0026-895X/97/030415-06$3.00/0

MOLECULAR PHARMACOLOGY, **52:**415–420 (1997).

Studies of Dual Promoters of Mouse κ-Opioid Receptor Gene

SHENGLI LU, HORACE H. LOH, and LI-NA WEI

Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

Received February 20, 1996; Accepted May 8, 1997

SUMMARY

Dual promoters were identified in the mouse κ-opioid receptor (KOR) gene. The distal promoter was located in the 5′-upstream region of exon 1 and the proximal promoter was located in the first intron of this gene. The transcription initiation site of the proximal promoter was mapped to the −93rd nucleotide position from the ATG codon in a primer extension experiment. The expression of KOR mRNAs transcribed from these two promoters in mouse central nervous system and an embryonal carcinoma cell line P19 was confirmed in a ribonuclease protection assay. In non-neuronal tissues, only the transcripts initiated from the distal promoter were detected. The biological activities of these two promoters were determined in transient transfection of P19 cells with a series of reporters, each truncated at various 5′-upstream regions. It was concluded that the distal promoter was located between nucleotide positions −990 and −570, and the proximal promoter was located between nucleotide positions −330 and −93, relative to the translation initiation codon. The presence of dual promoters in the KOR gene suggested potential regulation of KOR expression by using different promoters.

Several opioid receptor types are present in the central and peripheral nervous systems, distinguished by their preference for different ligands (for reviews, see Refs. 1 and 2). Autoradiographic and *in situ* hybridization studies indicate that these opioid receptors and their mRNAs can be found in brain and spinal cord areas that are involved in pain sensation (for reviews, see Ref. 3 and 4). In addition to the spatial specificity, the expression of these receptors also exhibits temporal specificity, being differentially expressed in various developmental stages. The κ receptor binding sites can be detected as early as gestation stage E14.5, followed by an increase during early postnatal life and a gradual decline to the adult level (5). To study how the expression of the κ-opioid receptor gene is regulated for its tissue and developmental specificity, we have previously isolated the mouse gene, designated as KOR, and determined its entire genomic structure (6).

The mouse KOR gene spans a distance of approximate 16 kb and contains 4 exons. Based upon the DNA sequence of its 5′-upstream region, this gene is characteristic of a housekeeping gene, containing multiple transcription initiation sites but no TATA box sequences in the 5′-upstream region. By comparing its 5′-upstream sequence with a TFDB, many potential regulatory DNA elements are located in this region. Interestingly, the translation of KOR mRNA starts within the second exon, and several clusters of potential regulatory elements are present in the first exon and the first intron.

This report follows our previous study by characterizing two promoters and their upstream regulatory region of the mouse KOR gene. Specifically, three questions are addressed: 1) Can the first intron function as a promoter, and if yes, where is the transcription initiation site of this promoter? 2) Are the KOR mRNAs transcribed from the two promoters differentially expressed in mouse tissues? 3) Where are the two KOR promoters and what are their biological activities?

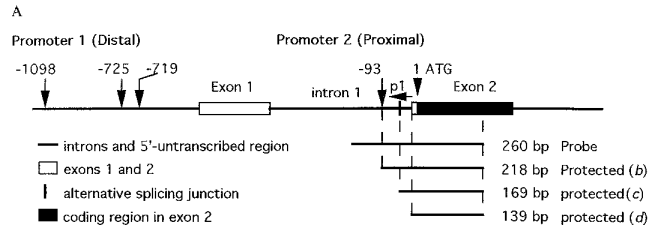
Experimental Procedures

Sequence of the 5′-regulatory region and promoter mapping. DNA sequences of the 5′-upstream region were determined from a subclone of the mouse KOR gene containing the 5′-upstream region using the dideoxy nucleotide chain termination method (7). The second (proximal) promoter was determined in a primer extension experiment using a specific primer (P1, 5′-CTGGAAAGCGAGAAGGTG-3′; see Fig. 1 for its position) complementary to a sequence in the intron. The procedure was as described previously (6).

Analyses of KOR mRNA expression. RNA was isolated from mouse tissues and cell lines as described previously (6). To detect different KOR mRNA species, RPA was modified from a procedure established previously (8), using an RPAII kit from Ambion (Austin, TX). To differentiate KOR transcripts that were overlapping in the coding region (beginning from exon 2), a DNA fragment of approximate 260 bp was selected from a genomic segment containing a portion of intron 1 and exon 2 as indicated in Fig. 1. This genomic

This study was supported by National Institutes of Health Grants DA01583, DA05695, K05-70554, and the F & A Stark Fund of Minnesota Medical Foundation (H.H.L.) and DK46866 and MMF-975-95 (L.N.W.)

