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# Transcriptional Modulation of Mouse μ-Opioid Receptor Distal Promoter Activity by Sox18

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## ABSTRACT

Previously, we reported the presence of dual promoters, referred to as distal (DP) and proximal, with a negative regulatory element between them in the mouse μ-opioid receptor (*mor*) gene. Here we have identified a positive regulatory element influencing *mor* DP transcription, which contains multiple consensus binding motifs for Sox factors (sex-determining Sry-like high mobility group box-containing genes). In gel supershift assays, the Sox family member Sox18 bound directly to the multiple Sox consensus binding motifs of the *mor* DP enhancer. Overexpression of Sox18 cDNA increased luciferase activity regulated by the *mor* DP, and did so in a Sox18 concentration-dependent manner. In contrast, overexpression of another Sox member, Sox5, triggered no such *trans*-activation of *mor* DP-driven luciferase activity or DNA-protein binding activity. These results suggest that Sox18 directly and specifically stimulates *mor* gene expression, by *trans*-activating the *mor* DP enhancer.



Opioids have been widely used as analgesics after major surgery or to manage severe pain associated with cancer. However, prolonged clinical use triggers side effects of opioids such as tolerance and physical dependence, limiting their effectiveness. Three major types of opioid receptors, μ, δ, and κ, which mediate the pharmacological effects of opioids, have been cloned, and shown to belong to the G protein-coupled receptor superfamily (Mansour et al., 1995). The μ-opioid receptor (mor), the major molecular target of morphine, exhibits discrete expression in different brain regions with variable receptor density (Matthes et al., 1996). The presence of *mor* in differing regions and at varying densities in the brain suggests different functional roles (Delfs et al., 1994a; Nestler et al., 1994; Mansour et al., 1995; Maldonado et al., 1997). For example, mor located in the periaqueductal gray has been suggested to mediate analgesia (Delfs et al., 1994a), whereas mor located in the locus ceruleus and ventral tegmental areas may be involved in the development of tolerance and physical dependence (Nestler et al., 1994; Maldonado et al., 1997). Likewise, the differential role of mor in different CNS areas may also depend on variable regulation of *mor* gene (Delfs et al., 1994b; Azaryan et al., 1996a,b; Ronnekleiv et al., 1996). For example, *mor* is inducibly expressed in response to a variety of physiological and neuronal activities (Delfs et al., 1994b; Simantov and Levy, 1986, 1989), suggesting that *mor* is temporally, spatially, or developmentally regulated.

Characterization of the mouse *mor* gene (Min et al., 1994) has revealed the presence of dual promoters, referred to as distal (DP) and proximal (PP) (Ko et al., 1997), that may contribute to its differential temporospatial, activity-dependent, and developmental expression. RT-PCR analysis using total RNA extract from adult mouse whole brain showed that the expression ratio of two promoters is 20 (PP):1 (DP) (Ko et al., 1997), suggesting that PP may be a dominantly expressed *mor* promoter. However, the detailed mechanisms governing *mor* expression, including the role of PP and DP, remain largely unknown. We could not exclude the possibility that DP may have important role(s) in a particular brain region or condition of the brain, controlling the overall balanced mor expression in vivo. The proportional activities of the DP and PP might vary, depending on the influence of a 34-bp negative element residing in a bridging regulatory between the two promoters, known as the 5′-DP negative regulatory sequences (5′-DPRS) (Ko et al., 1997; Choe et al., 1998). Therefore, understanding the regulatory system and the mecha-

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**ABBREVIATIONS:** mor, μ-opioid receptor; *mor*, μ-opioid receptor gene; CNS, central nervous system; DP, distal promoter; PP, proximal promoter; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); 5′-DPRS, 5′-distal promoter regulatory sequence; Sox, Sry-like HMG-box containing transcription factor (Sox, mouse; SOX, human); PCR, polymerase chain reaction; SV40, simian virus 40; CHO, Chinese hamster ovary; N2A, Neuro2A; EMSA, electrophoretic mobility shift assay; E, enhancer; S, silencer; HMG, high mobility group; wt, wild-type; mut, mutated; Basic, pGL3-Basic.

nism(s) of *mor* DP might be critical for better understanding of the dual promoter system in the *mor* gene.

In the present study, we show that the *mor* DP is regulated by a 15-bp *cis*-acting enhancer element containing multiple Sox consensus binding motifs in the 5′-DPRS. Moreover, we demonstrate that the *mor* DP is *trans*-activated by binding of the Sox18 directly to the enhancer element without affecting the PP activity. Sox proteins have critical roles in the regulation of numerous developmental pathways, such as those of epithelium-derived tissues, including the nervous system (Connor et al., 1995; Lefebvre et al., 1997; Tani et al., 1997; Wegner, 1999), T-cell differentiation (Van de Wetering et al., 1993; Wotton et al., 1995), and bone formation and gonadogenesis (Lefebvre et al., 1997; Pevny and Lovell-Badge, 1997; Wegner, 1999). Recently, the expression of Sox18 in the CNS has been reported (Azuma et al., 2000), although its role in regulating particular CNS genes has hitherto been unknown. Therefore, the *mor* gene is, to the best of our knowledge, the first reported Sox18 target gene expressed in brain.

## Materials and Methods

**Plasmid Construction.** pL1.3K/444, pLup, pL450, pL800, pL1.3K/687, pL1.3K/721, L3, and L7 constructs have been described previously (Ko et al., 1997; Choe et al., 1998). For progressive site-directed PCR mutagenesis, PCR with the sense and antisense primers bearing the restriction enzyme site *Xba*I (the positions are indicated by the construct name) and template pL1.3K/721 was performed. For the construction of p4x25s/SV40 and p4x25as/SV40, four copies of tandem repeats of double-stranded oligonucleotides (−748 to −724) (5′-GAAAAAGACAATTGTTTCTTTGAAC-3′) were cloned at 5′ upstream of SV40 promoter (pGL3-Promoter) at *Bgl*II site. The resulting constructs were referred to as p4x25s/SV40 with sense direction of the inserted double-stranded oligonucleotide and p4x25as/SV40 with antisense direction. mutp4x25s/SV40 and mutp4x25as/SV40 were constructed using *Xba*I-bearing-mutated (−739 to −734) four copies of tamdemly repeated double-stranded oligonucleotides as an insert and screened for the both orientations. All the resulting constructs were verified by restriction digestion and PCR DNA sequence analysis.

**Cell Culture, Transfection, and Reporter Gene Assay.** Human neuroblastoma NMB cells were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum and antibiotics in an atmosphere of 5% $CO_2$ at 37°C. HeLa cells were grown in basic minimal essential medium containing 2 mM L-glutamine, 1.0 mM sodium pyruvate, fetal bovine serum, and antibiotics, penicillin/streptomycin (Life Technologies, Gaithersburg, MD). Fibroblastoma of Chinese hamster ovary (CHO) cells, mouse neuroblastoma NS20Y, and N2A (Neuro2A) were maintained in Dulbecco's modified Eagle's medium in an atmosphere of 10% $CO_2$ at 37°C. For transfection, cells were plated 24 h before transfection at a density of $1 \times 10^6$ cells/plate in six-well plates. Cells were transfected with construct plasmids using the SuperFect Transfection reagent (QIAGEN, Valencia, CA) as described by the manufacturer. All transfection experiments were repeated four or more times with similar results, using plasmids that were independently prepared at least twice. To correct the differences in transfection efficiency, one-fifth molar ratio pCH110 plasmid (Amersham Pharmacia Biotech, Piscataway, NJ) containing β-galactosidase gene under the SV40 promoter was included in each transfection and used for normalization. The luciferase and β-galactosidase activities were determined using Luciferase Assay System (Promega, Madison, WI) as described by the manufacturer.

**Quantification of mRNA by Northern Analysis.** RNA was prepared using RNeasy Maxi kit (QIAGEN) as described by the manufacturer. The method for Northern blot hybridization was as described previously (Lee et al., 1998). Briefly, the prepared RNA was resolved on 1% agarose-formaldehyde gels followed by blotting onto Hybond membrane (Amersham Pharmacia Biotech) under conditions recommended by the supplier. Blots were incubated with random primed probes, washed, and subjected to autoradiography.

