Transcriptional Regulation of Mouse μ-Opioid Receptor Gene*

Jane L. Ko‡§, Hsien-Ching Liu‡, Sharon R. Minnerath, and Horace H. Loh

From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Previously, the existence of dual promoters was reported in mouse μ-opioid receptor (mor) gene, with mor transcription in the mouse brain predominantly initiated by the proximal promoter. In this study, we further analyzed the proximal promoter region, base pairs −450 to −249, to identify cis-DNA regulatory elements and trans-acting protein factors that are important for mor promoter activity. The results revealed that a mor inverted GA (iGA) motif and a canonical Sp1 binding site are required for the promoter activity. Using electrophoretic mobility shift analysis, we identified nuclear proteins that specifically bind to the mor iGA motif and that are immunologically related to Sp1 and Sp3. Mutation of the mor iGA motif, resulting in a loss of Sp binding, led to a 50% decrease in activity. Mutation of the canonical Sp1 binding site yielded a lesser (approximately 25%) loss of activity. Mutation of both motifs together resulted in an approximately 70% decrease in activity. In cotransfection assays using Drosophila SL2 cells, Sp1 trans-activated the promoter in a manner dependent on the presence of mor iGA and canonical Sp1 binding motifs. Sp3 can also trans-activate the promoter, and furthermore, Sp1 and Sp3 can trans-activate the mor promoter additively. Our results suggest that combined or cooperative interaction of Sp transcription factors within the proximal promoter is necessary for activation of mor gene transcription.

Opioids can relieve pain without affecting sensory modalities such as vision and hearing, making these drugs the preferred clinical analgesics for severe pain. Three major types of opioid receptors, μ, δ, and κ, have recently been cloned and shown to belong to the G-protein-coupled receptor superfamily (1). Based largely on pharmacological and clinical observations, μ-opioid receptor (mor) has traditionally been considered the main site of interaction of the major clinically used analgesics, particularly morphine (2). The critical role of mor in analgesia, as well as in the development of tolerance and dependence, has been further confirmed by pharmacological analysis of knockout mice in which this receptor has been eliminated (3–4). Mor is mainly expressed in the central nervous system, with receptors varying in densities in different regions and perhaps playing different roles (5–7). For example, mor located in the periaqueductal gray has been suggested to mediate analgesia (2, 8), whereas mor located in the locus coeruleus and ventral tegmental areas may be involved in the development of tolerance and physical dependence (9–11).

The development of different roles for mor in different central nervous system areas must ultimately depend on how its expression is regulated in these areas. It is likely that this expression is modified by fluctuating levels of various agents in certain brain regions (12–15), as well as in certain disease states (16). This raises the possibility of increasing the clinical efficacy of morphine and other opioids, and perhaps also of treating certain diseases, by manipulation of mor levels.

We have previously isolated and partially sequenced the mouse mor gene, which is over 53 kilobases long and its coding sequence divided into four exons (17). Using deletional and transient transfection assays, a distal and a proximal promoter of mouse mor gene were identified in the cell lines endogenously expressing mor (18, 19). The distal promoter initiated the mor transcription from a single transcription initiation site, which was located 794 bp upstream of the translation initiation site (19). The proximal promoter initiated mor transcription from four major transcription initiation sites, located in a region 291–268 bp upstream of the translation initiation site (17). Furthermore, utilizing the reverse transcription-polymerase chain reaction (RT-PCR) assay, we found that mor mRNA in the brain was predominantly initiated by the proximal promoter, in a ratio of approximately 20:1 (proximal:distal) (18).

Thus, the proximal promoter appears to be the principal promoter of regulation of the mor gene in the brain.

DNA sequence analysis indicates that the proximal promoter (17) of the mouse mor gene lacks a consensus TATA box (20), a consensus initiator (21, 22), or GC-rich sequences (23). However, analysis indicated that this promoter does possess a canonical Sp1 binding site, (G/T)(G/A)GGC(G/T)(G/A)(G/A)(G/T) (24). It has been suggested that the Sp1 transcription factor could tether preinitiation complexes to the promoter by interacting with TFIIID (20, 25, 26), which is essential for the transcription event at both TATA and TATA-less promoters (27).

Recently, a family of Sp1-related proteins, Sp2, Sp3, and Sp4, were identified, which recognize GC and/or GT or CACCC boxes (28, 29). In the present study, our objective was to identify cis-acting DNA elements and their trans factors, which were required for the expression of mouse mor gene, with particular focus on the proximal promoter.

We report here that a mor iGA motif-containing region, positioned near the cluster of transcription start sites, is essential for mor gene expression. This site binds Sp1 and Sp3 or antigenically related proteins. In addition, a canonical Sp1 binding site, located 40 bp upstream of the mor iGA motif, also contributes to mor promoter activity. Binding of Sp transcription factors to both sites simultaneously has an additive effect on activation of transcription. Because Sp proteins are the
ubiquitous transcription factors (28–30), they are reasonable candidates as regulators of the basal expression of the mor gene. Furthermore, recent identification of brain-enriched regulatory proteins (31–33) or cell/tissue-specific proteins (34, 35), which may interact with Sp binding motifs or with Sp transcriptions factors, raises the possibility that these Sp binding sites play a role in establishing the cell-specific pattern of mor expression in vivo.

