bp downstream of the translation start site and the 5′-end of the primer-1 was at −107 bp, thus the TIS was located at about 110 bp upstream from the translation start site.

Verification of pLuc Plasmid Constructs

To analyze the function of the putative LMOR promoters, a series of 5′-deleted LMOR-luc plasmids were constructed with the 3′ends terminating at −2. All six constructs were verified by restriction digestion (data not shown). The digested fragments with expected size were observed after *HindIII*/*SacI* and *Bgl II*/*Pst I* digestion for pLuc-1, *HindIII*/*SacI* for pLuc-2, *HindIII*/*KpnI* for pLuc-3, *XhoI*/*PstI* for pLuc4 and 5, and *SacI*/*HindIII* for pLuc-6. The expected sizes of PCR products creating the construct pLuc-4 and pLuc-5 have been confirmed by sequencing (data not shown).

Transcriptional Activity of Putative LMOR Gene Promoter

Six LMOR-luc constructs were transiently transfected into CEM ×174 cells to analyze the function of LMOR promoters. The ratio of pRL-SV 40 promoter driven Renilla luciferase activity to the pLuc promoter driven firefly luciferase activity was used to show the activity of each pLuc construct. As shown in Figure 5, the fold changes of pLuc 2-6 and pGL3 basic compared to pLuc 1 were 0.6, 1.5, 1.6, 2.3, 0.3, and 0.2 respectively. Deletion of the 5′-region to -(pLuc-2) resulted in a decreased activity compared to −2279. The increased activities of promoters were significant in both regions of the deletion to −984 and −372 ($P < 0.01$). pLuc 5 (deletion to −372) exhibited the highest promoter activity. Further deletion to −252 (pLuc 6) caused a loss of promoter activity that was not different from the pGL3-basic. These results indicated that there were two enhancer elements within the regions from −2279 to −1372 and from −372 to −254 respectively. Similarly, two potential silencer elements might be located within the regions from −1371 to −985 and from −652 to −373.

DISCUSSION

The existence of mu opioid receptors in immune cells of several species has been repeatedly reported and has been shown to subserve effector functions of these cell types. In contrast to the well-defined mu opioid receptor in the brain, the expressional characteristics of human LMOR are unclear. The purpose of this study is to verify the morphine-induced expression of LMOR and to analyze the potential mechanism behind it. To achieve this purpose, the 5′-flanking region of the LMOR gene was cloned and analyzed in the CEM ×174 cells.

Although it is now doubtless, based on the pharmacological and immunological observation, that the mu opioid receptor exists and may also be identified in lymphocytes, the evidence at the molecular level is still lacking. It was once generally difficult to prove this in certain studies owing to the lower copies of mu transcripts in cells of the immune system [Salter et al., 1985; Gavériaux et al., 1995; Wick et al., 1996]. In one study, a high affinity morphine binding site was detected. However neither human MOR nor any mu related mRNA could be detected in human T-lymphocytes using any of primer sets. The failure to detect mu mRNA led to the presumption that the morphine-binding site on human activated T-cells was not related to the mu opioid receptor [Madden et al., 2001]. However, our experiment in this study confirmed that the expression of LMOR was induced by morphine in a dose-dependent manner with the optimal response time at 12 h. The blocking of naloxone, the specific antagonist, on the morphine-induced expression indicated the involvement of a classical opioid receptor in lymphocytes.

Primer extension analysis in this study revealed that only one transcription initiation site (TIS) was detected to be mapped at 110 bp upstream from the ATG translational start codon. The result has been repeatedly verified in our lab. In other studies, several potential TISs were reported using a rodent brain model and human neuroblastoma SK-N-SH cells. In mice, four TISs were observed 268, 276, 284, and

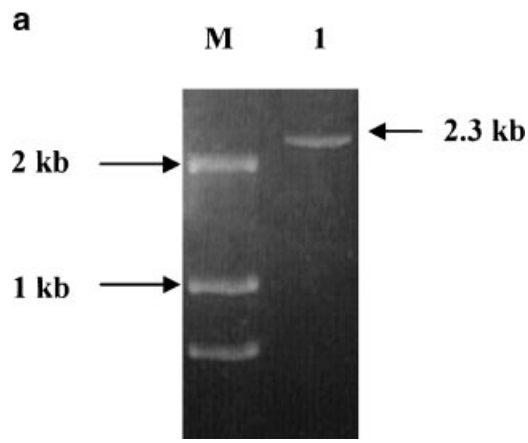

Fig. 2. Amplification of the 5′-flanking region of the LMOR gene by PCR assay. **a**: A fragment with size 2,298 bp was visualized by amplification from CEM ×174 cells. M: DNA marker; **lane 1**: PCR product. **b**:. Nucleotide sequence of the LMOR gene flanking the 5′ end of the coding region. The sequence presented was amplified using the gene-specific and adapter primers. Nucleotide +1 corresponds to the transcription start codon, the boldfaced ATG. The numbering of the LMOR gene sequence is relative to the translation start codon. Underlined sequences represent the nucleotides as primers used for primer extension and PCR. Additional nucleotides with restriction sites in the end of primers are shown above or below the sequence. The orientation of primers is indicated as horizontal arrows above the sequence. Restriction sites used for making deletion reporter constructs and PCR are indicated as the shaded nucleotides. The nucleotides with possible *cis*-acting transcription elements are also shaded. Arrow downwards at nucleotide position −110 indicates the approximate transcription site of the LMOR gene.

Fig. 2. (*Continued*)

291 bp upstream of the translation site [Min et al., 1994]. Other laboratories have reported that one proximal TIS was at −268 nucleotide from the translational initiation coden ATG (designated as +1) and the distal TIS was at −794 nucleotide from ATG [Liang et al., 1995]. In rats, a proximal TIS and a distal TIS were mapped 230 and 880 bp upstream of the ATG codon, respectively [Kraus et al., 1995]. Interestingly, the previous studies have identified the different number of TISs in the human genome. One of them, which used the entire human brain poly $(A)^+$ RNA, identified that four TISs were mapped at sites 216, 285, 358, and 373 bp upstream of the ATG codon by primer extension [Wendel and Hoehe, 1998]. The major

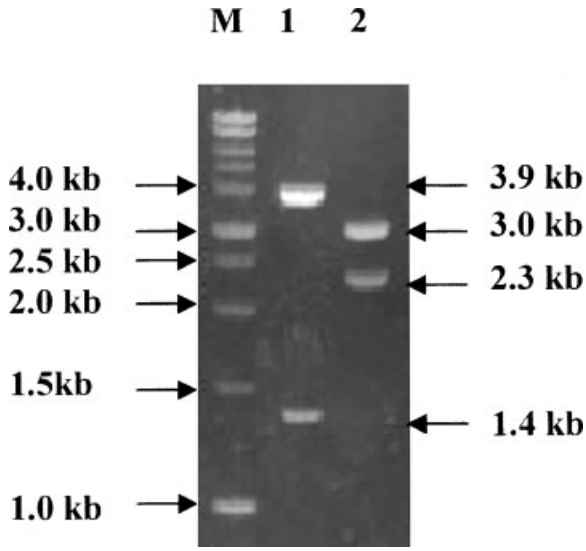

Fig. 3. Analysis of pGEM-2.3 digested by restriction enzymes. The PCR product from amplification of CEM ×174 cells was collected and cloned into pGEM-T vector to create the pGEM-2.3 which was verified by restricted digestion. M: DNA marker; **lane 1**: product digested with BglII/NcoI. **Lane 2**: product digested with EcoRI.

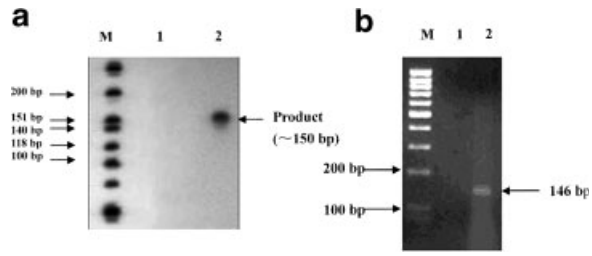

Fig. 4. Maping of the 5′-end of LMOR by primer extension. **a**: primer extension assay was carried out to identify the TIS of the LMOR gene. The primer extension product was generated using an antisense oligonucleotide as primer (Materials and Methods). M: DNA marker; **lane 1**: negative control (without reverse transcriptase); **lane 2**: primer extension product. The arrow indicated the 150 bp product. **b**: RT-PCR product validating the transcription start sites as infered from the primer extension products. These data were representative of three experiments.

initiation site was at −216. In another study, TISs were mapped at −313, −306, and −288 by 5′-RACE using the entire human brain poly (A)+ RNA [Andria and Simon, 1999]. In human neuroblastoma SK-N-SH cells, TISs were mapped at sites −252, −566, −586, −598, −663, and −827 [Xu and Carr, 2000]. Although it is not fully clarified at the level of molecular mechanisms why different cell types have different sequence features, such as various multiple TISs, the alternative promoter usage is considered as an important means to create more diversity in regulating developmental and tissue specific gene expression. Thus, it is likely that the quite dissimilar regulation of the expression of the mu opioid receptor exists in lymphocytes. Our results may partly account for the lower copies of mu transcripts that may contribute to the difficulty in detection of mu mRNA in lymphocytes.

To further clarify the possible mechanism for LMOR expression, we analyzed the promoter region of the gene encoding the LMOR. In the

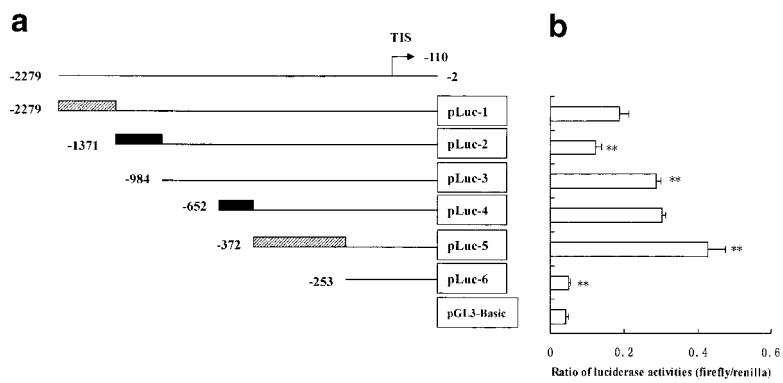

Fig. 5. LMOR promoter activity in transient transfection assays. **a**: A schematic diagram corresponds to the LMOR gene from nucleotide −2279 to −2. The TIS, −110, is indicated by an arrow. Various deletion promoter fragments were fused to the luciferase reporter gene. The promoterless luciferase reporter vector, pLuc-basic, served as the negative control. Black box represent potential enhancer region. Diagonal box represent potential silencer region. **b**: The activities of LMOR promoters. The promoter activity of each construct was standardized by cotransfection of the internal control plasmid, renilla luciferase, and was expressed as the ratio of pRL-SV 40 promoter driven renilla luciferase activity to the pLuc promoter driven firefly luciferase activity. The bars show the mean ± s of the result from three independent transfection experiments. $^{**}P < 0.01$ was considered statistically significant between construct pLuc-1 and pLuc-2; pLuc-2 and pLuc-3; pLuc-4 and pLuc-5; pLuc-5 and pLuc-6.

present experiment, a series of 5′ deleted LMOR promoter-luciferase constructs were made and transiently transfected into CEM ×174 cells. These results from LMOR-luc activities indicated that there were two enhancer elements (−2279 to −1372 and −372 to −254) and two potential silencer elements (−1371 to −985 and from −652 to 373). This finding is consistent with that in neuroblastoma cells [Xu and Carr, 2000]. In a murine mode, two promoters were identified, which demonstrated that the proximal promoter in directing MOR transcription during murine development play the pivotal role [Ko et al., 2002]. The *cis*-element in human neuroblastoma SH-SY5Y cells and murine cells were also analyzed in detail [Ko et al., 1998, 2003]. The further analysis of the sequence upstream of the ATG start codon revealed that the promoter region of LMOR lacked a classical TATA box and a CCAAT box at the expected region 25 to 30 bases upstream of TIS. The TATA-less promoters have been found in several genes including G-protein coupled receptors [Bogomolski-Yahalom et al., 1997; Gopalkrishnan et al., 1998; Huber et al., 1998; Janne and Hammond, 1998].

Taken together, we concluded that the expression of the mu opioid receptor gene is elaborately regulated in lymphocytes. The LMOR gene is controlled by constitutive promoters, the activity of which may be modulated by sequence-specific enhancer and/or silencer binding protein to produce restricted patterns. These findings in this study are not reported in other studies. Further efforts will focus on identifying the *cis*-elements involved in the promoter activity of LMOR. These experiments are currently under way in our laboratory. The primary results have confirmed the binding site of SP1 on the promoter located in the Luc-5 (data not shown). The precise elucidation of the expressional mechanism of the LMOR gene will help to better understand the immunological effect of morphine on lymphocytes.

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