**Nuclear Extract Preparation, in Vitro Translation, and Electrophoretic Mobility Shift Assays (EMSA).** Nuclear extract preparation and EMSA were performed as described previously (Ko et al., 1998). In vitro translation was conducted using TNT Quick Coupled Transcription/Translation Systems (Promega) as described by the manufacturer. Briefly, nuclear extracts or in vitro translated protein was incubated with 1 ng of $^{32}$P-labeled double-stranded oligonucleotides probe in 10 μl of reaction solution containing 10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM dithiothreitol, and 0.1 mg/ml poly(dI-dC). For competition analysis, the competitor oligonucleotides were also added to the mixture. After incubation at 22°C for 15 min, the mixture was analyzed on 5% nondenaturing polyacrylamide gels. For antibody supershift assays, 2 μl of anti-Sox18 antibody was added to the mixture. The reaction was then incubated at room temperature for 30 min followed by fractionation of the DNA-protein complexes and free DNA on 5% polyacrylamide gels in 0.5× Tris borate-EDTA buffer at 4°C and were visualized by autoradiography. The double-stranded oligonucleotides used as probes are shown in Table 1.

**Construction of Sox Protein Expression Vector.** RT-PCR was performed using ThermoScript RT-PCR System (Life Technologies) as described by the manufacturer. The cDNAs obtained from RT-PCR were gel-purified and cloned into pCRII-TOPO cloning vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) by following the instructions provided by the manufacturer. The coding sequences in pCRII-TOPO cloning vector were separated by an *Eco*RI digestion followed by running on a 1% agarose gel, and the cDNA fragments were gel purified to subclone into an *Eco*RI site of pcDNA3 expression vector. The resulting mammalian expression plasmids were verified by restriction enzyme digestion and the orientation was confirmed by PCR sequencing. Primer sets used for RT-PCR are as follows: Sox5-f: 5′-AGA GGT GAC CCT TAC CCT GTT CAG CTG ATC-3′; Sox5-r: 5′-CTT GGC CAC TGG GAA GGA TGA ACC GGA CA-3′; Sox18-f: 5′-CAT CAG ACC TCC GTA CTT GGC TTT GCA GTG-3′; and Sox18-r: 5′-TTA GCT TCT TCA CCA CCA ATC CTG GCA GAG-3′.

**Antisense Experiment.** Morpholino oligonucleotides SOX18 antisense (Gene Tools, LLC, Corvallis, OR) was transfected using SuperFect Transfection reagent (QIAGEN). Antisense- and plasmid-delivered cells were harvested after 24- or 48-h incubation at 37°C. Luciferase assay was performed using Luciferase Assay System (Promega) by following the instructions provided by the manufacturer. Nuclear extract from antisense delivered-cells was analyzed by EMSA. The antisense SOX18 sequences used in this experiment is as follows: SOX18 antisense, 5′-CGTAGCCGGG-CGGCGATCTC-TGCATTCCAG-3′; and nonspecific antisense, 5′-CTAAGCCGAGGC-GGCTATCTGTGCTATCCGA-3′.

**Antibodies.** anti-Sox18 antibody:rabbit antibodies directed against Sox18 were obtained by using a peptide (SRTRPDATTLPY-HVACISG) corresponding to the C terminus of mouse Sox18. It was conjugated to Diphtheria toxoid through the cysteine side chain

TABLE 1
Double-stranded synthetic oligonucleotides used as probes for EMSA
The mutated base pairs were indicated by bolded lowercase letters.

| Double-Stranded Oligonueotides | Sequence |
|---|---|
| wt(−748/−724) | GAAAAAGACAATTGTTTCTTTGAAC |
| wt(−756/−735) | CTCTTTTAGAAAAAGAACAATTG |
| wt(−741/−724) | ACAATTGTTTCTTTGAAC |
| wt(−744/−724) | AAGACAATTGTTTCTTTGAAC |
| mut(−742/−741) | GAAAAA**tc**CAATTGTTTCTTTGAAC |
| mut(−739/−737) | GAAAAAGAC**tga**TGTTTCTTTGAAC |
| mut(−738/−736) | GAAAAAGACA**gaa**GTTTCTTTGAAC |
| mut(−734/−733) | GAAAAAGACAATTG**aa**TCTTTGAAC |

using maleimide chemistry (Lee et al., 1980). The conjugated peptide was emulsified with 2 volumes of complete Freund's adjuvant. The total volume of the emulsion per animal was 1 ml containing 0.47 mg of peptide, which was injected subcutaneously. A second similar immunization followed 2 weeks later, using incomplete Freund's adjuvant, and the rabbits were bled 3 to 5 weeks after the second injection. Specific antibodies were affinity purified using the same peptide, covalently coupled to thiopropyl-Sepharose 6B gel and acid elution. Antibodies against Sox5/liter-Sox5, Sox6, and Sox9 were generously provided by Dr. Veronique Lefebvre (University of Texas, Houston, TX).

## Results

**Identification of an Enhancer in the 5′-Untranslated Region in Mouse μ-Opioid Receptor Gene.** In a previous study, we narrowed down the 5′-DPRS of mor to a 34-bp segment (−721 to −687) by detailed 3′ deletion mapping of the 5′-DPRS (Choe et al., 1998; Fig. 1A). Further deletion analysis showed that a DNA fragment from −721 to −775 maximized DP activity (pL1.3K/721) by 300% compared with those of pLup, which does not contain the DNA fragment (Fig. 1B). The significantly reduced DP activity after the deletion of the DNA sequences from −721 to −775 implied that this DNA segment might contain an enhancer, thereby increasing the basal DP activity. The luciferase reporter constructs, pL1.3K/444 and pL1.3K/687 containing DP + enhancer (E) + 34-bp silencer (S), showed no luciferase activity driven by DP. It suggests that the enhancer may not be able to function in the presence of the silencer (Fig. 1B). We next addressed the question whether this newly identified positive element could influence the PP activity. In a previous study, we already demonstrated that the silencer inhibits only the DP but not the PP activity at the level of transcription (Choe et al., 1998). As shown in Fig. 1B, cells transiently transfected with luciferase construct containing E + S + PP (pL800) showed similar luciferase activity to those of basal PP (pL450), suggesting the enhancer in the mor promoter