MATERIALS AND METHODS

**Plasmid Construction**—Luciferase fusion plasmids were constructed containing a 450-bp upstream sequence (pL450 construct; bp −450 to −249 relative to the translation start site, designated +1) or shorter upstream regulatory sequences of the mouse mor gene. The 5’-deletional (pL450, pL400, and pL299) and 3’-deletional (pL450/300, pL450/400, pL400/340, pL340/300, and pL300/340) constructs were generated by recombinant PCR with all of the upstream primers bearing the KpnI site and the downstream primers bearing the XhoI site. All of the PCR fragments were cloned into PCRII vector (Invitrogen). The sequences of PCR products were confirmed by sequencing and then subcloned into polylinker sites of a promoterless luciferase vector, pGL3-basic (Promega). The linker mutation was introduced into the pL450 construct to generate pLm1b and pLm2b constructs. The 34-bp mutagenic oligonucleotides, containing 10-bp linker bearing the XhoI restriction site with a 2-bp arm at each site, was substituted for the wild type sequence (pL450) by MORPH™ mutagenesis kit (5 Prime→3 Prime, Inc., Boulder, CO). Mutant clones with the correct digestion pattern were further confirmed by sequencing and then subcloned into pGL3-basic.

**Point Mutation Introduction**—The point mutation was introduced by PCR into pL450 to generate pLmGA, pLmGA1, pLmSp, and pLmGA/Sp constructs. A mutagenic oligonucleotide for pLmGA containing three base substitutions (underlined), 5’-CTT CTC TCT CCT GTT CCC TCT CT-3’, was used in the reaction. The mutagenic oligonucleotide for pLmGA1 also contained three base substitutions (underlined): 5’-CTT GTT CGT TCT CCC TCT CTT-3’. A mutagenic oligonucleotide for pLmSp contained three base substitutions (underlined): 5’-AGA GAA TGG TGA TAT CCT GGA AGG AGG-3’. The double mutation construct pLmGA/Sp was generated by introducing mutations into pLmSp construct. All constructs carrying PCR mutations were sequenced across the −450 to −249 bp region to ensure introduction of base changes at only the intended positions.

To prepare a promoter construct containing the mor iGA motif positioned upstream of the SV40 promoter, two oligonucleotides containing the mor iGA motif were prepared and annealed: upper, 5’-CTG CTT CTC TCT CCC TCT CTT CT-3’; and lower, 5’-AGA GAG GGG GAG GGA GAG GAG AGG AGG-3’. The resulting double-stranded oligonucleotide was inserted into the polylinker site of the pGL3-promoter (Promega), containing the SV40 promoter.

**Cell Culture**—Human neuroblastoma SH-SY5Y cells were grown in RPMI 1640 with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO2 and 95% air at 37 °C. Schneider’s Drosophila line 2 (SL2) cells were purchased from ATCC were grown at 22–24 °C in Schneider’s Drosophila medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal calf serum.

**Transient Transfection and Reporter Gene Activity Assay**—SH-SY5Y cells were transfected using the DOTAP (Boehringer Mannheim) lipofection method as described previously (18). Briefly, cells with approximately 40% confluency were transfected with an equimolar amount of expression plasmid and transfection method as described previously (18). Briefly, cells with approx-40% confluency were transfected with an equimolar amount of expression plasmid and transfection method as described previously (18). Briefly, cells with approx-

**RESULTS**

**Identification of the Minimum Sequences Required for the Proximal Promoter Activity of the Mouse µ-Opioid Receptor Gene**—We previously showed that deletional constructs containing only the proximal promoter (pL450, −450 to −249 bp) exhibited activities similar to the activities of constructs containing both distal and proximal promoters in SH-SY5Y, mor-expressing cells (18). Because the pL450 construct contains the entire region of multitranscription initiation sites (Fig. 1), or shorter upstream regulatory sequences of the mouse mor gene, we reasoned that elements important for the basal proximal activity are positioned in the −450 to −249 bp region. In order to identify the minimum sequences, modules, and trans-acting proteins required for the basal activity of proximal promoter, 5’- and 3’-deletional analyses were performed in this region.

As shown in Fig. 1B, a 5’-deletion to position −400, producing a pL400 (−400 to −249) construct, resulted in a small decrease (21%) of activity relative to pL450, whereas the pL299 (−299 to −249) construct displayed a major decrease (72%). The pL299 construct in fact showed no greater activity than pL450 to pL299 constructs were generated by recombinant PCR with all of the upstream primers bearing the KpnI site and the downstream primers bearing the XhoI site. All of the PCR fragments were cloned into PCRII vector (Invitrogen). The sequences of PCR products were confirmed by sequencing and then subcloned into polylinker sites of a promoterless luciferase vector, pGL3-basic (Promega). The linker mutation was introduced into the pL450 construct to generate pLm1b and pLm2b constructs. The 34-bp mutagenic oligonucleotides, containing 10-bp linker bearing the XhoI restriction site with a 2-bp arm at each site, was substituted for the wild type sequence (pL450) by MORPH™ mutagenesis kit (5 Prime→3 Prime, Inc., