Mouse $\mu$ Opioid Receptor Distal Promoter Transcriptional Regulation by SOX Proteins*

We have identified transcription factors that bind to specific sequences in 5'-distal promoter regulatory sequences of the mouse $\mu$ opioid receptor ($\text{mOR}$) promoter using the yeast one-hybrid system. The sequence between $\sim 746$ and $\sim 707$ in $\text{mOR}$ distal promoter was used as the bait because it acts as a functional promoter element and binds several DNA-binding proteins. From an adult mouse brain cDNA library, five cDNA clones encoding three Sox gene family ($\text{Sry}$ like high mobility group (HMG) box gene) transcriptional factors, mSOX18, mSOX21, and mSOX6, were isolated. Electrophoretic mobility shift assays confirmed the presence of a binding site for SOX proteins in the $\sim 731$-$\sim 725$ region. Additionally, we have also established that the flanking regions outside the core Sox-binding site play an essential role in high affinity binding. DNase I footprint analysis indicates that proteins from mouse brain interact with the Sox-binding site within the $\text{mOR}$ distal promoter. Finally, we demonstrated that overexpression of mSOX18 and/or mSOX21 was able to up-regulate mouse $\text{mOR}$ distal promoter activity in $\text{mOR}$-expressing neuronal cells (NMB). These data indicate that SOX proteins might contribute to the transcriptional activity of the $\text{mOR}$ gene and suggest that $\mu$ opioid receptor could mediate some of the developmental processes in which SOX proteins are included.

Opioids have many pharmacological and physiological effects, including analgesia, sedation, euphoria, and respiratory depression and also induce tolerance and physical dependence when administered chronically. Pharmacological, physiological, receptor binding, and molecular cloning studies (1) have revealed that opioids interact with three major types of opioid receptors in the mammalian central nervous system and peripheral tissues, $\mu$, $\delta$, and $\kappa$. All three types of receptors belong to the superfamily of G-protein-coupled receptors (2), and the $\mu$ opioid receptor ($\text{mOR}$) is known to play the essential role in morphine-induced analgesia, tolerance, and dependence as indicated from pharmacological studies. This has also been confirmed by additional in vitro pharmacological analyses of mice where $\text{mOR}$ was deleted by homologous recombination (3).

$\text{Mor}$ is expressed mainly in the central nervous system, where it exhibits distinct temporal and spatial patterns of distribution (1). Recently, in situ hybridization studies and other methods (e.g. RPA (RNase protection assay) and reverse transcriptase-PCR) indicate that expression of $\text{mOR}$ mRNA is regulated at the transcriptional level, especially during embryonic development (4–7). The molecular mechanisms underlying this regulation are not completely understood, but analysis of the 5'-upstream sequences from the translation initiation sites of the $\text{mOR}$ gene has shown that expression of $\text{mOR}$ is driven by two promoters, a distal and a proximal promoter (8). Both promoters exhibit characteristics of housekeeping genes lacking a TATA box (9, 10), with the proximal promoter regulating $\text{mOR}$ transcription from four major initiation sites located in a region 291–268 bp upstream of the translation initiation site. The distal promoter initiates $\text{mOR}$ transcription from a single transcription initiation site located 794 bp upstream of the translation initiation site (8) and is known to be 20-fold less active than the proximal promoter, based on quantitative reverse transcriptase-PCR using adult mouse brain mRNA (9). Thus, in adult mouse, the proximal promoter confers the largest part of the cell and tissue-specific expression of $\text{mOR}$ (9). It is also important to note that the 34-bp cis-acting element positioned between $\sim 721$ and $\sim 687$ possesses a strong inhibitory effect against the distal promoter transcriptional function (11). Because the exact role of the distal promoter in $\text{mOR}$ expression is largely unknown, our objective in the present study was to identify cis-acting DNA elements and their trans-acting factors that are associated with transcription from the mouse $\text{mOR}$ distal promoter. Secondly, we sought to determine the possible role of the distal promoter in regulating $\text{mOR}$ expression.

The results demonstrate the ability of the cis-acting element to augment the $\text{mOR}$ distal promoter activity in co-transfection analysis using deletional promoter constructs. Three kinds of Sox genes were isolated using the yeast one-hybrid system, which helped to identify the trans-acting factors that bind to the positive cis-acting element of the $\text{mOR}$ distal promoter. The Sox proteins belong to the high mobility group (HMG) box superfamily of DNA-binding proteins and are found throughout the animal kingdom (12). These proteins are involved in the regulation of such diverse developmental processes as germ layer formation, organ development, and cell-type specification. Hence, deletion or mutation of Sox proteins often results in developmental defects and congenital disease in humans. Sox proteins perform their function in a complex interplay with other transcription factors in a manner highly dependent on cell type and promoter context. They exhibit remarkable cross-talk and functional redundancy among each other (12, 13).
Therefore, Sox proteins may play an important role in regulating the expression of the mor gene, temporally, spatially, and developmentally as well as gender-dependently in brain. In this report, we characterized the nature and function of these nuclear proteins, which regulate the expression of μ opioid receptor gene.

**EXPERIMENTAL PROCEDURES**

**Yeast One-hybrid Screening for cDNAs Encoding Positive Regulatory DNA-Binding Proteins**—The MATCHMAKER One-Hybrid System (Clontech) was used according to the supplier’s protocol. Four tandem repeats of the −745 to −706 bp sequence (the positive regulatory element) of the distal promoter were ligated into the Saci- Xhol sites of pHis1 and EcoRl-Sacl sites of pLaCZi to generate pHis1-4x40 and pLaCZi-4x40, respectively (40 denotes the sequence between −745 and −706). These two bait constructs were then linearized with XhoI and NcoI, respectively, and integrated into the genome of yeast strain YM4271. The resultant yeast cells with the integrated pHsi1-4x40 were tested for growth on minimal medium lacking histidine (His+) in the presence of increasing concentrations of 3-aminol, 2,4-triazole. Background growth was inhibited in the presence of 30 μM 3-aminol, 1,2,4-triazole, and this concentration was then used when yeast cells were transformed with a mouse brain cDNA library for one-hybrid screening. Five positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids (5) from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct. Positive transformants grown on His- and leucine-negative (Leu-) plates were selected. To exclude false positive clones, plasmids recovered from these five clones were used to transform yeast cells harboring the pLaCZi-4x40 construct.

**Plasmid Construction and in Vitro Translation**—Constructs for the recombinant luciferase reporter gene plasmids pL1.3K/687, pL1.3K/775, pL1.3K/728, pL1.3K/721, and pL1.3K/717 have been previously described (11). To further define the positive regulatory region of the mor distal promoter, pL1.3K/747, pL1.3K/738, pL1.3K/731, and pL1.3K/725 were generated from pL1.3K/687 by PCR using the primers shown in Fig. 1; that is, forward (mor5′), 5′-GTGGTGGAAGTTTGAGTTAGTTAATTCTTTACAACC-3′ (EcoR1 site at nucleotide −1269 is underlined) and all the corresponding reverse primers bearing essential Kozak sequences (5′-CCATGGTG-3′) and NcoI site (located at start codon of luciferase). The resulting fragments were subcloned into the Bfr1 (-1105)-NcoI fragment of the pL1.3K/444 plasmid (11). The mutants of pL1.3K/721m123 were constructed by PCR using pL1.3K/687 as the template the same forward primer, mor5′, as described above and reverse primer, 5′-GGCCCATG-GGCTGTTTGTCGACAACTATGTCTTTTTCTAA-3′ for pL1.3K/721m2 and 5′-GGCCATGGAGTACAGTGATCCGTTGCTTTCTAAAAAGAAAAA-3′ for pL1.3K/721m123 (the NcoI site is underlined, the start codon is italicized, and mutated sites are in bold). The PCR products were inserted into a Ta cloning vector pcR2.1 (Invitrogen). The resulting fragments were subcloned into the Bfr1-NcoI sites of pL1.3K/721. The mammalian expression plasmids, pc1-Sox18 or pc1- Sox6, were constructed by inserting the EcoRl-Xhol fragment of the original clones, pcAT2-Sox18 or pCAT2-Sox6, into the same sites of pcDNA1.1 (amp, Invitrogen), respectively. To construct pc1-Sox21, the BamHI-Xhol fragment of the original clone was inserted into the same sites of pcDNA1.1. The integrity of all constructs was confirmed by restriction enzyme analysis and sequencing.

**In vitro translation** was carried out with pc1-Sox18, pc1-Sox21, and pc1-Sox6 in a reaction mixture containing [35S]methionine (Amersham Biosciences) using a TNT quick-coupled transcription/translation system (Promega). The labeled proteins were then electrophoresed by 10% SDS-PAGE, and their sizes were compared with the predicted sizes.

**Cell Culture, Transfection, and Reporter Gene Assay**—Human neuroblastoma NMB cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) at 37 °C in a 5% CO2 atmosphere of N2. Cells were plated at 106 cells/well and cultured overnight before transfection. Various plasmids at concentrations indicated in each figure were used with the SuperFect Transfection reagent (Qiagen) as described by the manufacturer. Briefly, for 3′ deletional analysis of the distal promoter, 2 μg of the reporter plasmids were mixed with SuperFect Transfection reagent for 15 min before being added to NMB cells. Forty-eight hours after transfection, cells grown to confluence were washed once with 1× phosphate-buffered saline and lysed with lysis buffer (Promega). To correct for differences in transfection efficiency, a one-fifth molar ratio of a βgalactosidase plasmid (Amersham Biosciences) containing the β-galactosidase gene under the SV40 promoter was included in each transfection for normalization. The luciferase and βgalactosidase activities of each lysate were determined as described by the manufacturers (Promega and Tropix, respectively). For co-transfection assays, the procedures were the same as above, except the plasmids were a mixture of the given amount of pc1-Sox18 or pc1-Sox21 and 1 μg of a corresponding reporter plasmid, pL1.3K/721 or pL1.3K/721m123. To further define the positive regulatory region of the distal promoter, 2 μg of in vitro translated proteins in a final volume of 50 μl of 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)) at room temperature for 30 min. For oligonucleotide competition analysis, a 25–100-fold molar excess of cold competitor oligonucleotide was added to the mixture before adding the probe. The reaction mixtures were electrophoresed in 4% polyacrylamide-SDS gels and subsequently stained with 4′,6-diamidino-2-phenylindole (0.5 μg/ml Tris borate and 1 mM EDTA) at 4 °C and visualized by autoradiography.

**DNase I Footprint Analysis**—The mor distal promoter region from −812 to −575 bp was generated by PCR using a 3′-labeled primer for the coding strand and using pL1.3K/506 (11) as template to create the 5′-labeled 236-bp probe. PCR primers used to prepare the probe for footprinting were: sense, 5′-GAAGATTTGAGAAGGTGCGGGCGAC-3′ (bases −812 to −788) and antisense, 5′-ATACTCTCAAACCTCCCGGACTCA-3′ (bases −575 to −557). All the oligonucleotides and probes were purified by PAGE. DNase I footprinting was performed according to the recommended manual using the Core Footprinting System (Promega). Nuclear extract from mouse brain was prepared according to the method described by Sonesen et al. (16). Binding reactions were carried out for 20 min on ice in a final volume of 50 μl. The binding solutions contained 100 fmol of labeled probe and the indicated amounts of the mouse brain nuclear extract in a final buffer concentration of 10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM dithiothreitol, and 1 μg of poly(dI-dC). After incubation, MgCl2 and CaCl2 were added to final concentrations of 5 and 2.5 mM, respectively, then 0.3 unit of DNase I (Promega) was added. The incubation was continued for 4 min at room temperature. The digestion was stopped by using 90 μl of stop solution containing 20 mM EDTA, 1% SDS, 0.2 mM NaCl, and 250 μg/ml RNA. DNA was extracted by using phenol-chloroform (1:1) and ethanol precipitation before loading on a 8% sequencing polyacrylamide gel for electrophoresis.

**RESULTS**

**Identification of Functional Cis-acting Elements of mor Distal Promoter**—Our previous results have shown that the 5′-flanking region of the mouse μ opioid receptor (mor) gene has two major promoters, designated as the proximal and distal (9). Choe et al. (11) from our laboratory have also reported a positive cis-acting element between the nucleotides −721 and −775 and a negative cis-acting element between the nucleotides −687 and −721. Both of these regulatory elements are present within the 5′-distal promoter regulatory sequences of mor promoter. To define the exact position of the positive cis-acting element, a further detailed 3′-deletional mutagenesis was performed in the region of −775 to −721 (Fig. 1). As shown in Fig. 1, a deletion of just 4 bp from the 3′ end (pL1.3K/725) exhibited a 50% decrease in luciferase activity relative to the pL1.3K/721 construct. A further deletion to −731 resulted in an additional 25% decrease in promoter activity. These results indicate that the deleted segment of −730 to −721 is important for the positive cis-acting element. Further deletions resulted in a slight increase in promoter activity, indicating the complexity of regulation of distal promoter activity.

To determine whether this positive cis-acting element of −730 to −721 could recruit transcription factors to regulate the...
Fig. 1. Identification of the positive cis-acting element using 3′ deletional analysis of distal promoter of the μ opioid receptor (mor) gene. A, schematic representation of a series of 3′ deletion constructs of mouse mor distal promoter. Fragments of distal promoter with varying lengths of the 3′ regulatory regions were inserted into the promoter-less luciferase vector, pGL3-Basic. The numbers in the name of each construct refer to the number of the nucleotide to the translation start site (designated +1) at the 5′ and 3′ ends of each inserted fragment, respectively. LUC represents the luciferase gene. B, 3′-deletion analysis of mouse distal promoter activity in transient transfection and luciferase assays in NMB cells. The promoter activity of each construct was expressed as relative luciferase activity, and transfection efficiencies were normalized to β-galactosidase activity by co-transfection of the internal control plasmid pCH110. The activities of the luciferase reporter were expressed as n-fold relative to the activity of pGL3-Basic, which was assigned an activity value of 1.0. The data shown are the means of three independent experiments with at least two different plasmid preparations. Error bars indicate the range of standard errors.

Promoter activity, we next performed EMSAs using nuclear extracts from mor-expressing NMB cells. We selected the segments O1 (−746 to −707) and O2 (−741 to −718) (Fig. 2A) as probes, because they both covered the region of −730 to −721 but with different lengths of their flanking sequences. As shown in Fig. 2, there are several protein-DNA complexes formed with the probe O1 and O2. The higher molecular weight complexes were efficiently inhibited by a 100-fold molar excess of cold self-competitors, indicating these complexes were sequence-specific, whereas the fastest running band with the O2 probe was not abrogated with self-competitor (Fig. 2C), indicating the presence of nonspecific interaction. In summary, the O1 and O2 sequences, containing the positive cis-acting element, can form sequence-specific DNA-protein complexes with NMB nuclear extracts, suggesting the presence of endogenous transcription factors. Based on the EMSA and luciferase reporter assay results, we hypothesize that the positive cis-acting element plays a role in regulating distal mor promoter activity.

NMB Nuclear Extracts Specifically Bind to a Sox-binding Motif within the Identifed Cis-acting Element—The SOX family consists of a group of transcription factors that recognize a specific common SOX protein binding site (5′-WWCAAWG-3′, where W = A or T) (17). These factors are thought to bind to the minor groove of double-stranded DNA and induce DNA bending, resulting in chromatin structure favorable to regulation of promoter transcription (18). Analysis of the O1 cis-acting element revealed at least four putative Sox-binding sites within this short DNA sequence, as indicated in Fig. 3. To further characterize Sox binding to O1, EMSAs were carried out using O1 as probe and a consensus Sox binding sequence, 5′-GATCCGCCGGCCTTTGTTTCTCCCA-3′ (SSC, as shown in Fig. 3B) (19) as a competitor. Efficient competition was observed with a 40-fold excess of the SOX consensus competitor and maximal competition with a 100-fold excess of the competitor, confirming the probe’s SOX factor binding property.

The O1 cis-acting element contains four putative Sox-binding sites in very close proximity to each other. Similar multiple Sox-binding sites have also been previously reported from several other genes (20, 21). To define exactly which of these Sox-binding sites is essential to the promoter activity, five additional oligonucleotides spanning the entire region were synthesized. They were designated as O3 to O7, as indicated in Fig. 3A. These fragments were then tested for their ability to compete with the DNA-protein complexes from the O1 element. The DNA-protein complexes were abolished by a 100-fold excess of competitors O2, O3, and O7 but not by O4, O5, and O6. These data suggest that the double-stranded DNA sequence shared by O2, O3, and O7 is probably important for binding of NMB nuclear extracts to the O1 cis-acting elements. Examination of this common sequence revealed a putative Sox-binding site, 5′-CTTTGAA-3′, as underlined and in bold in Fig. 3A. Furthermore, this putative Sox-binding site is located downstream of the distal promoter rather than upstream, as reported for other systems (18, 22). Therefore, its orientation to the distal promoter is also reversed. However, O6, which failed to block the formation of O1-protein complexes, also contains this entire sequence. This observation might be explained by a requirement of an additional two nucleotides at the 5′-flanking region of SOX core-binding site for the formation of the DNA-protein complex.

To obtain further insight into complex formation, we performed EMSAs using unlabeled mutant oligonucleotides to compete with 32P-labeled O2 for the complex formation with NMB nuclear extracts. We designed four mutated oligos; M7 and M8 have a mutation in each side of the Sox-binding consensus site, the 5′-flanking region was mutated in M6, and the 3′-flanking region was mutated in M9 (Fig. 4A). As shown in Fig. 4B, the probe O2 containing the complete Sox-binding site and both 5′- and 3′-flanking regions has formed DNA-protein complex bands in the presence of NMB nuclear extracts. The M6 competitor effectively blocked the appearance of the major migrating bands, but M9 did not affect the bands. In general, both flanking regions of the core consensus-binding site are required for protein binding in many transcription factors (22, 23). Together these data indicate that the specific sequence of
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Fig. 2. EMSA analysis of the cis-acting elements of mor distal promoter. A, schematic diagram representing the 5′-regulatory region of mouse mor gene, from nucleotide −1326 to the translation start site designated +1. Arrows indicate the distal and proximal transcription initiation sites (TIS). The box indicates the previously identified 34-bp pair negative cis-acting element from −721 to −687 (11). The solid left end of the box indicates the critical sequence for mediating the negative effects. The identified Sox binding motif is shown as an open oval. The positions of the oligos O1 and O2 arbitrarily selected for EMSAs are as indicated. B, EMSA was performed with nuclear extracts (NE) from NMB cells. The double-stranded O1 was selected as probe and 32P-labeled. Lane 1, probe alone; lanes 2 and 3, probe plus 5 μg of NMB nuclear extracts; lane 3, 100 molar ratio excess of unlabeled O1 as a self-competitor. The sequence-specific DNA-protein complexes are indicated by the symbol ▲, sequence nonspecific complexes are indicated by the symbol ▲. C, NMB nuclear extracts bind to O2 sequence. EMSA was performed similar to Fig. 2B, except that a shorter oligonucleotide O2 was 32P-labeled and used as the probe in EMSA. Lane 1, probe alone; lanes 2 and 3, probe plus 5 μg of NMB nuclear extracts; lane 3, 100 molar ratio excess of unlabeled O2 as a self-competitor.

four bases in the flanking region downstream of the core is required for high affinity binding (lane 7 in Fig. 4B). In addition, the presence of at least two bases without sequence specificity at the upstream flanking region is required for the DNA-protein complex formation (lanes 7 and 8 in Fig. 3C and lane 4 in Fig. 4B). As expected, the competitor M7 with the mutated 5′-core binding site, could not abrogate the DNA-protein complex. However, M8 with the mutated 3′-core binding site could compete partially but not as strongly as M6 and the self-competitor O2, indicating that the 5′-core binding site is more important than the 3′-core binding site for high affinity binding. The DNA-complexes were not inhibited by nonspecific competitors, Sp1 (24) and Ikaros (25) consensus sequences, confirming the specificity of the DNA-protein interaction (Fig. 4B). Taken together, these results suggest that NMB nuclear extracts contain proteins that bind sequence-specifically to the identified Sox binding motif of the mor distal promoter.

DNA-Protein Interactions in the Positive Cis-Acting Element of the Mouse mor Gene Determined by DNase I Footprinting—

Fig. 3. The Sox binding property of the identified cis-acting element. A, the O1 sequence of the mor distal promoter has four putative Sox-binding sites (underlined or lined above the O1 sequence). The identified Sox binding motif in this study is also underlined and in bold. The modified oligonucleotides, O2 to O7, are used as cold competitors in EMSA. The numbers in parentheses at both ends of O1 sequence represent the location of the sequence in mor promoter. B, EMSAs were performed as in Fig. 2. Lane 1, O1 probe alone; lanes 2–5, probe O1 and 5 μg of NMB nuclear extracts (NE). A published consensus Sox binding sequence, 5′-GATCCGCGCTTTTTGTCTCCCG-3′ (SSC, Sox binding motif is underlined) (19) was used as a competitor in 20 molar ratio excess (lane 3), 40 molar ratio excess (lane 4), and 100 molar excess (lane 5). The sequence-specific DNA-protein complexes are indicated by the symbol ▲. C, A putative Sox-binding motif (−731 to −725) within the O1 and O2 element is important for NMB nuclear extract binding. EMSA was performed with nuclear extracts from NMB cells. The O1 fragment was used as the probe in the absence or presence of 100-fold molar excess of unlabeled competitors. Lane 1, O1 probe alone; lanes 2–8, 5 μg of nuclear extract; lane 8, absence of competitor; lane 3, cold competitor O2; lane 4, cold competitor O3; lane 5, cold competitor O4; lane 6, cold competitor O5; lane 7, cold competitor O6; lane 8, cold competitor O7. The major bands representing the sequence-specific DNA-protein complexes are indicated by the symbol ▲.

The relationship between in vivo positive modulatory activity and in vitro DNA-protein interaction(s) was initially assessed by DNase I footprinting using a probe extending between nucleotides −812 and −575. As shown in Fig. 5, at a very high concentration of nuclear extract from mouse brain (80 μg), characteristic DNase I-hypersensitive sites below nucleotides −739 and above −725 and a protected region (−739 to −725) against DNase I digestion were observed. In contrast, relatively lower concentrations of the nuclear extracts (20 or 40 μg) hardly altered the DNase I digestion pattern of the probe (Fig. 4, compare lanes 2 and 3 with lane 4). This may be the reason why we did not observe the DNA-complex formation from mouse brain in EMSA, suggesting very low abundance of the endogenous transcription factor(s) that bind to this area in adult mouse brain.

Isolation of Transcription Factors That Interact with the Positive Cis-Acting Element Region in the mor Distal Promoter—To identify transcription factors that bind to the positive element of the distal promoter, we utilized the yeast one-hybrid system to screen an adult mouse brain MATCHMAKER cDNA library (Clontech). The O1 sequence was used as bait because it
contained more of the flanking regions potentially important for the transcription factor binding affinity. A double-stranded oligonucleotide containing four tandem repeats of the O1 sequence was subcloned into pHISi. The resulting plasmid, pHISi-4x40 was integrated into the yeast YM4271 genome. Using this yeast strain, 3 x 10^6 independent colonies from the library were screened. A total of five independent, histidine-positive clones were selected and sequenced. The results revealed that all five clones belonged to the SOX family of transcription factors. Two of the clones contained full-length sequences (2.7 kilobase pairs) encoding polypeptides with more than 80% sequence identity to human Sox21. These clones were designated mSox21 (GenBank™ accession number AY142959). The other two clones were identical to mSox18 and were designated mSox6 lacking the first 299 amino acids but encoding a DNA binding domain.

To verify that the three cloned SOX proteins bind to the O1-containing promoter in vivo, the three independent cDNA clones were transformed into yeast strains containing the control plasmid pHISi. All three SOX proteins could specifically activate the HIS3 gene containing the four tandem repeats of the O1 element but not the HIS3 gene lacking these repeats. This suggests that the cloned SOX proteins bind specifically to this O1 cis-acting element and not to the sequence of the HIS minimal promoter. To exclude the possibility that the addition of the O1 element may form some specific sequences with the minimal HIS3 promoter that favor binding of the SOX protein, we used another reporter yeast strain containing pLacZi-4x40. A control yeast strain without repeats of the cis-acting elements but with pLacZi integrated into its genome was also used. The cloned SOX plasmids were transformed separately into these yeast strains. All three SOX clones in yeast strains containing pLacZi-4x40 were found to be true positives after the LacZ expression test. In control yeast strains containing pLacZi transformed with SOX plasmids, there was no activation, indicating that these three cloned SOX proteins indeed have specific strong binding activity to the four tandem repeats of the O1 cis-acting elements in vivo.

Cloned SOX Proteins Bind Specifically to the Positive Cis-acting Element of mor Distal Promoter in Vitro—The ability of all three Sox cDNAs (Sox6, Sox18, and Sox21) to encode proteins was verified by in vitro translation, and the products were analyzed on SDS-PAGE. All the in vitro translated SOX proteins were electrophoresed and found to be the correct size, as expected from their calculated molecular weights (data not shown). To confirm whether the isolated SOX proteins can indeed bind to the positive cis-acting element, EMSAs were carried out using in vitro translated SOX protein products. The
SOX proteins were able to shift the target O2 oligonucleotide probe. The specificity of this DNA-protein interaction was verified by complete inhibition in the presence of cold wild-type O2 oligonucleotide in an EMSA using the SOX18 protein (Fig. 6A). Furthermore, competitor M7 or M8 containing a mutation in the Sox binding motif (lane 4 and 5 in Fig. 6A) failed to efficiently block the complex formation, whereas competitor M6 containing a mutation in the upstream flanking region of the core motif competed with the SOX18-DNA complex (lane 3). As we have observed similarly in the EMSA of Fig. 4B using nuclear extracts of NMB cells, competitor M9 containing a mutation in the downstream flanking region of the core motif was not able to block the complex (lane 6 in Fig. 6A). Similar results were obtained when using in vitro translated SOX21 (Fig. 6B). The competitive EMSA using the SOX6 protein has not been performed because of unavailability of the full-length SOX6 cDNA, although in vitro translated SOX6 protein using the partial cDNA was still able to bind to the O2 oligonucleotide probe (data not shown). We conclude that the in vitro translated SOX18 and SOX21 proteins have a higher binding preference to the identified Sox binding motif than other Sox-binding sites within probe O2. However, the identified Sox binding motif alone cannot account for 100% of the total Sox binding activity, because the competitor M9 containing a mutation of the downstream flanking region of the core motif could not bind to the SOX proteins seen in lanes 6 of Fig. 6, A and B.

SOX18 Binds Preferentially to the Sox-binding Site of mor Promoter among the Known Sox Binding Consensus Sequences from Several Different Promoters—To test the preferential binding of SOX18 to the known SOX family binding consensus sequences from several gene promoters, four different Sox-related binding sequences for T cell factor (TCF)-MW56 (T cell receptor) (26), SOX5-BS12 (26), TCF1α (T cell receptor α chain enhancer) (27), and SRY (sex region of the Y chromosome) (28) proteins were chosen for EMSA (Fig. 7A). Based on the sequence similarities among the Sox-binding sites between O2 and the related oligonucleotides (Fig. 7A), the competitor TCF1α containing the same core sequences as O2 showed the highest binding inhibition, i.e. a 40% reduction in band intensity relative to the band seen with only the O2 probe (Fig. 7B). The competitors, TCF-MW56 and SOX5-BS12, differing by 2 and 4 bases, respectively, from the core sequence of O2, are less effective at competing with the probe, showing 19 and 18% reductions in band intensity, respectively. The lowest competition was observed with SRY sequence, an 8% reduction in intensity, which is a similar competition ratio to the nonspecific binding sequence Sp1 (3% reduction). These results indicate that SOX18 has a preferential interaction with the cis-acting element of the mor promoter compared with other Sox-related binding sites, and the flanking sequences of the binding site may also be important for the DNA-protein interaction as described above (Figs. 3, 4, and 6).

The Identified Sox Binding Motif Is Required for the Regulation of mor Distal Promoter Activity—To further confirm the importance of the identified Sox binding motif in the regulation of the mouse mor distal promoter, two mutated constructs, pL1.3K/721m2 and pL1.3K/721m123, were cloned. In construct pL1.3K/721m2, the identified Sox binding motif of the distal promoter was mutated, whereas in construct pL1.3K/721m123, both the Sox binding motif and its flanking regions were mutated. As shown in Fig. 8, A and B, when compared with the wild-type distal promoter (pL1.3K/721), mutation of the Sox binding motif alone (pL1.3K/721m2) resulted in a two-thirds decrease in the relative luciferase activity. Additional mutation of the flanking region together with the Sox binding motif...
transfected with low concentrations of mSox18. On the other hand, mSox18 still has some trans-activation activity when co-transfected with mutant reporter constructs of either the identified Sox binding motif or its flanking region alone (data not shown). This indicates that the identified Sox binding motif together with its flanking region is important for the trans-activation by co-expressed mSox18. In addition, other multiple Sox-binding sites might be co-regulated by SOX18, although the physical interactions were not shown with the SOX proteins. Similar results were also obtained after co-transfection of cloned Sox18 and the distal promoter reporter plasmid into Chinese hamster ovary cells, which do not express mor endogenously (data not shown).

For the transcription analysis of mSox21, as in mSox18, co-transfection with a fixed non-saturated amount of the reporter pL1.3K/721 and increasing amounts of the expression plasmid pc1-SOX21 resulted in increased luciferase activity in a concentration-dependent manner (Fig. 9A). Five hundreds nanograms of mSox21, the maximum amount tested in this study, showed the highest promoter activity on pL1.3K/721, with a gradual increase in the activity, whereas 100 ng of mSOX18 showed 90% activity compared with the maximal promoter activity, as seen with 500 ng of mSOX18. This indicates that SOX18 at low expression levels is a more potent activator of mor promoter than Sox21. When these two proteins were co-expressed in NMB cells, Sox21 did not significantly enhance Sox18-induced activation of the promoter in pL1.3K/721 (Fig. 9B) (at most a 15% increase), suggesting that the amount of either DNA (500 ng) used alone might have been sufficient for maximum promoter activity, and/or these two SOX proteins might act independently in our system. Although the full-length cDNA of mSox6 was unavailable, the partial cDNA clone of mSox6 containing an N-terminal and internal deletions but retaining HMGI domain was inserted into the mammalian expression vector. The resultant plasmid, pc1-Sox6, did not show any significant effect when similarly co-transfected with the distal promoter construct into NMB cells, indicating the requirement of the full-length cDNA clone for the further study.

DISCUSSION

In the present report, we identified two transcription factors, SOX18 and SOX21, as potent transcriptional activators of the mouse μ opioid receptor (mor) gene. This regulatory activity is mediated by a Sox-binding site located in the downstream region of the mor distal promoter. Deletion or mutation of this Sox binding motif resulted in about a two-thirds decrease of the mouse mor distal promoter activity in transient transfection assays. Nuclear extracts from NMB cells endogenously expressing mor could specifically bind to this Sox binding motif, and a very high concentration of nuclear extract from mouse brain generated a protected region in this area against DNase I. We were able to isolate three SOX proteins (Sox6, Sox18, and Sox21) from mouse brain by the yeast one-hybrid system using the cis-acting sequence. Mutations of the Sox binding motif and its flanking regions blocked the transactivation effects of mSox18. Thus, the identified Sox binding motif plays an important role in the regulation of mor distal promoter, and we have identified mSOX18 and mSOX21 as possible candidates for this regulatory effect.

Cloning of these SOX proteins from the adult mouse brain cDNA library indicates that the expression of these SOX genes is not only restricted to early central nervous system development (31–34), but these genes are also expressed in the adult mouse brain. Although none of these proteins is strictly expressed in the central nervous system (31–34), the co-existence of SOXes and MOR in the central nervous system provides the

(pL1.3K/721m123) caused a slight further decrease of the promoter activity. The promoter construct pL1.3K/731 lacking the Sox-binding site showed 60% less activity than the wild-type promoter as shown in Fig. 8A and also in Fig. 1B, indicating consistency of the experiments. These results suggest that the identified Sox binding motif is important for up-regulating the mouse mor distal promoter activity.

SOX Proteins Activate Transcription through the Sox Binding Motif of mor Distal Promoter—To determine how the cloned SOX proteins regulate the transcription of the mouse mor gene, cDNAs for full-length mSox18 or mSox21 were cloned into a mammalian expression vector. These DNAs were co-transfected with the mouse distal promoter linked to the luciferase reporter gene into NMB cells endogenously expressing mor. Cells transfected with the mutant reporter construct pL1.3K/721m123 served as controls. As shown in Fig. 8C, mSox18 can specifically trans-activate the distal promoter activity (pL1.3K/721) in a concentration-dependent manner that is consistent with the previous reports that SOX18 is a trans-activator (29, 30). Mutations of the Sox binding motif and its flanking region resulted in a significant decrease of the trans-activation activity by mSox18. This decrease is rather obvious when cells were

![Fig. 8. The identified Sox binding motif is important for activation of the mor distal promoter by cloned mSOX18. A, wild-type and mutational reporter constructs used in the transient transfection and luciferase assays. The reporter constructs are: wild-type, pL1.3K/721; deletion mutant pL1.3K/731; mutant pL1.3K/721m2, mutated in the Sox-binding site; mutant pL1.3K/721m123, which mutated both the Sox-binding site and its flanking regions. The X mark in the filled oval indicates the mutated Sox-binding site or its flanking sequences. LUC represents the luciferase reporter gene. B, mutational analysis of mouse mor distal promoter activity by transient transfection assays in NMB cells. Transfection efficiencies were normalized as described in Fig. 1. The activities of the luciferase reporter were expressed as n-fold relative to the activity of pGL3-Basic, which was assigned an activity value of 1.0. The data shown are the means of three independent experiments with at least two different plasmid preparations. Error bars indicate the range of standard errors. C, NMB cells were co-transfected with the indicated amounts of pL-Sox18 and either wild-type pL1.3K/721 or mutant pL1.3K/721m123 plasmid. The relative luciferase activity was calculated as the activity of each reporter in the absence or presence of the indicated amount of pL-Sox18 and normalized by β-galactosidase activity as described above.

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Fig. 9. Sox18-induced activation of the mor distal promoter is enhanced by SOX21. A, NMB cells were co-transfected with pl.1.3K/721 and the increasing amounts of pc1-Sox21. Luciferase activities, normalized to β-galactosidase activity from a co-transfected LacZ vector (pCH110), are expressed as fold activation of the luciferase activity of the pl.1.3K/721 plasmid, which is arbitrarily defined as 1.0. Error bars indicate the range of standard errors. B, NMB cells were co-transfected with 0.5 μg of pc1-Sox18, 0.5 μg of pc1-Sox21, or 0.5 μg of pc1-Sox18 plus 0.5 μg of pl.1.3K/721. Luciferase activities are expressed as described in A. Results are the means of three independent experiments. Error bars indicate the range of standard errors. The empty vector pcDNA1.1/amp (Invitrogen) was added to make an equal amount of 2 μg of DNA for each transfection.

possibility that these SOXes might function in regulating mor gene expression.

Because of the unavailability of mouse cell lines that express endogenous mor, we used nuclear extracts from the human neuroblastoma NMB cell line as the source of transcription factors in EMSAs. Although the SOX proteins of human and mouse are slightly different, their amino acid sequences are highly conserved, especially in the HMG DNA binding domain (35). In the case of Sox18, human Sox18 contains an HMG box with 98% amino acid identity to the HMG domain of mouse Sox18 (34). Therefore, the DNA binding properties of these SOX proteins in NMB nuclear extracts should be highly similar to the corresponding transcription factors from mouse. We had tried to use nuclear extracts from mouse brain in EMSAs. However, because of strong background binding, we could not clearly distinguish any band representing specific Sox binding. In DNase I footprinting analysis, we could only detect the DNA-protein interactions on Sox-binding sites in very high concentrations of nuclear extract from mouse brain. This may reflect the relatively low amounts of mor expressed in the brain as well as presumably, of the transcription factors regulating them, which are not ubiquitous in their activity.