ABBREVIATIONS: KOR, κ-opioid receptor; TFDB, transcription factor database; RPA, ribonuclease protection assay; CNS, central nervous system; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; RT, reverse transcription; bp, base pair; kb, kilobase pair.



B

gtccccgGCGCACCTTGCTGATCCCAAACAGGCAGAGCTTCTTCCAGTCTTGGAAGGCACAAA

TTGAGCATCAGGAACGTGGACCCATCAGGGCTGAACAGCTACTCAGGATCTAAAGTGGTGAC

TTGGAAAGCTGACGGTGACTTGGGAAGGGAGGTCGCCAATCAGCGATCTGGAGgtgagcttggg

gctttcgaggcacagtggggaaattgctgtggctagtgcttcttggggttgcatgcacagacaggcagggtagagg

cagccacaggagtggacagcacaacgcccgagggtgaagcaacctcagggctccaaaaaggtgcaaatactctc

agtttaatagctgcagtggaggtaggggtaggggtgggaaggggatttcaacctgtctgctccttccttgggatg

ggcggccagactg agtcctgggg*(b, -93)ttgcgggctcctgcgcctccagccctgcctgtacaggcaaagtttgtctctct

gggttcaatctgttctctgctcctgcag*(c)ccccgcaagtgccaccttctcgctttccagCTGCAGCGCTCACCA*(d) 1

TGGAGTCCCCCATTCAGATCTTCCGAGGAGATCCAGGCCCTACCTGCTCTCCCAGTGCTTGCC

TTCTCCCCAACAGCTCTTGGTTCCCCAACTGGGCAGAATCCGACAGTAATGGCAGTGTGGGC

Fig. 1. A, The relative positions of dual promoters of the mouse KOR gene, the primer used in primer extension and the probe used in RPA. The first (*distal*) promoter is located at the 5′-upstream region of exon 1 and the second (*proximal*) promoter is located within intron 1. Nucleotide positions are numbered above the map, with respect to the translation initiation codon ATG. The primer used in the primer extension experiment for mapping promoter 2 is labeled as *p1*. Under the map, the position of the probe used in the RPA as well as the sizes of protected fragments are indicated. The fragment *b* (218 bp) represents the protected transcripts initiated from promoter 2, fragment *c* (169 bp) represents protected transcripts initiated from promoter 1 and containing 30 additional bases as a result of alternative splicing, and fragment *d* (139 bp) represents the protected transcripts initiated from promoter 1 and properly spliced. B, *Underlined,* the riboprobe DNA sequence in the genomic sequence spanning exon 1, intron 1, and exon 2 (6). *Capital letters*, exon sequences. The A residue of the initiation codon is numbered as 1. *, First base in each protected fragment.

segment was inserted into pSp73 (Promega, Madison, WI) for *in vitro* transcription into riboprobes with T7 RNA polymerase. An actin cDNA vector was provided in the kit for the synthesis of antisense riboprobes using SP6 RNA polymerase. Probes were labeled with [α-^{32}P]UTP and the full-length probes were further purified from a polyacrylamide gel. For each RPA reaction, 30 μg of total RNA were incubated with 2×10^5 cpm probes at 45° overnight. After hybridization, a mixture of RNase A and RNase T1 was added and incubated at 37° for 30 min. The digested RNA was denatured in boiling water and immediately separated in a denaturing 5% polyacrylamide gel. A DNA marker was prepared by labeling *Hpa*II-digested pBR322 DNA with [α-^{32}P]dCTP.

Reporters for KOR promoter activities. A reporter for the KOR promoter activity was constructed by inserting the *Escherichia coli* β-galactosidase (lacZ) structure gene (pMC 1871; Pharmacia, Piscataway, NJ) into a KOR genomic clone, at the 14th bp position after the translation initiation codon ATG. This construct generated an in-frame fusion gene that utilized the ATG codon of KOR and included four additional amino-terminal amino acid residues of the mouse KOR protein at the amino terminus of the lacZ protein. This construct, designated as KOR-lacZ (− 4590 construct), was used to generate a series of deletion mutants truncated from the 5′ end of the KOR gene by digesting with appropriate restriction enzymes or ligating the PCR-generated genomic fragments with the lacZ cassette. The −1320 construct was generated by *Bam*HI deletion of the parental construct and the −990 construct was generated by *Bgl*II deletion of the parental construct. The −570, −330, −280, and −170 constructs were all made by ligating PCR-generated genomic fragments into the lacZ cassette. The −570/del-Pr2 was made by ligating the amino terminus of KOR cDNA (beginning at the −570 position) to the lacZ cassette, thus deleting the entire intron 1.

