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Differential Expression of μ-Opioid Receptor Gene in CXBK and B6 Mice by Sp1

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ABSTRACT

It is well known that there are individual differences in the sensitivity to analgesics. The CXBK mice are characterized by reduced sensitivity to morphine and by partial deficiency in μ-opioid receptor (MOR) expression. The sequences of MOR genes in CXBK and B6 mice are identical in their coding regions but differ at 5′-untranslated region (UTR) nucleotide −202 (C nucleotide in CXBK, but A nucleotide in B6). In this report, we identified an Sp1 element (−211 to −204) immediately before the polymorphic nucleotide. In electrophoretic mobility shift assay, nuclear protein binding to the B6-Sp1 sequence was more efficient than to the CXBK-Sp1 sequence, and anti-Sp1 but not anti-CREB antibody interfered with the formation of the DNA-protein complex. In MOR-expressing cell lines SH-SY5Y, P19, and PC12, B6 MOR promoter possessed high transcription activity than the CXBK promoter, and Sp1 inhibitor PDTC reduced the promoter activities. In SL2 cells that lack endogenous Sp1 expression, B6 and CXBK MOR promoters demonstrated equal activity, whereas overexpression of Sp1 in SL2 cells enhanced B6 MOR promoter activity better than the CXBK promoter. Together, the A-to-C change at MOR 5′-UTR decreases Sp1 binding and MOR gene transcription, which could underlie the reduced morphine expression in CXBK mice.

Opioids relieve pain without affecting sensory modalities such as vision and hearing, making these drugs the preferred clinical analgesics. Three major types of opioid receptors, μ, δ, and κ, have been cloned and are part of the G-protein-coupled receptor superfamily (Kieffer, 1995). Previous pharmacological and clinical studies indicated that the μ-opioid receptor (MOR) was the main target of most of the clinically used analgesics, including morphine (Calo et al., 1998).

MOR is mainly expressed in the central nervous system, with varying densities in different regions of the brain where they may play different roles (Delfs et al., 1994; Mansour et al., 1995; Minami and Satoh, 1995). For example, MOR in the periaqueductal gray has been suggested to mediate analgesia (Monteillet-Agius et al., 1998; Commons et al., 1999), whereas MOR in the locus coeruleus and ventral tegmental areas may be involved in the development of tolerance and physical dependence (Nestler, 1996). It is likely that MOR expression is regulated by fluctuating levels of various agents in specific regions of the brain (Delfs et al., 1994; Azaryan et al., 1996; Ronnekleiv et al., 1996), and these regulation mechanisms could be implicated in diseases (Ronnekleiv et al., 1996). This raises the possibility to improve the efficacy of morphine and other opioids by manipulating MOR expression.

Individual difference in sensitivity to analgesics has been attributed to levels of MOR expression (Ikeda et al., 2001). Mice lacking the *mor* gene (MOR-KO) are insensitive to morphine (Matthes et al., 1996; Sora et al., 1997; Tian et al., 1997; Loh et al., 1998) and less sensitive to δ- and κ-opioid agonists despite normal expressions of δ- and κ-opioid receptors in the brain (Matthes et al., 1996; Kitchen et al., 1997; Sora et al., 1997). Heterozygous MOR-KO mice also show a reduced sensitivity to morphine (Sora et al., 1997; Loh et al., 1998). These studies suggest that MOR expression level may affect the sensitivity to opioid analgesics.

CXBK mice, a cross between C57BL/6By and BALB/cBy mice (Bailey, 1971), show reduced sensitivity to morphine and κ-agonist U-50488 (Ikeda et al., 1999). Previous studies suggested that CXBK mice had a partial deficiency of MOR (Ikeda et al., 1999), and the level of MOR mRNA in the brain of CXBK mice was reduced to 60% of B6 or BALB/c mice. The sequence of the coding region (1197 bases) of MOR mRNA of CXBK mice was identical to that of B6 mice. Within the 726 bases of 3′-untranslated region (UTR) and 241 bases of 5′-

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ABBREVIATIONS: MOR, μ-opioid receptor; EMSA, electrophoresis mobility shift assay; nt, nucleotide; kb, kilobase(s); Ab, antibody; CRE, cAMP response element; CREB, CRE-binding protein; MAZ, Myc-associated zinc finger protein; PCR, polymerase chain reaction; PEMT, phenylethanolamine *N*-methyltransferase; bp, base pair(s); UTR, untranslated region; PDTC, pyrrolidine dithiocarbamate; U-50488, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methane-sulfonate.

UTR of the MOR gene, a C/A polymorphism at nt −202 of 5′-UTR was the only difference between CXBK (C nucleotide) and B6 (A nucleotide) mice (Ikeda et al., 2001).

We have shown that the 5′-UTR is essential for MOR gene expression (Lee and Lee, 2003). In this study, we characterized the molecular mechanism underlying the difference between CXBK and B6 MOR gene expression. The A to C change at nt −202 lowered the affinity of an Sp1 element to Sp1 and then decreased the expression MOR gene.

Materials and Methods

Plasmid Construction. Mouse MOR gene has been isolated (Min et al., 1994), and its promoter was defined in a cell line expressing endogenous MOR (Ko et al., 1997). pCXBK-4.7/ATG-Luc containing a 4.7-kb MOR promoter from BALB/c was described in detail elsewhere (Lee and Lee, 2003). The nucleotide −202 is C in both BALB/c and CXBK and is A in B6 (Min et al., 1994). B6-4.7/ATG-Luc was constructed from pCXBK-4.7/ATG-Luc by replacing the 0.3-kb BamHI/NcoI 5′-UTR fragment with the B6 sequence obtained by PCR with primers mMORF-527D: 5′-GGGGTACCATGAAACAG-GCTTCTTT and mMOR-ATG: 5′- GGAATTCGCCGGTGCTGCT-GTCCAT. The B6 5′-UTR sequence was confirmed by sequencing.

Cell Culture. SH-SY5Y, PC12, P19, and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO_2 and 95% air at 37°C. PC12 cells were a generous gift from Dr. Yijuang Chern (Institute of BioMed Science, Academia Sinica, Taiwan) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal calf serum. *Drosophila melanogaster* Schneider cells SL2 were kindly provided by Dr. Z. L. Zhuang (National Health Research Institute, Taiwan) and were grown in S2 insect medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 25°C.

Transient Transfection and Reporter Gene Activity Assay. SH-SY5Y, PC12, 293T, SL2, and P19 cells were transfected using the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instruction. In brief, cells with approximately 40% confluence were transfected with an equimolar amount of each plasmid. The amount of DNA used was justified to allow a linear expression of the luciferase activity. Forty hours after transfection, cells were washed and lysed in buffer (0.25 M Tris-HCl, pH 7.6). Luciferase activity was determined according to the manufacturer's instruction (Promega, Madison, WI). To control for transfection efficiency, a 1:5 M ratio of pCH110 plasmid (Amersham Biosciences, Inc., Piscataway, NJ) containing a β-galactosidase gene driven by the SV40 promoter was included in each transfection. β-Galactosidase activities were used to normalize the data. Pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma-Aldrich (St. Louis, MO).

Nuclear Extract Preparation. Nuclear extracts were prepared from SH-SY5Y, PC12, and SL2 cells (Schreiber et al., 1989). In brief, cells were grown to confluence, harvested, and washed with phosphate-buffered saline. The following steps were all performed at 4°C. Cells were first lysed in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). The lysate was centrifuged at 500*g* for 5 min to pellet the nuclei. After washing with sucrose buffer without Nonidet P-40, the nuclei were suspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) to extract the nuclear protein with incubation for 20 min on a rotary platform. The sample was microcentrifuged at 13,690*g*. Aliquots of the supernatant (nuclear extract) were stored at −80°C.