SOX proteins are found throughout the animal kingdom and have been shown to be intrinsically involved in the regulation of the development of germ layer formation, the central nervous system and other organs, and cell-type specification (35). SOX factors are characterized by the presence of a SRY box, a 79-amino acid protein motif (12, 35) that encodes an HMG-type DNA binding domain. Thus, the SOX family falls into a subclass of HMG box proteins, the members of which show highly restricted tissue distribution and bind to specific sequences at high affinities (12). The HMG domain has been known to interact with the minor groove of the DNA helix and induce a dramatic bend in the DNA molecules (35, 36), in contrast to the majority of types of DNA binding domains that have access to DNA through the major groove. These HMG-box proteins are classified into two broad categories; those proteins that carry multiple HMG domains (ribosomal transcription factor UBF (upstream binding factor) and the abundant non-histone chromosomal proteins HMG1 and HMG2) and show little DNA sequence preference and those having a single HMG domain that bind DNA in a sequence-specific manner including the TCF/lymphoid enhancer binding factor (LEF) family and the SOX proteins (36). To date, more than 20 SOX proteins have been identified in vertebrates, and they have been grouped into Groups A-F, Group A being assigned to SRY (sex-determining region of Y chromosome), which is the prototype of Sox genes, encoding the mammalian testis-determining factor (37). Sox18 belongs to the F sub-group of SOX proteins along with Sox7 and Sox17. Sox21 and Sox14 are classified into subgroup B2 (18). Within an individual group, the amino acid sequence identity of the DNA binding (HMG-box) domain remains very high (over 90%), although it decreases to ~60% between distant groups. Then, how do individual SOX proteins regulate the target gene temporally and spatially? The present theory is that combinatorial protein interactions of several SOX proteins and other transcription factors is often necessary to promote target gene expression (13, 38–40). Sox2 and the POU domain transcription factor, OCT-3, bind adjacent sites and participate together for the transcriptional activation of the PGP4 gene through protein-protein interactions in teratocarcinoma cells (38, 41), whereas either factor alone is ineffective. Three different SOX proteins, a long form of SOX5, SOX6, and SOX9, are co-expressed in chondrocytes and cooperatively activate the chondrocyte-specific enhancer of the type II collagen gene (39). The activation is facilitated by the dimerization of the long form of SOX5 and SOX6. SOX6 contains a leucine zipper motif that allows dimerization of the protein, and homodimers fail to bind DNA (40). These data suggest that, in testis, Sox6 may bind to another protein as a heterodimer to show transactivation properties. Coincidently, it was reported that a POU domain protein OCT-1 could regulate the mouse mor distal promoter activity by binding to a functional OCT-1 binding site (a negative cis-acting element), which is 94 bp upstream of the identified Sox binding motif (42). It would be interesting to investigate whether these two sites could cooperate with each other to confer cell- or tissue-specific regulation of the mouse mor expression.

In our EMSAs, we observed the sequence-specific DNA-protein complexes on the identified Sox binding motif with nuclear extract of NMB cells (Fig. 2). Interestingly, the identified Sox binding motif and its flanking regions together are responsible for the complex formation. Because a mutation of 4 bp downstream of the core binding motif abolished the formation of the major band in EMSAs (Figs. 4 and 6), it is unlikely that either of them alone could account for the major band formation. On the contrary, there would be some extent of cooperation between the identified Sox-binding motif and its flanking regions.
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As previously mentioned, there are theoretically three additional SOX-binding sites adjacent to the 5'-side of the identified SOX binding motif. Because the O2 probe lacking the first SOX site still was able to form the same major bands (Figs. 2C and 4B) as O1 probe containing all four SOX-binding sites, the first SOX-binding site is unlikely to be a major site for influencing the major band formation. Based on the results from EMSAs using mutant or deleted oligonucleotides (Fig. 3C, 4B, and 6), the flanking sequences of the core-binding site also affect the formation of SOX-protein-DNA complexes to the SOX binding motif. In addition, the requirement of specific flanking sequences for SOX protein binding was confirmed by another EMSA using different SOX binding consensus sequences of several SOX proteins (Fig. 7). The only competitor TF1α containing the same core-binding site as O2 but having different flanking sequences competed for about 40% with the probe O2. The others showed minor interactions, although they have very similar core binding sequences compared with O2 (Fig. 7A). It was previously reported that SOX proteins achieve DNA sequence specificity through subtle preferences for flanking nucleotides and that this is likely to be dictated by signature amino acids in their HMG domains (43, 44). For example, the optimal SOX9 binding sequence, AGAACAATG, contained a core DNA binding element AACAT, flanked by 5'-AG and 3'-GG nucleotides. The 5'-AG and 3'-GG-flanking nucleotides enhance binding by SOX9 HMG domain but not by the HMG domain of another SOX factor, SRY. For SRY, different 5'- and 3'-flanking nucleotides are preferred (43). Therefore, in the SOX binding motif of mor distal promoter, the presence of at least two bases at the 5'-flanking region and a specific four bases at 3'-flanking region of the core-binding site are required for SOX protein binding (e.g. Sox18 and Sox21).

It is very interesting to note that both mor and Sox genes are associated with different effects depending on sex. Recent studies indicate that the relative efficacy of μ opioids (e.g. morphine) as an antinociceptive agent is greater in male than in female rodents and monkeys (45, 46). Perinatal exposure to opioid drugs produced changes in binding and density of μ opioid receptor that differed regionally and that were mostly different as a function of sex (47). Sex-related differences in the experience of both clinically and experimentally induced pain have been widely reported (48). Several papers have reported a critical role for SOX proteins in sex determination (49) as well as in the proper development of the central and/or peripheral nervous system (12, 32, 50). In addition, Sox18 is expressed in fetal brain (34) and also expressed weakly in adult brain (33). The Sox21 gene is highly conserved and specifically expressed in the brain (51, 52). Sox6 is also specifically expressed in the developing nervous system (32). Collectively, it is feasible that SOX proteins may mediate sex differential responses of the opioid system through the mor distal promoter. Therefore, further studies will help to elucidate the mechanism of association between the sex differential response of the opioid system and opioid receptor gene regulation by SOX proteins.

In conclusion, the foregoing observations indicate that an activating cis-acting element can be used to regulate the target gene transcription (mor) by transcriptional activators like SOX18 and SOX21. Here we contribute to the characterization of the μ opioid receptor transcriptional machinery by identifying two members of the SOX family as strong transcriptional activators of this gene. Future experiments on the regulatory processes that control the activation effect of the positive cis-acting element on the mor distal promoter by SOX proteins will help to understand how the mor gene is expressed temporally, spatially, and developmentally as well as gender-dependently in brain.

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