Cell culture techniques and the determination of reporter activities in cell lines. P19 cells were maintained in α minimal essential medium supplemented with 2.5% fetal calf serum and 7.5% calf serum as described previously (9). CHO cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as described elsewhere (10). The calcium phosphate precipitation method (9) was used for transfections. Cells were transfected with each reporter DNA at equal molecular ratio, and cytosolic extracts were collected at 24 hr after transfection to determine lacZ activities. The lacZ activities were determined using a solution assay as described previously (9) and represented as $A_{420}/10^5$ cells. As an internal control, a ptK-luc vector was included in each transfection experiment. This luciferase reporter has been shown to be expressed at a constant basal level in P19 cells (9). Luciferase activities were determined using a luciferase assay kit from Promega (Madison, WI) and represented as arbitrary luciferase units. The specific lacZ activity was represented as A_{420}/arbitrary luciferase unit for each reporter.

Results

Dual promoters of the mouse KOR gene. In our previous study (6), we identified three major transcription initiation sites of the mouse KOR gene, approximately 200–500 bp upstream from the 5′ end of the mouse KOR cDNA. We later detected a different KOR mRNA species, in a RT-PCR, which was transcribed from the first intron of this gene. To validate this novel species of KOR transcript, we conducted primer extension experiments using a primer (P1) complementary to the first intron to examine RNA isolated from both mouse brain and a mouse cell line, P19, which expressed the KOR gene. The expression of this novel transcript in mouse tissues was further confirmed in RPA (see the following). Fig. 1A showed the KOR genomic region where the relative position of the P1 primer used in primer extension, as well as the probe and the protected fragments in RPA experiments, was indicated. The sizes of the probes and the protected fragments in RPA were indicated on the right. Fig. 1B shows the DNA sequence spanning exon 1, intron 1, and exon 2, from which the riboprobe (Fig. 1B, *underlined*) was prepared. The first base in each protected fragment was indicated with an asterisk (*).

The result of a primer extension experiment is shown in Fig. 2. By using the intron-specific primer, P1, a major band was detected in the lanes of RNA samples isolated from mouse brain (Fig. 2, *lane Br*) and P19 (Fig. 2, *lane P*). By aligning this extended band to a sequencing ladder, the initiation site was mapped to the A residue at the −93rd nucleotide upstream from the translation initiation codon ATG. For a negative control, yeast tRNA (Fig. 2, *lane Y*) was included in the experiment. Based upon this result and the results of our previous study (6), it was concluded that the mouse KOR gene utilized two promoters, one located in the 5′ upstream of the first exon and the other located within the first intron.

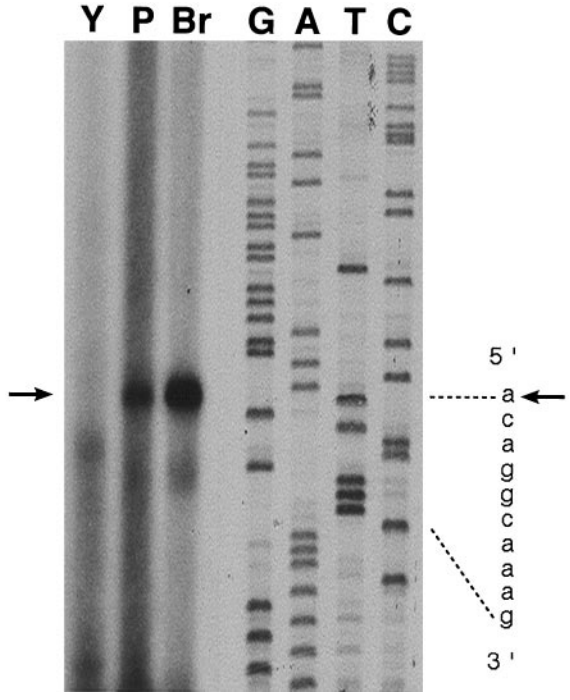


Fig. 2. Determination of the transcription initiation site from the proximal promoter. A primer extension experiment was conducted using primer 1 (see Fig. 1) to anneal the yeast tRNA (*Y*), or 150 μg each of brain (*Br*) and P19 (*P*) total RNA as described previously (6). A DNA sequence ladder of a KOR genomic clone using the same primer is run on the same gel for alignment. The initiation site is aligned to the *A* residue; *arrows,* −93rd nucleotide position relative to the translation initiation codon ATG.

Differential expression of two types of KOR mRNAs in mouse tissues and P19 cells. The size of KOR message expressed in mouse brain was estimated to be approximately 6 kb based upon Northern blot analysis in our previous study (6). Later, we also detected the expression of KOR mRNA in Northern blots, approximately 6 kb in length, in a mouse embryonal carcinoma cell line P19 (data not shown). Because the sizes of KOR mRNAs transcribed from these two promoters were too close to be distinguished in a typical Northern blot analysis, we established an RPA that allowed different types of KOR transcripts to be differentiated in the same reaction. By using riboprobes prepared from a genomic DNA segment as indicated in Fig. 1, three types of KOR transcripts were detected in this type of RPA. The first type was transcribed from the distal promoter and spliced at the 14th nucleotide upstream from the initiating ATG codon of exon 2 (transcript 1), the second type was transcribed from the same distal promoter but spliced at the 30th nucleotide upstream from the first splicing junction (transcript 2) and the third type was transcribed from the proximal promoter located in intron 1 (transcript 3). The band protected by transcript 1 was 139 (fragment *d*) bp in length because only the sequence transcribed from exon 2 could be protected. The band protected by transcript 2 was 169 bp (fragment *c*) in length because 30 additional bases were protected. The band protected by transcript 3 was 218 bp (fragment *b*) in length, covering 79 bp from intron 1 and 139 bp from exon 2. For an internal control, actin probes were hybridized with each sample and a band of approximate 250 bp (fragment *a*) was expected. Fig. 3 shows a typical result of differential KOR mRNA expression detected in the RPA. Three major protected bands of approximately 218, 169, and 139 bp in size (Fig. 3, *b, c,* and *d,* respectively) were detected in P19, brain, and spinal cord (Fig. 3, *lanes 6, 7,* and *8,* respectively) samples. A relatively constant level of actin expression (fragment *a*) was detected in all the samples (Fig. 3, *lanes 12–17*). Using a PhosphorImager to quantify the densities of these bands,

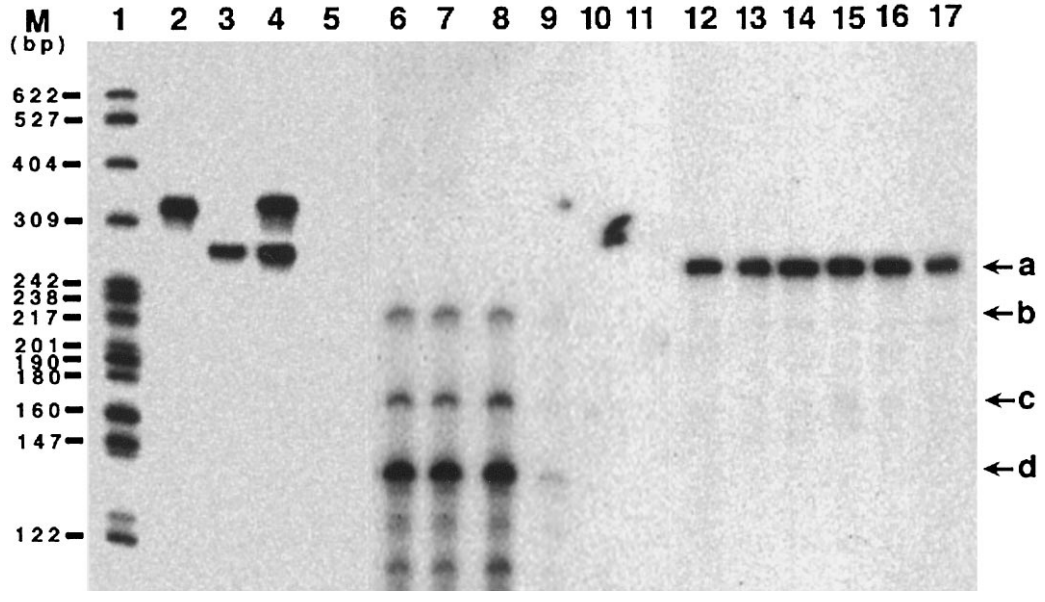


Fig. 3. Detection of different KOR mRNAs in P19 and mouse tissues by RPA. Thirty micrograms of the RNA sample were used in each reaction. KOR probes were used in *lanes 6–11* and actin probes were used in *lanes 12–17*. Samples in each lane were: DNA size marker (*lane 1*); actin probe (lane 2), KOR probe (*lane* 3); yeast tRNA without RNase digestion (*lane 4*); yeast tRNA after RNase digestion (*lane 5*); P19 (*lanes 6* and *12*); brain (*lanes 7* and *13*); spinal cord (*lanes 8* and *14*); stomach (*lanes 9* and *15*); spleen (*lanes 10* and *16*); and kidney (*lanes 11* and *17*). Band *a,* protected actin messages (250 bp); band *b,* protected KOR messages initiated from promoter 2 (the proximal promoter) (218 bp); band *c,* protected KOR messages initiated from promoter 1 (the distal promoter) and alternatively spliced (169 bp); band *d,* protected KOR messages initiated from promoter 1 and properly spliced (139 bp).

the ratio of b/c/d was estimated to be approximately 1/2/10 for these three samples. Thus, it was concluded that the distal promoter was much stronger than the proximal promoter because the transcripts initiated from the distal promoter, represented by the 139- and 169-bp bands, were approximately 12-fold more abundant than the transcripts initiated from the proximal promoter represented by the 218-bp band.

Among other mouse tissues tested, only the stomach sample showed the 139-bp band (*lane 9*), indicating KOR mRNA expression from the distal promoter (promoter 1) in the stomach. Thus, it was concluded that the mouse KOR gene can be transcribed into at least three types of transcripts, two initiated from the distal promoter and one initiated from the proximal promoter. In addition, the KOR gene was expressed primarily in the CNS, including the brain and the spinal cord. In P19 cells, the three types of KOR transcripts were also expressed at a ratio similar to that detected in the CNS.

The determination of dual KOR promoter activities. To determine the biological activities of the two promoters, the series of lacZ reporters were transiently co-transfected into P19 and CHO cells with an internal control luciferase vector. The lacZ activity of each reporter was normalized to the internal control for transfection efficiency, and represented as the specific lacZ activity as described in Experimental Procedures. The amount of DNA used in each transfection was carefully determined based upon the molecular size of each construct, so that each relative reporter activity was presented as the specific activity from an equal number of molecules for all the constructs. The results were obtained from four independent experiments, each conducted with duplicate cultures, to determine the means and standard errors of the means (mean ± standard error).

As shown in Fig. 4B, the shortest reporter that still retained the proximal promoter activity was the −330 construct, whereas deletion for another 50 bp (−280 construct) or 160 bp (−170 construct) resulted in the complete loss of reporter activity in P19. Addition of 250 bp more (exon 1 and a portion of intron 1) to the −330 construct generated the −570 construct, which contained the entire KOR cDNA sequence. Addition of the first exon sequence alone (−570) appeared to have no effects on this proximal promoter, whereas a further extension to the −990 position, which covered two of the three transcription initiation sites of the distal promoter, stimulated the reporter activity by approximately 50%. In the construct containing additional 330-bp sequence (the −1320 construct), the reporter activity increased for another 50%, which is approximately 2-fold the proximal promoter activity. Interestingly, the reporter activity decreased to about the basal proximal promoter (the −320 construct) activity in the longest construct, which contained approximately 4.6 kb of the 5′-upstream region (the −4590 construct). In addition, deletion of the proximal promoter from the −570 construct, the −570/del.Pr2 construct, abolished all the reporter activity, confirming the biological activity of promoter 2 encoded in this intron. All of these reporters were also transfected into CHO cells, and none of these reporters expressed lacZ activity, indicating that the lacZ activities detected in P19 cells were specific to the KOR gene. From the reporter activities of these deletion mutants, it was concluded that the proximal promoter of the KOR gene was located between nucleotide positions −93 and −330, with the sequence between −280 and −330 positions encoding the essential biological activity for this promoter. The distal promoter was located between nucleotide positions −570 and −990. The sequence between positions −990 and −1320 encoded some stimulatory activity whereas the sequence 5′ to the −1320 position encoded a slightly suppressive activity.

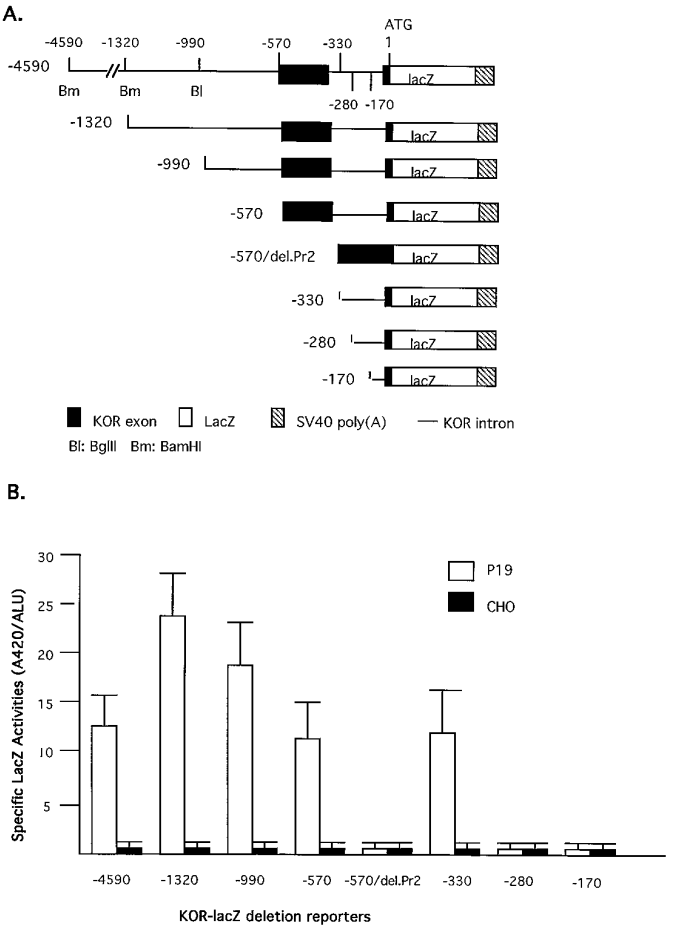


Fig. 4. KOR-lacZ reporters and their biological activities. A, The first reporter (−4590) was made by inserting the lacZ structural gene, in frame, into the 14th nucleotide position of a KOR genomic clone. All the deletions were made by using either restriction enzyme fragments (such as the *Bam*HI and *Bgl*II deletions) or PCR-generated fragments (such as −570, −570/del.Pr2, −330, −280, and −170). B, Each reporter was co-transfected with the internal control, ptK-luc, into P19 cells as described elsewhere (8). LacZ and luciferase activities were determined 24 hr after transfection. The reporter activities, represented as specific lacZ activity, were determined for an equal number of molecules for each construct. Four independent experiments, each conducted with duplicate cultures, were carried out to obtain the means ± standard error.

Discussion

We isolated several overlapping genomic clones covering the entire mouse KOR gene, and identified a major promoter located in the 5′-upstream region of exon 1 in a previous study (6). In this study, KOR mRNA initiated from a promoter located in intron 1 was first detected in RT-PCR experiments (data not shown) and the transcription initiation of this new RNA species was located, in a primer extension experiment, to the −93rd nucleotide position with respect to the ATG codon. The expression of transcripts initiated from

the two promoters was further confirmed in RPA experiments. P19 and all the major mouse organs were examined to determine differential expression of these KOR transcripts. It appeared that KOR transcripts initiated from the distal promoter could be spliced at two different positions 30 bp apart, generating two types of mRNAs (represented by protected band *c* and *d* in RPA). KOR transcription could also initiate from the proximal promoter, resulting in the mRNA species represented by the protected band *b* in the RPA. All three types of mRNAs were detected in P19, mouse brain, and spinal cord, but only the transcript represented by band *d* was detected in non-CNS tissues such as the stomach. In addition, mRNAs represented by band *d* appeared to constitute the major KOR mRNAs in either mouse CNS tissues or the P19 cell line. Thus, it was concluded that promoter 1 (the distal promoter) was the primary promoter for KOR expression in both CNS and non-CNS tissues. In contrast, promoter 2 (the proximal promoter) encoded a much weaker activity and appeared to be active only in the CNS. As expected, KOR expression, from either the proximal or the distal promoter, was strongest in the CNS.

Based upon the signals from PhosphorImager analyses of the protected fragments, the ratio of the mRNAs initiated from the distal promoter (represented by bands *c* and *d*) to the mRNAs initiated from the proximal promoter (represented by band *b*) was estimated to be approximately 12 to 1 in P19 and mouse CNS. By studying a lacZ reporter gene and its deletion mutants in P19 cells, the biological activities of these two promoters were confirmed and the positions of these two promoters were located. However, the distal promoter was only approximately 2-fold stronger than the proximal promoter based upon this type of reporter analysis. It is possible that some regulatory information for the distal promoter may be missing in the constructs. Alternatively, the dissected proximal promoter activity as detected by transient transfection experiments could be stronger than its physiological activity as a result of deleting some regulatory sequences from the genomic locus or the accumulation of transient reporter activity in transfected cells. Finally, it is also possible that mRNA stability varies among the different types of KOR transcripts. The reporter activities may not reflect mRNA stability, which could vary among the three types of transcripts.

The activity of two promoters used by the KOR gene was confirmed, based upon the expression of endogenous KOR mRNA from these two promoters and the reporter assays. Addition of exon 1 sequence to the proximal promoter had no effects on the proximal promoter, suggesting that no regulatory information was encoded within exon 1 sequence (the −570 construct) in the P19 system. Deletion of promoter 2 from the −570 construct abolished its promoter activity, confirming the activity of promoter 2 located inside the intron. Addition of the sequence containing two of the three transcription initiation sites of the distal promoter increased the reporter activity (the −990 construct), confirming the distal promoter (promoter 1) activity encoded between nucleotide positions −570 and −990. Addition of another 330 bp (the −1320 construct, containing another transcription initiation site of the distal promoter) increased the reporter activity to the highest level, which was approximately 2-fold of the proximal promoter activity (the −320 construct), based upon the transient transfection experiments. Interestingly, the addition of the entire 4.6 kb of the 5′-upstream region suppressed the reporter activity to about the basal proximal promoter activity. Thus, the sequence 5′ to the −1320 position probably contained some negative regulatory activity for this gene. However, it remains to be determined if this slightly suppressive activity is transferable and if more regulatory sequences are present in the further upstream sequence.

By comparing this series of reporter activities, it was concluded that the distal and the proximal promoters were located between nucleotide positions −570 and −990 and between positions −93 and −330, respectively. The differential use of dual promoters and the presence of an untranslated exon in the 5′ region of the coding sequence in KOR gene raised an interesting issue about its regulation. Three major KOR mRNA isoforms were detected, including the major RNA species as shown in the first cloned cDNA (11, 12) that spans the 4 exons, an isoform containing a 30-nucleotide insert derived from intron 1 as detected in mouse R1.1 cells (13) and P19 (this study), and another isoform transcribed from a promoter located in intron 1 (this study). Based upon the genomic sequence, it was shown that a rat KOR isoform (14) could be transcribed from intron 1 sequence, starting at a region further upstream from the proximal promoter identified in this study. However, using RT-PCR, we could not detect additional mouse KOR transcripts initiated from the region corresponding to this particular rat initiation site (data not shown).

KOR expression could be detected not only in nervous system, but also in some immune cell types by more sensitive assays such as RT-PCR (15, 16). We detected KOR expression in the stomach in RPA, suggesting that KOR neurons were present in this organ in relatively high levels. By RT-PCR analysis, we also detected some KOR expression in other organs such as the heart (data not shown). KOR expression in these non-CNS tissues was mostly from the distal promoter, indicating that the proximal promoter was probably CNS-specific. Utilization of alternative promoters to generate mRNA isoforms that can be differentially regulated has been widely observed in the nuclear hormone receptor gene families (17). It has also been observed in several membrane receptor genes more recently, such as the human D_{1A} dopamine receptor gene (18) and the human A_1 adenosine receptor gene (19). This study has demonstrated, for the first time, the specific sequence requirement and the biological activities of the two KOR promoters in mouse tissues and cell lines. However, it remains to be answered as to the physiological significance of the use of alternative promoters for the KOR gene and differential regulation of these two KOR promoters.

Within the 5′-untranscribed region of the distal promoter, no typical TATA or CAAT boxes were present, but multiple initiation sites of transcription were identified (6). In addition, many putative transcription factor binding sites were found in this sequence as compared with the TFDB, such as NF-μE1, GATA, NF-IL6, granulocyte/macrophage-colony stimulating factor, glucocorticoid response element, and SP-1. Interestingly, the proximal promoter used a single transcription initiation site, although it contained no TATA box. By comparing with the TFDB, several putative transcription factor binding sites were also found in the upstream region of the proximal promoter, such as heat shock factor and a zinc finger-type transcription factor Adr1. However,

the authenticity of these regulatory elements and their biological functions await further studies. This study unambiguously demonstrated different expression levels of the KOR mRNAs transcribed from two promoters in mouse tissues and P19 cell line and the biological activities of the two KOR promoters. These findings raised interesting questions about KOR gene expression with respect to alternate promoter usage and differential regulation in various tissues.

Acknowledgments

We thank Dr. L. Chang for help in enzyme assays and P19 cultures. We thank the core B of a program project DA08131 for providing the oligonucleotides.