Electrophoretic Mobility Shift Assay. EMSA was performed with ^{32}P-labeled double-stranded oligonucleotides that have been incubated with nuclear extract in EMSA buffer [10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml poly(dI-dC)]. For oligonucleotide competition analysis, a 40, 100, or indicated molar excess of unlabeled competitor oligonucleotides was added to the binding mixture. After incubation at 22°C for 20 min, the mixture was analyzed on 5% nondenaturing polyacrylamide gels. Oligonucleotide sequence for CRE in FMR1 gene was used as a control (Hwu et al., 1997). For gel supershift assays, 1 or 2 μl of anti-Sp1 and anti-CREB antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the mixture. The reaction was then incubated at room temperature for 15 min. The DNA-protein complexes and free probe were fractionated on 5% polyacrylamide gels in Tris-glycine buffer (50 mM Tris, pH 8.3, 380 mM glycine, and 2 mM EDTA) at room temperature and visualized by autoradiography.

Results

Differential Expression of CXBK and B6 MOR Promoter in SH-SY5Y Cells. To explore if the −202C/A polymorphism will alter the expression of MOR, we used an intact 4.7-kb MOR promoter reporter (CXBK-4.7/ATG-Luc). We performed mutagenesis to change the −202C nucleotide to A to obtain B6-4.7/ATG-Luc (Fig. 1A). Thus, this nucleotide is the only difference between these two constructs. In

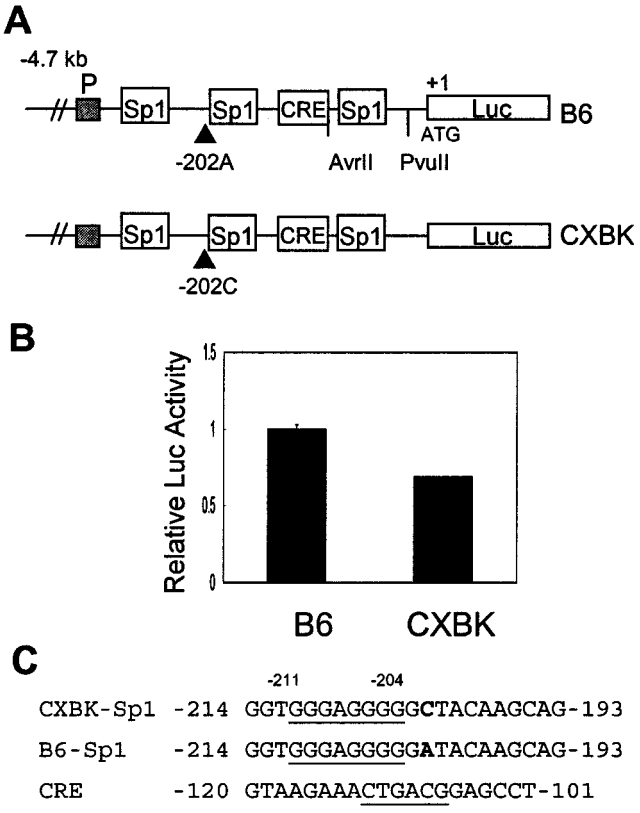

Fig. 1. Comparison of B6 and CXBK MOR promoter activity. A, a schematic diagram of the B6 and CXBK 4.7-kb MOR promoter constructs, with emphasis on the 5′-UTR. +1 represents the translational start site. There are three putative Sp1 binding elements and one CRE on the 5′-UTR. Box P denotes the MOR proximal promoter. The nt −202 polymorphism is also marked. B, SH-SY5Y cells transfected with MOR promoter reporter gene plasmids. Luciferase activities of CXBK-4.7/ATG-Luc were expressed against those of B6-4.7/ATG-Luc. The error bar indicates 1 S.D. derived from four independent experiments. β-Galactosidase activities, a control for transfection efficiency, were used to normalize the data. C, the sequences of oligonucleotides used in the electrophoretic mobility shift assay. The consensus binding sequences are underlined and the nt −202 polymorphisms are shown in bold.

SH-SY5Y cells that express endogenous MOR, luciferase activity of B6-4.7/ATG-Luc was higher than that of CXBK-4.7/ATG-Luc (Fig. 1B).

A search for transcription factor binding elements using the Signal Scan program (http://bimas.dcrt.nih.gov/molbio/signal/) revealed three Sp1 elements on the 5′-UTR (Fig. 1A). One Sp1 element (GGGAGGGG, nt −211 to −204) immediately flanks the C/A polymorphism at −202. To study the role of this Sp1 element, oligonucleotide probes corresponding to either B6 or CXBK Sp1 sequences were synthesized (Fig. 1C).