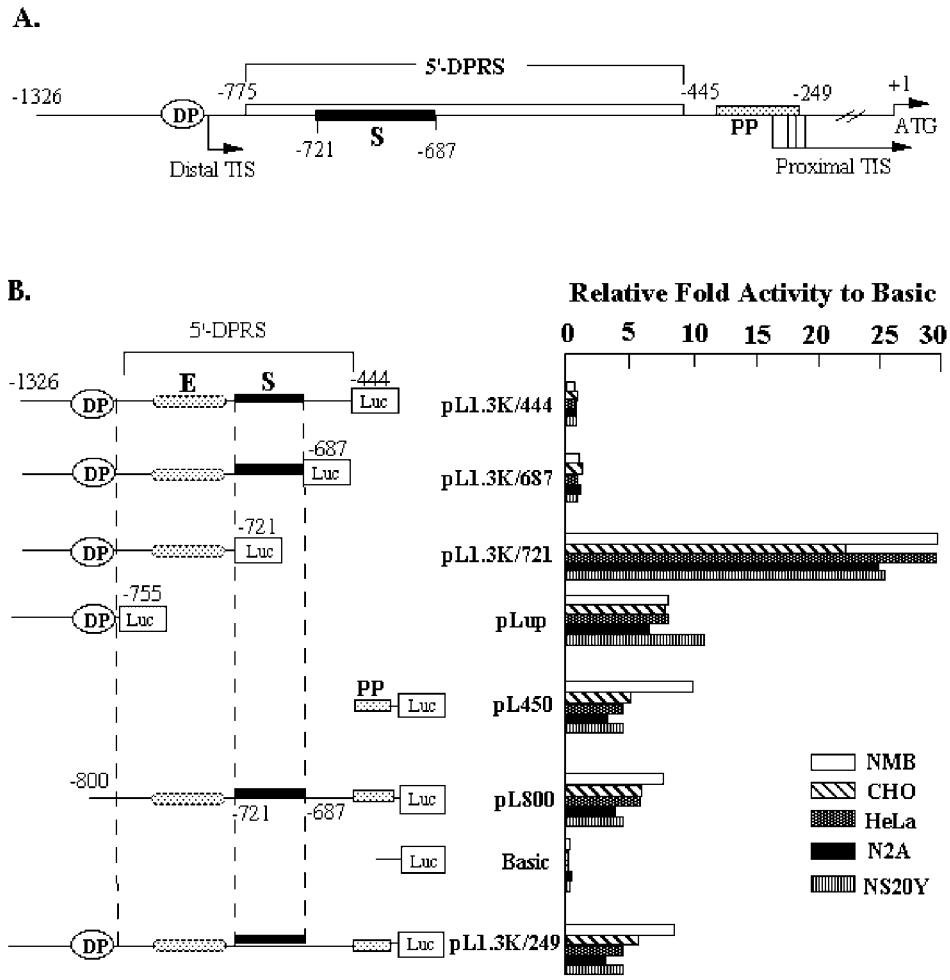


**Fig. 1.** The mouse *mor* promoter. A, structural features of 5′-flanking sequences (nucleotides −1326 to +1) of *mor*. Nucleotide +1 corresponds to the translation start site (ATG). DP, minimal functional domain of the mouse *mor* distal promoter. PP, minimal functional domain of the mouse *mor* proximal promoter. TIS, transcriptional initiation site. Black bar (S), 34-bp DP silencer. B, identification of the enhancer (E) by 5′- and 3′-deletion analysis. Schematic representation of a series of 5′- and 3′-deletion constructs of the mouse *mor* in the recombinant luciferase (Luc) reporter gene system (left) and the summary of luciferase reporter assay results (right). Each construct was transfected into the endogenous *mor*-expressing neuronal cell line NMB (□, and into various *mor* nonexpressing cells, including non-neuronal CHO (▨) or HeLa (▨) cells and neuronal N2A (■) and NS20Y cells (▥). The cells were harvested 24 h after transfection and a luciferase reporter assay was performed. "Basic" construct (pGL3-Basic), which contains no enhancer/promoter, was used as a negative control. Transfection efficiencies were normalized to the β-galactosidase activity by cotransfecting with the internal control *lac* gene plasmid pCH110. The activities of the luciferase reporter were expressed as *n*-fold relative to the activity of the Basic construct, which was assigned an activity value of 1.0. The data shown are means of three independent experiments with at least two different plasmid preparations.

does not affect PP activity. Similar results were obtained when luciferase construct containing DP + E + S + PP (pL1.3K/249) was transiently transfected in all cell lines tested. Collectively, our results suggest that the two opposite regulatory elements (positive and negative) within the mor promoter region exert their effect only on DP activity.

To determine whether the enhancing activity is regulated in a tissue- or cell-specific manner, transient transfection studies were performed in several neuronal and non-neuronal cell lines. These cell lines include a human neuronal cell line, NMB, which expresses endogenous mor (Baumhaker et al., 1994) and mouse neuronal cell lines NS20Y and N2A. NS20Y cells express only δ-opioid receptor, whereas N2A is not known to express any opioid receptor. Parallel experiments were also carried out using non-neuronal cells such as CHO and HeLa. Compared with pLup, which contains DP only, construct pL1.3K/721 containing the enhancer region showed increased luciferase activity in all the cell lines tested. These results indicated that the enhancer element is not tissue- or cell-type specific, because its presence elicits the similar effect on the luciferase activity in neuronal (NMB, NS20Y, and N2A) and non-neuronal (CHO and HeLa) cells (Fig. 1B).

To define critical sequences within the enhancer region, the DNA segment from −721 to −776 was subjected to site-directed mutagenesis analysis. Mutants that contain progressively replaced base pairs of the DNA sequences from −721 to −776 were generated by PCR using pL1.3K/721 as a template. Luciferase construct pL1.3K/721 contained intact DNA fragment from −721 to −775 with a maximum DP activity. The resulting mutant constructs were transiently transfected into NMB cells (Fig. 2). Compared with pL1.3K/721, the mutant constructs showed progressively decreased luciferase activity. Especially, mutations between −730 and −744 (15-bp) (mu730, mu732, mu735, mu737, mu739, mu742, and mu744) showed progressive and dramatic decreases in luciferase activity, reaching the activity of basal DP (pLup). Mutations further upstream from −750 to −776 (mu755 and mu776) showed no significant reductions in the luciferase activity. These findings suggest that the location of the critical enhancer element was between −730 and −744 (15-bp), 10-bp upstream of the 34-bp negative *cis*-acting element, and that the critical enhancer element was responsible for the enhanced DP activity. When those mutants were transiently transfected into other cell lines, such as CHO, NS20Y, N2A, and HeLa, the decreased pattern of the luciferase activities were similar to those of NMB cells (data not shown).

**Increased DP Activity Is Not Due to Translational Modification.** Although the 5′-untranslated region is known to be predominantly involved in translational control, several studies suggested that this region could also influence transcriptional activity (Hershey, 1991; Choe et al., 1998). Because the 34-bp negative element inhibits the DP at the level of transcription, we hypothesized that the stimulating effect of the enhancer element on luciferase activity driven by the *mor* DP may occur at the transcriptional level. To verify this hypothesis, the amount of luciferase mRNA generated by the individual mutants after transient transfection into CHO cells was compared by Northern blotting.

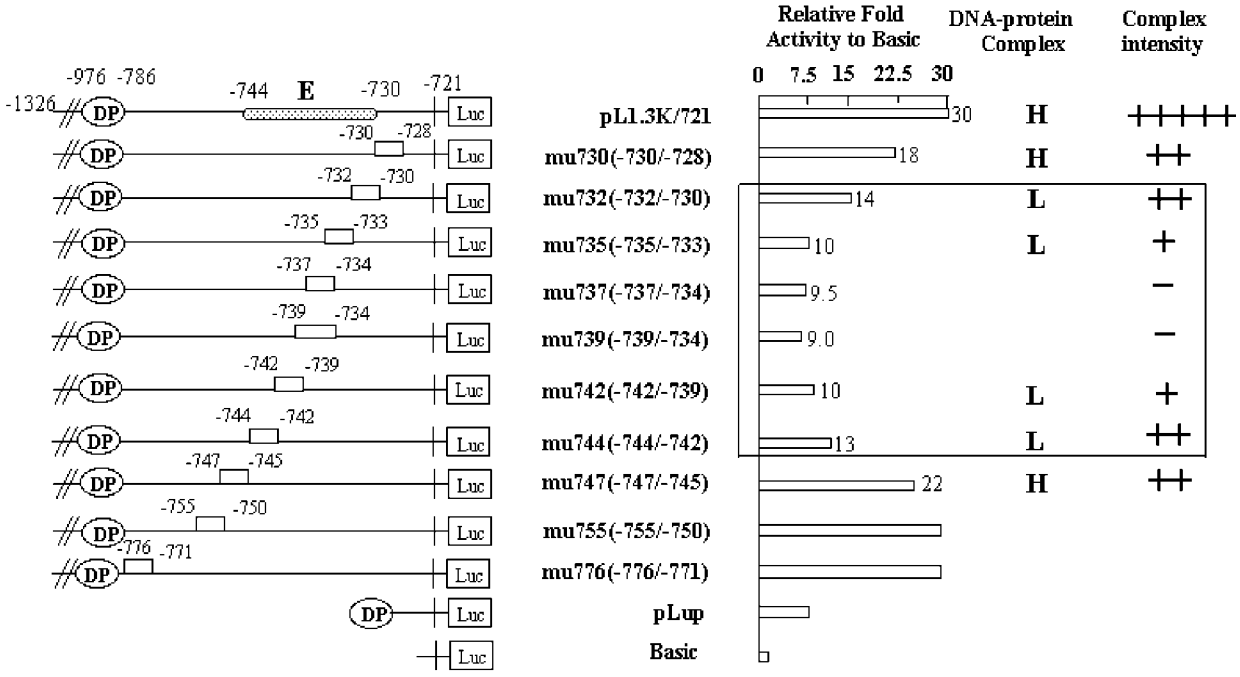


**Fig. 2.** Operational characterization of the *mor* DP enhancer region by progressive site-directed PCR mutagenesis. Schematic representation at left shows the relative locations of mutated enhancer region. Each white rectangle represents the position of a clustered region of two to six nucleotide substitutions introduced by PCR. The positions of the mutated sequences are indicated by the mutant construct name and marked at the top of each white rectangle. pL1.3K/721, the wild-type enhancer construct expressing the maximum *mor* DP activity, was used as the template for PCR mutagenesis. The critical enhancer region (−744/−730) identified is indicated by the gray bar (E). The enhancer mutants were then transfected into NMB cells. Transfection efficiencies were normalized as described in Fig. 1. The relative luciferase activity data, cross-compared with the extent of enhancer DNA-protein complex formation (from data in Fig. 4), shown at right, are means of five independent experiments with at least three different plasmid preparations. H, high-molecular-weight DNA-protein complex; L, low-molecular-weight DNA-protein complex. The intensity of the DNA-protein complex was shown by cross symbols. +++++, very strong; ++, weak; +, very weak; −, no binding activity.

The results showed that the increased promoter activity was associated with the increased mRNA (Fig. 3A).

Northern blot analysis is a reflection of a steady-state level of mRNA. Therefore, we examined the possibility of the increased DP activity associated with the 15-bp enhancer element resulting from post-transcriptional event, such as, mRNA stability by a nuclear runoff assay. Nuclei from transiently transfected CHO cells with pL1.3K/721 and its mutant mu739(−739/−734) were prepared and labeled with [$^{32}$P]UTP. Basic plasmid (pGL3-Basic) was used as a negative control. As demonstrated in Fig. 3, B and C, the transcription of mu739(−739/−734) showed 80% decreased transcription activity compared with that of wild-type, pL1.3K/721. These results clearly demonstrated that the increased mRNA associated with the enhancer element of *mor* results from an accelerated transcription rate.

**Multiple DNA-Protein Interactions Are Required for Maximized Enhancing Activity.** Our site-directed mutagenesis analysis suggested that the DNA sequence from −744 to −730 (15 bp) of *mor* gene is critical for the enhancement of DP activity. DNA sequence analysis revealed that the enhancer region of the *mor* contains multiple consensus binding motifs for Sry-like HMG transcription factors, such as Sox proteins. These multiple Sox core binding sequences are located from −742 to −733 (10-bp DNA stretch) and are present in a 2-bp overlapped form (Fig. 4A). The possible binding of Sox protein(s) within the enhancer region was performed by EMSA using different lengths or various mutant oligonucleotides and nuclear extract from NMB cells (Fig. 4B). The double-stranded synthetic oligonucleotides used as probes for EMSA are described in Table 1. The EMSA results were analyzed and are shown in a schematic diagram (Fig. 4C). The competition EMSA using unlabeled specific or nonspecific oligonucleotides showed that the DNA-protein interactions are sequence-specific (data not shown). As shown in Fig. 4, the enhancer region between −748 and −724 (wt 25 bp), which contains two distinct Sox consensus binding motifs in a 2-bp overlapped pattern, formed two DNA-protein complex bands, high (H) and low (L) molecular weight. When the oligonucleotide probe was divided in half to include only one intact Sox binding motif at a time (wt−756/−735 and wt−741/−724), the high-molecular-weight complex disappeared and only the low-molecular-weight complex remained. Similar results were observed when either half of the binding motif was mutated (mut−742/−741 and mut−734/−733). The formation of low-molecular-weight complex when mut(−742/−741) was used as a probe, suggested that the first Sox binding site, GACAAT, was unable to bind a protein due to the mutation. Similarly, when the second Sox consensus binding motif, ATTGTT, was mutated (mut−734/−733), only a low-molecular-weight complex was detected. When the oligonucleotides were mutated at the 2-bp overlapped region, and used as probes, DNA-protein interactions were completely abolished (mut−739/−737 and mut−738/−736 in Fig. 4, B and C). These results suggested that the mutation of this area destroyed both binding recognition sites.

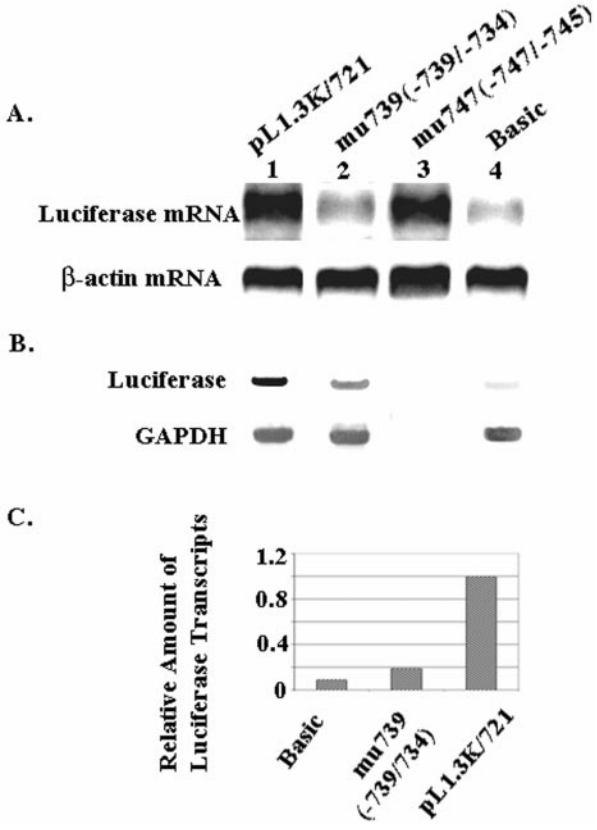


**Fig. 3.** Transcriptional activity of the *mor* DP. A, Northern blot quantification of *mor* DP enhancer-induced transcripts. The amount of mRNA transcripts from pL1.3K/721 and its PCR mutants was compared by northern analysis. mRNAs (5 $\mu$g) from CHO cells transiently transfected with plasmids pL1.3K/721, mu739, mu747, and Basic construct (pGL3-Basic) were loaded to a denaturing agarose gel, transferred to a nylon membrane, and hybridized with a luciferase cDNA probe. The Basic and actin probes were used as negative and quantitative controls, respectively. B, nuclear runoff assay quantification of *mor* DP enhancer-induced transcription. Nuclei from CHO cells transiently transfected with plasmids pL1.3K/721, mu739, and Basic construct were prepared and used to generate [$^{32}$P]UTP labeled runoff transcripts. The labeled RNA was hybridized to the slot blots carrying 3-$\mu$g quantities of luciferase cDNA. Basic and hamster GAPDH cDNA samples (5 $\mu$g) were included as negative and quantitative controls, respectively. C, relative amount of the luciferase transcription rate from the runoff assay is depicted, after normalization to GAPDH transcription rate (represented as relative intensity).

The formation of the DNA-protein complexes shown in EMSA strikingly corresponded to the promoter activity of the constructs presented in the mutagenesis analysis of the enhancer element region in NMB cell systems (Fig. 2). In the mutagenesis results, the mutation of the promoter region between −730 and −744 that included both Sox consensus binding sites showed dramatically reduced DP activity. Furthermore, the mutated constructs between −739 and −734, the region around the overlapped junction of two binding motifs, completely abolished the enhancing activity (Fig. 2). Taken together, these results strongly suggest that multiple DNA-protein interactions in both Sox consensus binding motifs may be required to exert maximum enhancing activity.

**Sox18 Binds to Sox Consensus Motif ATTGTT.** Our results suggested that Sox protein(s) could affect the DP activity via the multiple Sox consensus binding motifs of the *mor* promoter region. Supershift experiments were performed using nuclear extract from the *mor*-expressing cell line NMB cells, so as to elucidate the identity of the Sox protein involved. We first tested whether Sox18 could be one potential Sox protein to bind to the Sox putative binding sites. An anti-Sox18 antibody was able to supershift the DNA-protein complexes formed with a radiolabeled 25-bp

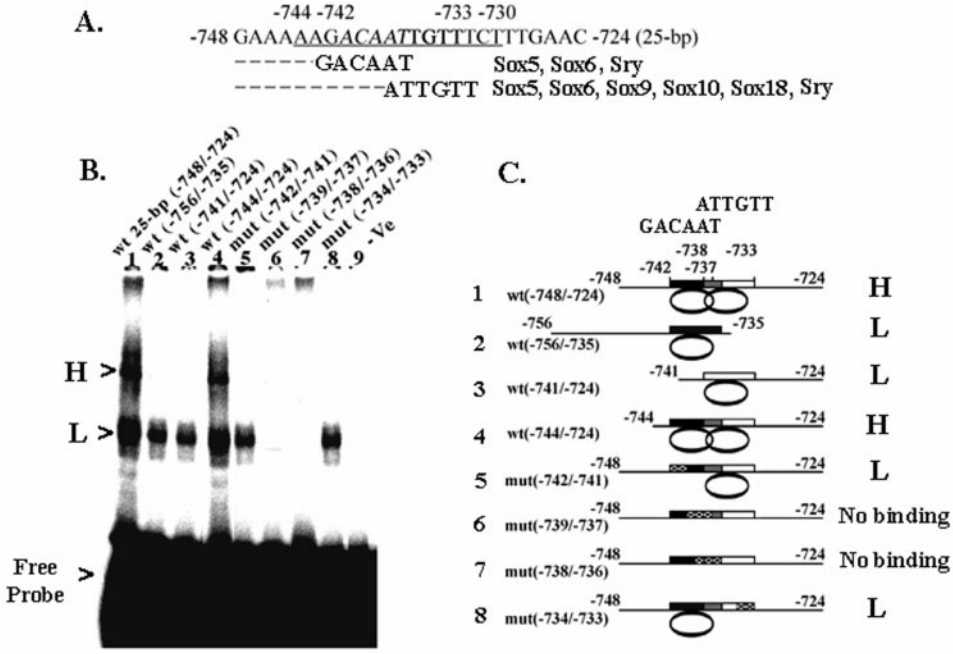


**Fig. 4.** Analysis of the multiple Sox consensus binding motifs. A, DNA sequence analysis shows putative multiple Sox binding sites between −744 and −730 in 2-bp overlapped form. High-affinity binding Sox proteins to the right of the sequence. The critical enhancer region (−744/−730) identified from mutagenesis analysis was underlined. B, EMSA was performed using different-length wild-type and mutant enhancer oligonucleotide sequences as probes (indicated by the probe names), which were incubated with endogenous *mor*-expressing NMB cell nuclear extracts. Mutations within the overlap of the two Sox consensus sites abrogate binding activity, indicating that both binding sites lose binding ability. Arrows indicate detection of both a high-molecular-weight DNA-protein complex (H) and low-molecular-weight DNA-protein complex (L). C, schematic illustration of EMSA results. The first Sox binding site, GACAAT (−742 to −737, black bar) and the second site, AATGTT (−738 to −733, white bar) overlap by 2-bp (gray bar), forming high- (H) or low- (L) molecular-weight complexes. Crossed boxes indicate the location of mutated positions in the mutant enhancer oligonucleotides.

oligonucleotide containing both Sox consensus binding motifs (−748/−724) (Fig. 5, lane 4). A similar result was obtained when oligonucleotide (−741/−724) containing the second Sox consensus binding motif, ATTGTT, was incubated with nuclear extract from NMB cells. However, oligonucleotide (−756/−735) containing the first Sox binding motif, GACAAT, failed to supershift the DNA-protein complex. In parallel, in vitro-translated Sox18 was incubated with an anti-Sox18 antibody as a positive control. This in vitro translated Sox18 protein was able to supershift the DNA-protein complex. On the other hand, no supershifted complex bands were observed when antibodies against Sox5/L-Sox5, Sox6, and Sox9, which are endogenously expressed in adult mouse brain, and NMB cells (data not shown) were added. Taken together, these results strongly suggested that Sox18 is one of the transcription factors responsible for the stimulated DP activity by directly interacting with the consensus motif ATTGTT of the *mor* promoter region. Furthermore, the observation that Sox18 protein did not interact with the first Sox consensus binding motif, GACAAT, suggests that other Sox or HMG proteins might interact with this binding motif.

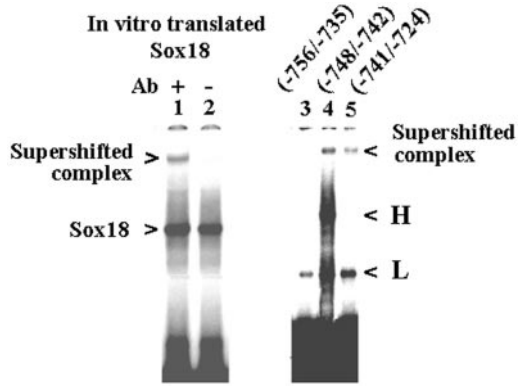


**Fig. 5.** Supershifting of DP enhance-protein complex by anti-Sox18 antibody. Lanes 1 and 2, in vitro translated Sox18 complexed with a $^{32}$P-labled oligonucleotide probe representing the 25-bp wild-type enhancer sequence (−748/-742) is supershifted when Sox18 is first incubated with anti-Sox18 antibody (lane 1), compared with no antibody incubation (lane 2). Lanes 3 to 5, anti-Sox18 antibody supershifts the complexes formed between NMB cell nuclear extract and oligonucleotide probes representing either the wild-type enhancer sequence (−748/−742) containing the both Sox binding sites (lane 4), or a partial enhancer sequence (−741/−724) containing only the second Sox binding site, ATTGTT (lane 5), but not the other partial enhancer sequence (−756/−735) containing only the first Sox binding site, GACAAT, where no supershifting was observed (lane 3). H, high molecular weight; L, low molecular weight.

**Sox18 *trans*-Activates *mor* DP through the Enhancer Element.** To test the ability of Sox18 to *trans*-activate transcription of *mor* DP through the Sox consensus binding site, Sox18 was overexpressed in cells transfected with luciferase reporter constructs. These luciferase reporter constructs contained four copies of the multiple Sox consensus binding motifs of the *mor* gene (25-bp) at 5′ upstream of SV40 promoter of pGL3-Promoter vector. The resulting constructs were referred to as p4x25s/SV40 with sense direction and p4x25as/SV40 with anti-sense direction (Fig. 6). When the construct p4x25s/SV40 was transiently transfected in the cell lines tested (NMB, HeLa, CHO, NS20Y, and N2A), a 4-fold increased luciferase activity was observed compared with that of pGL3-Promoter. In parallel, mutant constructs containing four copies of mutated multiple Sox binding motifs (25 bp) were also transiently transfected as a negative control. These mutated 25-bp–bearing constructs (under *Mate-*

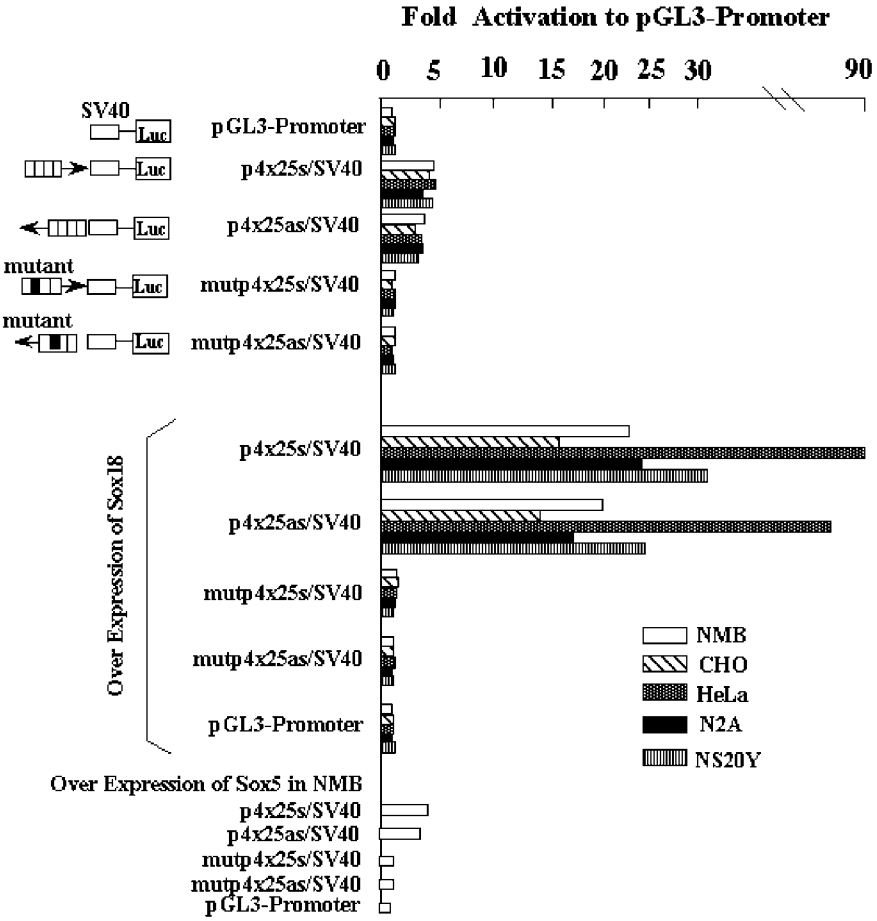


**Fig. 6.** DP *trans*-activation by Sox18 via the multiple Sox binding motifs of *mor*. Sox18 cDNA subcloned into the mammalian expression vector pcDNA3 was cotransfected into neuronal and non-neuronal cells, such as NMB, CHO, HeLa, N2A, and NS20Y cells, together with the "p4x25s/SV40" and "p4x25as/SV40" luciferase reporter constructs, which, respectively, contain four copies of the 25-bp multiple Sox binding motifs (−748/−724) inserted 5′ upstream of SV40 promoter (pGL3-Promoter) in either the sense or antisense orientation. Identical cotransfections were also performed with the "mutp4x25s/SV40" and "mutp4x25as/SV40" luciferase plasmid constructs, which are identical to those described above except for the presence of mutated Sox binding motifs (done by insertion of *Xba*I site between −739 and −734). Arrows shown in the wild-type or mutant constructs indicate the orientation of the Sox binding sites. Luciferase activity was measured after 24-h incubation. Transfection efficiencies were normalized according to β-galactosidase activity from the cotransfected internal control plasmid pCH110 and luciferase reporter activities were expressed as *n*-fold relative to the activity of the pGL3-Promoter, which was assigned an activity value of 1.0. The data shown are means of three independent experiments with at least two different plasmid preparations. In parallel, overexpression of Sox5 was performed to test for specificity of the *trans*-activation ability of Sox18 via the multiple Sox consensus binding sites of the *mor* DP. Sox18 *trans*-activated cotransfected luciferase reporter constructs containing the wild-type Sox consensus binding sites from the *mor* promoter, but not the mutated Sox binding sites, nor did Sox18 *trans*-activate the pGL3-Promoter construct in which Sox binding sites were absent. Sox5 did not *trans*-activate any of the wild-type or mutant promoter constructs.

*rials and Methods*) were referred to as mutp4x25s/SV40 and mutp4x25as/SV40 for both orientations. As expected, the mutated 25-bp–bearing constructs showed no stimulated promoter activity compared with the pGL3-Promoter control (Fig. 6.).

Overexpression of Sox18 cDNA increased luciferase activities with a range from 6- to 30-fold, dependent on cell type used. Interestingly, up to 30-fold induction was observed in HeLa cells when cotransfected with the Sox18 expression vector. However, cotransfection of sense- and antisense-oriented constructs (mutp4x25s/SV40 and mutp4x25as/SV40), and pGL3-Promoter, showed no significant changes in the luciferase activity in all cell lines tested. In addition, overexpression of Sox5, which did not show a supershifted complex by the addition of anti-Sox5 antibody in our EMSA analysis, also showed no changes in the luciferase activity when overexpressed in MNB cells. These results strongly indicated a specific *trans*-activation of Sox18 through the enhancer element in the *mor* DP. Taken together, these results demonstrated that the 25-bp DNA fragment could mediate *trans*-activation not only within the context of the *mor* promoter but also within a heterologous promoter. Moreover, these results clearly showed that such *trans*-activation by Sox18 was dependent on the presence of the 25-bp DNA sequences, because Sox18 protein failed to promote luciferase expression from a reporter gene lacking this sequence, pGL3-Promoter. In addition, mutp4x25/SV40 in sense and antisense directions completely abolished *trans*-activity of Sox18, indicating that the *trans*-activation of Sox18 was mediated through the 25-bp binding motif of *mor* DP region.

We next wanted to know whether overexpression of Sox18 protein also could increase the DNA-protein binding activity, subsequently leading to the stimulation of the DP activity. Nuclear extracts from various cell lines in which Sox18 protein was overexpressed were subjected to EMSA. Indeed, overexpression of Sox18 showed an increased DNA-binding activity (Fig. 7). On the other hand, there was no significant change in DNA-binding activity when Sox5 was overex-

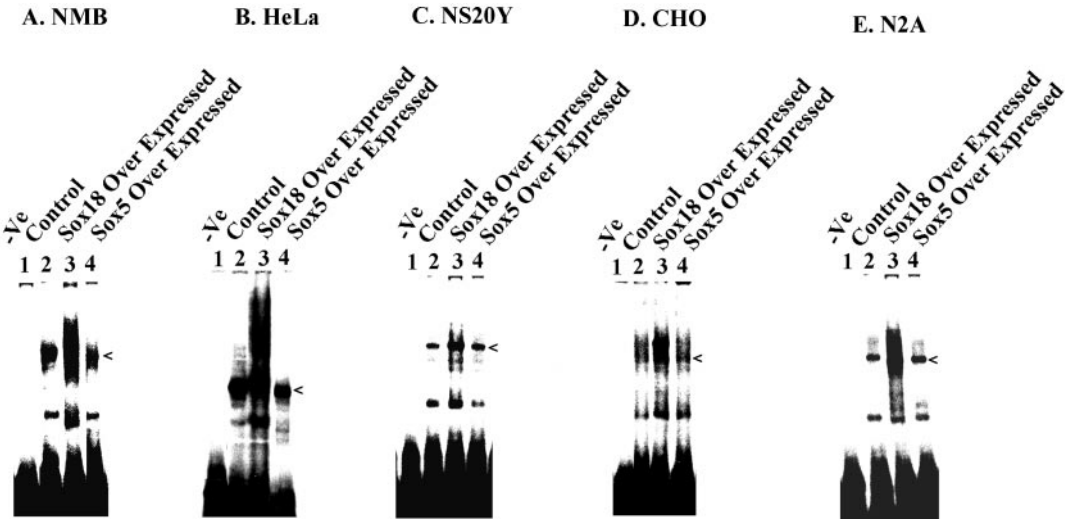


**Fig. 7.** DP enhancer DNA-protein complex formation stimulated by overexpressed Sox18. Stimulation of DP enhancer DNA-protein complex formation by overexpressed Sox18 was examined by EMSA using nuclear extracts from Sox18 expression vector transfected NMB (A), HeLa (B), NS20Y (C), CHO (D), and N2A (E) cells, with the probe representing the 25-bp wild-type DP enhancer sequence (−748/−724), which contains intact multiple Sox consensus binding sites. In parallel to overexpression of Sox18 (lane 3), examination of the extent of stimulation of DNA-protein complex formation by overexpressed Sox5 (lane 4) was also performed as a negative control to confirm the specificity of *trans*-activation by Sox18. Nontransfected cells were used for nuclear extract preparation for controls with the 25-bp probe (lane 2, Control) or without the 25-bp probe (lane 1, −Ve). After transient overexpression of Sox proteins, cells were harvested after 24-h incubation at 37°C. Over-expression of Sox18 (*lane 3*) showed a significantly increased extent of DNA-protein complex formation compared with that of nontransfected cell extracts (lane 2) or Sox5-transfected cell extracts (lane 4).

pressed (Fig. 7). These results suggested the corresponding relationship between the DNA-binding activity and the increased promoter activity. It indicated that the enhancer element-bearing DP could be stimulated by the direct binding of Sox18 to the enhancer region in the *mor* promoter region.

**Enhancer-Mediated *trans*-Activity by Sox18 Is Dose-Dependent.** We investigated whether the increased luciferase activity after overexpression of Sox18 is dose-dependent. An increasing amount of Sox18 cDNA was transfected in NMB and HeLa cells. As shown in Fig. 8, Sox18 can specifically *trans*-activate the DP activity through the *cis*-acting element of enhancer region in a concentration-dependent manner. Mutation of the Sox consensus binding sequence (mutp4x25s/SV40) or pGL3-Promoter vector alone abolished the *trans*-activity mediated by Sox18. Overall,

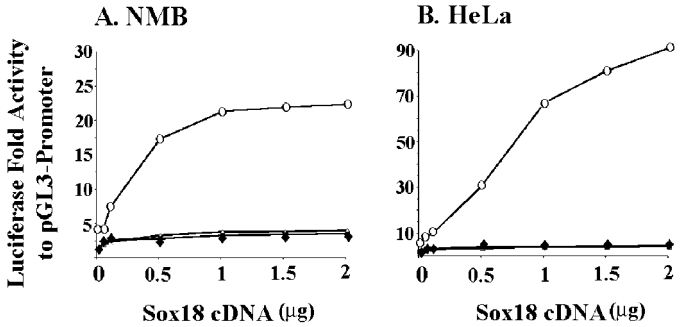


**Fig. 8.** *Trans*-activation by Sox18 of the *mor* DP enhancer is concentration-dependent. Sox18 cDNA-based expression vector was cotransfected with the wild-type DP enhancer luciferase reporter construct containing four tandem repeats of the multiple Sox consensus binding sites of the *mor* DP (p4x25s/SV40). As the amount of transfected Sox18 cDNA is increased, the promoter activity of p4x25s/SV40 (●) is also increased in dose-dependent manner in both of two tested cell lines, NMB (A) and HeLa (B). Reporter constructs with either a mutated form of the Sox binding site of the *mor* DP enhancer [mutp4x25s/SV40, (♦)] or without the Sox binding site [pGL3-Promoter, (△)] did not show the *trans*-activation by Sox18.

these results confirmed that the enhancer element can mediate *trans*-activation through direct interaction with Sox18 in a dose-dependent manner.

**Antisense Sox18 Decreases Enhancing Activity of DP and DNA-Protein Complex Formation Mediated by Sox Consensus Binding Site.** We demonstrated that Sox18 could be involved in the enhanced promoter activity through binding to the second Sox consensus binding site in the *mor* promoter region (Figs. 4 and 5). If this is the case, then depleting the endogenous Sox18 by blocking its translation should result in both reduced luciferase activity driven by pL1.3K/721 that expresses maximum DP activity (Fig. 2) and decreased DNA-protein complex formation. Specific human Sox18 antisense oligonucleotides were delivered together with pL1.3K/721 into human cell lines NMB and HeLa, which were shown to endogenously express Sox18 by RT-PCR analysis (data not shown). In parallel, controls were carried out with a nonspecific antisense oligonucleotide (scrambled Sox18 antisense oligonucleotide) or with transfection reagent only. As expected, delivery of antisense Sox18 decreased the luciferase activity driven by pL1.3K/721 in both cell lines, NMB and HeLa (Fig. 9A). On the other hand, the nonspecific antisense or the transfection reagent alone failed to show any detectable changes in the luciferase activity when cotransfected with pL1.3K/721. Similar results were observed when the incubation time after transfection was varied from 24 to 48 h (data not shown). Cotransfection of antisense Sox18 with pLup, containing only DP with no enhancer element, showed no changes in the luciferase activity compared with pLup activity without antisense Sox18. This suggests that Sox18 protein has no influence in the absence of multiple Sox consensus motifs in the *mor* DP.

The effect of antisense Sox18 oligonucleotide on its DNA-protein binding activity was also investigated. In cell lines NMB and HeLa, reduced DNA-protein binding activity was observed when the endogenous Sox18 protein synthesis was

blocked (Fig. 9B, NMB, and C, HeLa, lane 2). When the nonspecific antisense or only the transfection reagent was delivered, however, no detectable changes in the intensity of DNA-protein complex were detected, compared with the control in which nuclear extracts from nontreated cells were used (Fig. 9, B and C, lanes 3 and 4). The incubation time after delivery of the antisense was also varied from 24 to 48 h with similar results (data not shown). Considering the strongly enhanced promoter activity observed when Sox18 was overexpressed in HeLa cells (~30-fold) (Fig. 6), and the abolished the DNA-protein complex observed when Sox18 was depleted these results may suggest that Sox18 might be critical for the enhanced *mor* DP activity in the environment of HeLa cells. Taken together, these data showed that *mor* DP expression is modulated by the direct binding of the Sox18 to the enhancer region of the *mor* DP.

## Discussion

Because our identification of a 34-bp DP negative *cis*-acting element between the DP and PP of the *mor* gene (Choe et al., 1998), continuous molecular dissection of the transcriptional regulation of the *mor* gene has identified an another regulatory element of DP positive *cis*-acting element. Our data indicate that the activity of the *mor* DP is regulated by the presence of this enhancer element (−744/−730) at the transcriptional level and that this element has no effect on the PP activity. DNA sequence analysis of the *mor* gene's 5′-flanking region (−2188 to +1) revealed that multiple Sox consensus binding motifs reside in the DP enhancer element, and nowhere else. From EMSA studies, we identified that Sox18 binds directly to the second Sox consensus binding site, ATTGTT, of the DP enhancer. Thus, we report here that the activity of the mouse *mor* DP is regulated by Sox18.

Previously RT-PCR analysis using adult mouse whole brain total RNA extracts showed that the "adult whole brain" expression ratio of the *mor* gene's two promoters is 20 (PP):1 (DP) (Ko et al., 1997) although in the presence of the enhancer region containing multiple Sox binding sites, the expression level of DP is approximately 300% of that of PP (Fig. 1). These in vitro results could reflect, in vivo, a biphasic regulation of less-regulated, predominant expression from the PP in most *mor* expressing neurons, CNS, or adult developmental stages, and *mor* expression from the DP in a smaller subset of neurons, CNS regions, or earlier developmental stages. Using in vivo approaches to identify distinct subsets of brain regions or developmental stages showing stronger DP than PP expression should be carried out in the future. More understanding of the in vivo consequences of *mor* DP activity may contribute to better understanding of the overall role of dual promoters in the *mor* gene. In this regard, we have so far not observed cell line specificity of the DP enhancer's activity, among those cell lines tested in this study. Similar results were obtained from our previous study on the 34-bp DP silencer, which also showed no cell line specificity of its activity (Choe et al., 1998). However, a comprehensive survey of other cell lines remains to be performed.

From our EMSA results and promoter DNA sequence anal-

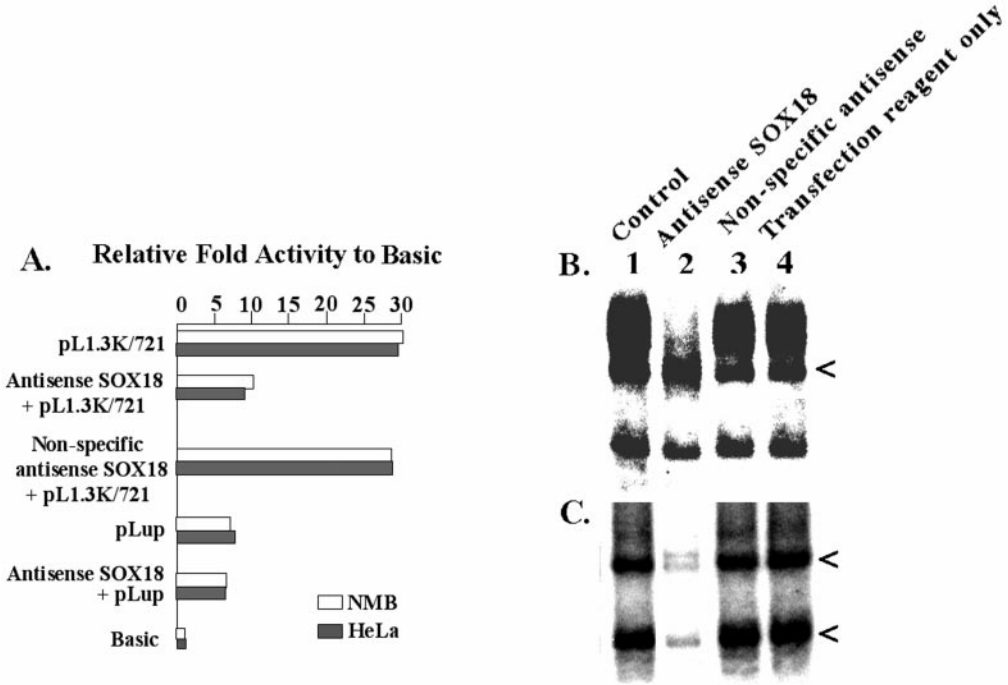


**Fig. 9.** Antisense Sox18 inhibits the *mor* DP activity and DP enhancer-protein complex formation. A, Sox18-specific antisense oligonucleotide or nonspecific antisense oligonucleotide was cotransfected into neuronal NMB cells (white bar) and into non-neuronal HeLa cells (gray bar), along with either the luciferase reporter construct pL1.3K/721 (containing the intact, multiple Sox binding sites of the wild-type *mor* DP enhancer) or the pLup reporter construct (containing the DP without the enhancer). Cell incubation time variations from 24 to 48 h after transfection showed no significant difference. Delivery of the antisense Sox18 oligonucleotide, but not the nonspecific oligonucleotide, significantly reduced the luciferase activity of pL1.3K/721. No detectable changes were observed in the promoter activity of pLup when antisense Sox18 was cotransfected. B and C, EMSA using the 25-bp wild-type DP enhancer sequence (−748/−724) as probe complexed with nuclear extracts from NMB (B) and HeLa (C) cells. Extracts from types of cells treated with antisense Sox18 dramatically decreased enhancer DNA-protein complex formation (lane 2), compared with those of control untreated cells (lane 1), or cells treated with nonspecific (scrambled) antisense oligonucleotide (lane 3) or with SuperFect transfection reagent only (lane 4).

ysis, at least two different proteins appeared to bind to each binding site within the 15-bp DP enhancer DNA fragment (−742/−737 and −738/−733). These multiple DNA-protein interactions in the enhancer element seem to be critical for it to exert maximal enhancement of *mor* DP activity, as shown by the exact correlation between reporter luciferase *trans*-activation and multiple enhancer DNA-protein complex formation. Whether the other protein(s) that binds to the DP enhancer directly dimerize with Sox18 is under investigation. Identification of these other factors that may interact with Sox18 and/or the DP enhancer element will increase our understanding of the mechanism of *mor* DP transcriptional regulation.

Most of the natural target genes of Sox factors have been found to contain multiple binding sites (Lefebvre et al., 1997; Wegner, 1999). It was suggested that the target genes regulated by one group of Sox proteins may have regulatory sites bound by multiple Sox proteins expressed in the same cells, and that the expression level of the genes is determined by the overall effect of the bound proteins. Multiprotein interactions to plural Sox binding sites for sufficient *trans*-activation of the target gene have been demonstrated (Lefebvre et al., 1997). That study reported that the *Col2a1* gene for pro$\alpha$1(II) collagen was activated by the combination of three Sox proteins, L-Sox5, Sox6, and Sox9, bound to the *Col2a1* enhancer, whereas one of the Sox proteins alone was not sufficient to account for the full expression pattern of the *Col2al*. Similar cooperative activation by L-Sox5, Sox6, and Sox9 has also been shown for a second chondrocyte marker, the aggrecan gene (Lefebvre et al., 1997).

Surprisingly, the Sox6 overexpression results obtained from our experiments demonstrated a *trans*-repressive effect (data not shown). We were unable to observe supershifted DP enhancer DNA-protein complex with anti-Sox6 antibody; thus, it is possible that Sox6 interferes with the *trans*-activation by other Sox protein(s) such as Sox18, probably via formation of inactive heterodimers. Previous study suggested Sox6 as a potential transcriptional repressor (Connor et al., 1995); however, we did not further investigate on this possibility. Collectively, these data suggest that Sox proteins perform their function in a complex interplay with other factors, in a manner highly dependent on cell type and promoter- and/or gene context.

Direct protein-protein interaction of Sox proteins with other non-Sox transcription factors is also well documented (Bulfone et al., 1993). For example, early embryonic expression of the *FGF4* gene requires binding of both Sox2 and Oct-3/4 to their respective recognition sites separated by 3 bp. The function of *FGF4* enhancer is critically dependent on the synergistic interaction between these two transcription factors bound to their respective sites, and to their additional engagement in direct protein-protein interactions, thereby forming a ternary complex with exact stereospecific requirements. Because DNA sequence analysis shows that the *mor* DP contains a recognition element for POU (−826/−833) proteins upstream of the DP transcription initiation site, it would be plausible to further investigate the possible protein-protein interactions between Sox and POU proteins in the modulation of *mor* DP activity.

Anti-Sox18 antibody successfully supershifted nuclear extract protein complexed to the sequence (−741/−724) that contained one of the two Sox consensus core sequences in the DP enhancer, ATTGTT, whereas no supershifted complex was observed with the sequence (−756/−735) containing the other Sox consensus core sequence, GACAAT. This result indicates that these two different consensus binding sites for Sox proteins may be recognized by different Sox proteins with unique specificities and preferences. Previously, it was reported that Sox proteins achieve DNA sequence specificity through flanking nucleotide sequences that are likely to be dictated by signature amino acids in their HMG domains (Blaise et al., 1999), subtle preferences for restricted tissue distribution (Pevny and Lovell-Badge, 1997), and combinatorial protein interactions (Bulfone et al., 1993; Peirano and Wegner, 2000). Hence, the preferential affinity of Sox18 for only one of the two Sox consensus binding sites in the *mor* DP enhancer could be specified by either flanking nucleotide sequences, or architectural DNA-protein interactions leading to DNA conformation changes preferential for Sox18 binding to one particular Sox consensus binding site.

This role for Sox18 in *mor* gene expression is intriguing, in light of the knowledge that sex-dependent developmental changes occur in mor expression, nociception, and opioid effects (Dahan et al., 1998; Sarton et al., 1999; Zubieta et al., 1999; Fillingim and Ness, 2000). Other studies have shown a critical role for Sox proteins in sex determination (Jennifer and Marshall, 1998), as well as in the proper development of the central and/or peripheral nervous system (Connor et al., 1995; Tani et al., 1997; Pevny and Lovell-Badge, 1997). Evidence of the expression of Sox18 in the brain (Azuma et al., 2000), as well as Sry in midbrain (Wegner, 1999), makes it plausible that Sox18 regulation could mediate known sex differences in *mor* expression. Therefore, it is tempting to propose that dual promoters could be a mechanism for sexually dimorphic gene regulation by Sox factors, to provide sex differences in pain responses and in the effectiveness of various analgesic agents that act on opioid receptors.

In conclusion, our results suggest that HMG superfamily Sox18 is one of the responsible transcription factors for the enhanced expression of *mor* DP at the level of transcription, by directly binding to the multiple Sox consensus binding motifs in the DP enhancer element. Further studies will be necessary to identify other transcription factor(s) that probably contribute along with Sox18 to activation of the *mor* DP via multiple DNA-protein and/or protein-protein interactions. Continuing studies on the regulatory processes that control the *mor* gene's dual promoters, and their individual regulation by enhancer(s) and silencer(s), should help elucidate the mechanisms conferring temporal, regional, and developmental regulation as well as gender-dependent regulation of cellular *mor* expression.

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