References

1. Goldstein, A., and A. Naidu. Multiple opioid receptors: ligand selectivity profiles and binding signatures. *Mol. Pharmacol.* **36:**265–272 (1989).
2. Loh, H. H., and A. P. Smith. Molecular characterization of opioid receptors. *Annu. Rev. Pharmacol. Toxicol.* **30:**123–147 (1990).
3. Mansour, A., C. A. Fox, H. Akil, and S. J. Watson. Opioid receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci.* **18:**22–29 (1995).
4. Elde, R., U. Arvidsson, M. Riedl, L. Vulchanova, J. H. Lee, R. Dado, A. Nakano, S. Chakrabarti, X. Zhang, and H. H. Loh. Distribution of neuropeptide receptors. New views of peptidergic neurotransmission made possible by antibodies to opioid receptors. *Ann. N. Y. Acad. Sci.* **757:**390–404 (1995).
5. Kitchen, I., M. Kelly, and P. M. Viveros. Ontogenesis of kappa opioid receptors in rat brain using ^{3}H-U69593 as a binding ligand. *Eur. J. Pharmacol.* **175:**93–96 (1990).
6. Liu, H.-C., S. Lu, L. B. Augustin, R. F. Felsheim, H.-C. Chen, H. H. Loh, and L.-N. Wei. Cloning and promoter mapping of mouse κ opioid receptor gene. *Biochem. Biophys. Res. Commun.* **209:**639–647 (1995).
7. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:**5463–5467 (1977).
8. Wei, L.-N, W. S. Blaner, D. S. Goodman, and M. C. Nguyen-Huu. Regulation of the cellular retinoid-binding proteins and their messenger ribonucleic acids during P19 embryonal carcinoma cell differentiation induced by retinoic acid. *Mol. Endocrinol.* **3:**454–463 (1989).
9. Wei, L.-N., and L. Chang. Promoter and upstream regulatory activities of the mouse cellular retinoic acid binding protein-I gene. *J. Biol. Chem.* **271:**5073–5078 (1996).
10. Maggio, R. Sodium nitroprusside induces internalization of muscarinic receptors stably expressed in Chinese hamster ovary cell lines. *J. Neurochem.* **65:**943–946 (1995).
11. Chen, Y., A. Mestek, J. Liu, and L. Yu. Molecular cloning of a rat κ opioid receptor reveals sequence similarities to the μ and δ opioid receptors. *Biochem. J.* **295:**625–628 (1993).
12. Li, S., J. Zhu, C. Chen, Y. W. Chen, J. K. Deriel, B. Ashby, and L. Y. Liu-Chen. Molecular cloning and expression of a rat κ opioid receptor. *Biochem. J.* **295:**629–633 (1993).
13. Belkowski, S. M., J. Zhu, L. Y. Liu-Chen, T. K. Eisenstein, M. W. Adler, and T. J. Rogers. Sequence of κ opioid receptor cDNA in the R1.1 thymoma cell line. *J. Neuroimmunol.* **62:**113–117 (1995).
14. Yakovlev, A. G., K. Krueger, and A. I. S. Faden. Structure and expression of a rat κ opioid receptor gene. *J. Biol. Chem.* **270:**6421–6424 (1995).
15. Lawrence, D. M. P., and J. M. Bidlack. Kappa opioid binding sites on the R1.1 murine lymphoma cell lines: sensitivity to cations and guanine nucleotides. *J. Neuroimmunol.* **41:**223–230 (1992).
16. Belkowski, S. M., J. Zhu, L. Y. Liu-Chen, T. K. Eisenstein, M. W. Adler, and T. J. Rogers. Detection of κ opioid receptor mRNA in immature T cells. *Adv. Exp. Med. Biol.* **373:**11–16 (1995).
17. Mangelsdorf, D. J., K. Umesono, and R. M. Evans. The retinoid receptors, in *The Retinoids* (M. B. Sporn, A. B. Roberts, and G. S. Goodman, eds.). Raven Press, New York, 319–350 (1993).
18. Lee, S.-H., M. T. Minowa, and M. M. Mouradian. Two distinct promoters drive transcription of the human D_{1A} dopamine receptor gene. *J. Biol. Chem.* **271:**25292–25299 (1996).
19. Ren, H., and G. L. Stiles. Separate promoters in the human A_1 adenosine receptor gene direct the synthesis of distinct messenger RNAs that regulate receptor abundance. *Mol. Pharmacol.* **48:**975–980 (1995).

Send reprint requests to: Dr. Li-Na Wei, Department of Pharmacology, University of Minnesota Medical School, 3-249 Millard Hall, 435 Delaware Street S.E., Minneapolis, MN 55455. E-mail: weixx009@maroon.tc.umn.edu