Decreased Sp1 Binding to the CXBK-Sp1 Element. To determine whether Sp1 bound to the −211 to −204 Sp1 element, we performed EMSA using PC12 nuclear extract and ^{32}P-labeled oligonucleotide probes of this Sp1 element, and a protein-DNA complex was seen (Fig. 2A). When we calculated the bound to free ratio of the oligonucleotide probe, the nucleic factor was shown to bind to the B6 sequence more efficiently than to the CXBK sequence (Fig. 2B). The protein-DNA complex involving CXBK-Sp1 was competed out by unlabeled B6-Sp1 oligonucleotides (Fig. 2C, lanes 8–14), but the B6-Sp1 complex was barely competed by unlabeled CXBK-Sp1 oligonucleotide (Fig. 2C, lanes 1–7), which also suggests a higher affinity of the nuclear factor to B6-Sp1. To further confirm Sp1 binding to the element, we preincubated anti-Sp1 or anti-CREB antibody with SH-SY5Y nuclear extract, and the observation that anti-Sp1 but not anti-CREB Ab interfered with the protein-DNA complex formation indicated that Sp1 is the nuclear factor responsible for DNA binding (Fig. 2D, lanes 1–3).

The *D. melanogaster* Schneider (SL2) cell is deficient in Sp1. In EMSA employing SL2 nuclear extracts and the Sp1 probe, no binding complex could be seen (Fig. 2D, lane 3). In comparison, a protein-DNA complex could be seen with nuclear extracts from SL2 cells transfected with an Sp1 expression vector (lane 5), and this protein-DNA complex could be supershifted by anti-Sp1 Ab (lane 6, arrowhead).

Sp1 Is Responsible for the Differential Expression of MOR. To explore whether Sp1 transactivation determines the difference in MOR gene expression between the B6 and CXBK mice, we expressed the two promoter reporter plasmids in cells with (PC12, P19, and 293T cells) or without (SL2 cells) endogenous Sp1. In these cells, PC12 and P19 express MOR (Chen et al., 1999). In cells containing Sp1, including the PC12, P19, and 293T cells, luciferase activity of B6-4.7/ATG-Luc was higher than that of CXBK-4.7/ATG-Luc; however, in SL2 cells, the luciferase activities of the two promoters were the same (Fig. 3).

We further used PDTC, an inhibitor reducing Sp1 binding to the *cis*-element (Lee et al., 2001), to verify the role of Sp1 on MOR promoter activity in Sp1-expressing cells SH-SY5Y. As shown in Fig. 4A, PDTC reduced MOR promoter activity. On the other hand, we also restored Sp1 in the Sp1-nonexpressing SL2 cells by transfecting an Sp1 expression vector Pac-Sp1. The results showed that Pac-Sp1 transfection activated B6-4.7/ATG-Luc in a dose-dependent manner; however, the CXBK-4.7/ATG-Luc activity was not affected by restoring Sp1 in SL2 cells (Fig. 4B). The expression of Sp1 after transfection in SL2 cells was confirmed by Western blot analysis (Fig. 4C).

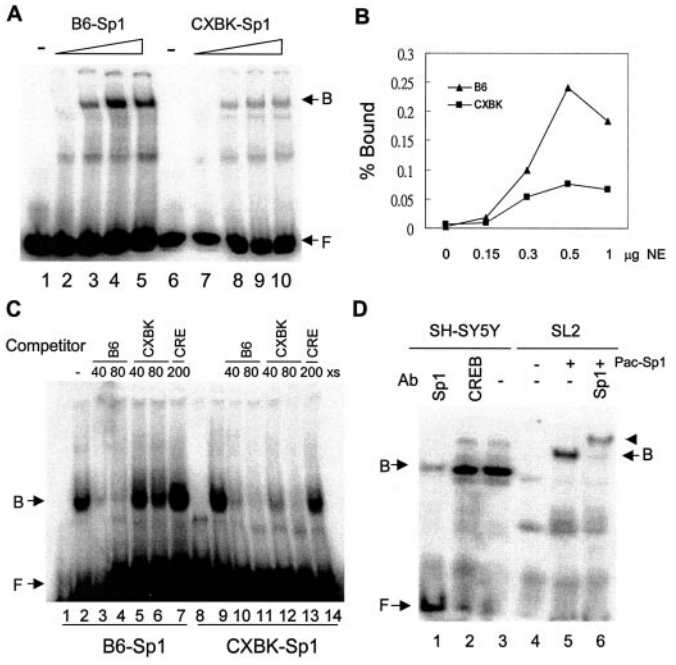

Fig. 2. Sp1 binds to the MOR 5′-UTR. A, EMSA using oligonucleotide B6-Sp1 (lanes 1–5) and CXBK-Sp1 (lanes 6–10) as the probes. Either 0 (lanes 1 and 6), 0.15 (lanes 2 and 7), 0.3 (lanes 3 and 8), 0.5 (lanes 4 and 9), or 1 (lanes 5 and 10) μg of PC12 nuclear extract was used in the assay. B indicates the DNA-protein complex whereas F indicates the free probe. B, histogram of the ratios of bound to free oligonucleotides in Fig. 3A. C, EMSA using B6-Sp1 and CXBK-Sp1 as probes and unlabeled oligonucleotide B6-Sp1 (B6), CXBK-Sp1 (CXBK), and CRE as competitors. Competition experiment: 0.5 μg of PC12 nuclear extract was used in lanes 2 through 7 and 9 through 14. Molar excesses (xs) of the competitors were marked. To give a similar intensity of the DNA-protein complex, we added three times more CXBK-Sp1 probe in the binding reaction (lanes 8–14). D, EMSA using B6-Sp1 probe and 0.5 μg of SH-SY5Y nuclear extracts (NE, lanes 1–3) or SL2 extract (lanes 4–6). Either anti-Sp1 (Sp1, lanes 1 and 6) or anti-CREB (CREB, lane 2) was added to the binding reaction. In lanes 5 and 6, SL2 cells were transfected with Pac-Sp1. An arrowhead indicates the supershifted band. We repeated the experiments three times with similar results, and one experiment was presented.

Discussion

Our data provide a cause for the difference in MOR expression between CXBK and B6 mice. The C/A polymorphism at

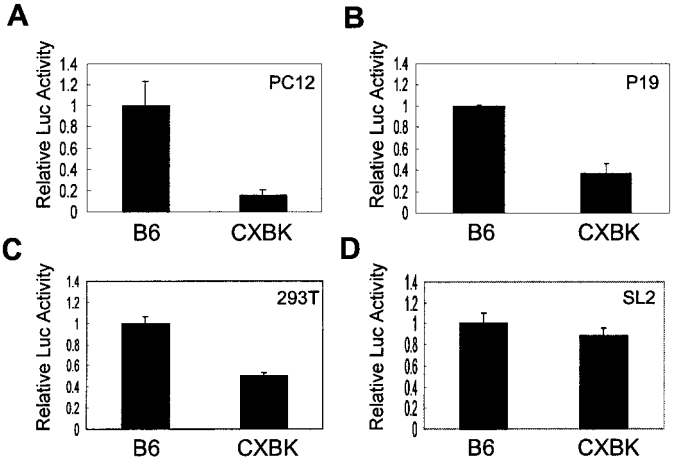

Fig. 3. Activity of B6 and CXBK MOR promoter in different cell lines. Transient transfection of B6-4.7/ATG-Luc and CXBK-4.7/ATG-Luc in PC12, P19, 293T, and SL2 cells. CXBK MOR promoter activity was expressed relative to that of B6. The error bars indicate 1 S.D. derived from three experiments in duplicate. β-Galactosidase activities, a control for transfection efficiency, were used to normalize the data.

5′-UTR of MOR gene closely flanks an Sp1 element. Although the consensus Sp1 sequences are the same, Sp1 bound to the B6-Sp1 element (Sp1 with flanking A) more efficiently than to the CXBK-Sp1 element (Sp1 with flanking C). MOR promoter with A nucleotide at nt −202 revealed higher activity than that with C nucleotide in SH-SY5Y, PC12, P19, and 293T cells, but not in SL2 cells, indicating that Sp1 is responsible for the difference. Because Sp1 is expressed in most tissues including brain, it is likely that Sp1 maintains the constitutive expression of MOR. There, the C/A polymorphism could underlie the lower expression of MOR in CXBK than in B6 mice. We did these experiments in cells expressing endogenous MOR (SH-SY5Y, PC12, and P19), and the results were similar.

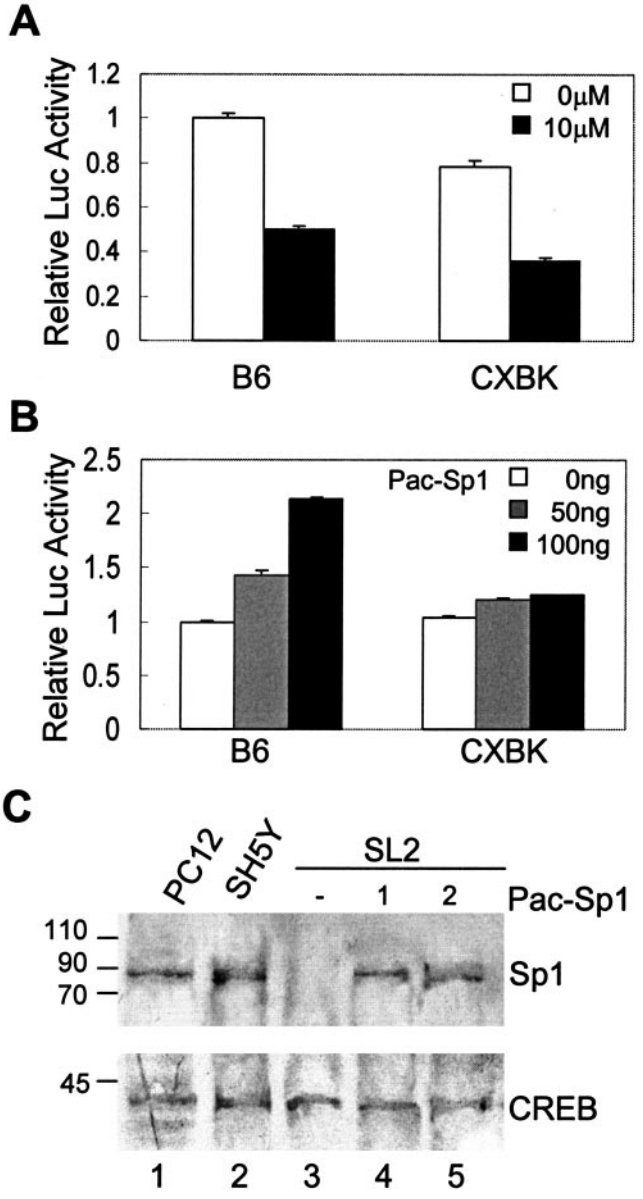

Fig. 4. Sp1 is required for the differential expression of B6 and CXBK MOR promoter. A, SH-SY5Y cells were transfected with plasmids B6-4.7/ATG-Luc and CXBK-4.7/ATG-Luc with or without the treatment of 10 μM PDTC for 4 h. Luciferase activities were expressed relative to that of untreated B6-4.7/ATG-Luc. The error bars indicate 1 S.D. derived from a triplicate. β-Galactosidase activities, a control for transfection efficiency, were used to normalize the data. The experiments have been repeated three times with similar results. B, cotransfection of B6-4.7/ATG-Luc or CXBK-4.7/ATG-Luc with 50 or 100 ng Pac-Sp1 plasmid in SL2 cells. Luciferase activities were expressed relative to that of transfection of B6-4.7/ATG-Luc only. β-Galactosidase activities, a control for transfection efficiency, were used to normalize the data. C, Western blot analysis. 0, 1, or 2 μg of Pac-Sp1 plasmid was transfected into SL2 cells (lanes 3–5). Fifteen micrograms of PC12, SH-SY5Y, and SL2 nuclear extract was used in the assay blotted with anti-Sp1 Ab (top) and anti-CREB Ab (bottom).

Constitutive transcription of housekeeping genes is often based on the interactions between the GC-rich DNA-binding proteins Sp1 (binds to GGGCGG) and the Myc-associated zinc finger protein (MAZ, binds to GGGAGG). MAZ serves as negative regulator on MAZ promoter (Song et al., 2001b). Independent regulation of MAZ and Sp1 was also reported in MAZ and phenylethanolamine *N*-methyltransferase (PEMT) gene expression (Song et al., 2001a; Her et al., 2003). MAZ has higher affinity for its binding element, but it is a less effective activator for PEMT gene (Her et al., 2003). MAZ is ubiquitously expressed but is less abundant in brain (Song et al., 1998). Although the sequence of the Sp1 element in MOR 5′-UTR is closer to the MAZ binding sequence, MAZ may not play an important role in MOR transcription.

PDTC has been used to inhibit Sp1 and nuclear factor-κB (Ziegler-Heitbrock et al., 1993; Lee et al., 2001) in experiments studying gene expression. Because a dominant-negative Sp1 plasmid is missing, we used PDTC to bring down Sp1 action in cells expressing endogenous Sp1. On the other hand, we also restore Sp1 to different levels in Sp1-deficient cells SL2. By use of these experiments, we can clearly demonstrate that Sp1 could be a determining factor for differential expression of MOR promoters with the C/A polymorphism. Because this region is not a consensus Sp1 site, other transcription factors such as Sp3 could also interact with this site. Kraus et al. (2003) also demonstrated that nuclear factor-κB plays a role in MOR expression under tumor necrosis factor induction through this region. The interplay of different transcription factors may add more complexity to MOR gene expression. Ikeda et al. (2001) proposed that the low amount of MOR mRNA caused the reduced sensitivity to opioids. Our results further extend those finding.

In this study, the 4.7-kb MOR promoter was used in the experiments for optimal promoter activity, although we focused on the −202 C/A polymorphism. There could be other polymorphisms at the upstream promoters of the two strains of mice studied, which also contribute to the difference in MOR expression. Although, a knock-in animal would be required to complete resolve the etiology of MOR deficiency in the CXBK mice, this study is the first time to prove the role of the −202 C/A polymorphism.

The UTR plays a role in regulation of mRNA stability, control of mRNA poly(A) tail length, influence on mRNA localization, and regulation of translational initiation and its efficiency (Decker and Parker, 1995). For example, the 3′-UTR of the Ca^{2+} channel α1B mediates calcium-dependent stabilization of the mRNA (Brook et al., 1992), and the 3′-UTR of the growth-associated protein of 43 kDa is required for the stabilization of the mRNA in response to treatment with phorbol esters (Fu et al., 1992). The κ-opioid receptor uses two poly(A) signals in its 3′-end, and utilization of different poly(A) of the κ-opioid receptor gene produces different mRNA stability, transcription efficiency, and ability to regulate by retinoic acid (Hu et al., 2002). CXBK MOR mRNA possesses longer 3′-UTR than other strains whereas two parental strains possess normal sized MOR mRNA (Ikeda et

al., 2001). Therefore, other mechanisms may also contribute to MOR mRNA stability in CXBK mice.

Humans differ in their individual responses to pain and opiate drugs, and MOR has been discussed as one of the candidates in search of a physiological basis of drug abuse (LaForge et al., 2000; Mayer and Hollt, 2001). Hoehe et al. (2000) have defined a characteristic pattern of sequence, variants in the MOR gene, which was associated with substance dependence. Natural sequence variations in MOR gene could modify receptor density and signaling (Befort et al., 2001), the A118G variant of MOR binds β-endorphin three times more tightly than the common allelic form (Bond et al., 1998). Crowley et al. (2003) also mapped a significant portion of the genetic variance in morphine preference to the vicinity of the MOR locus by using quantitative-trait-locus approaches (Crowley et al., 2003). CXBK mice show reduced sensitivity to morphine. Although we may have offered a genetic basis underlying morphine sensitivity in CXBK mice in this study, in vivo morphine response might not be dependent on the receptor level as reflected in some with the μ-receptor knockout heterozygotes who do not reveal significant change in morphine sensitivity.

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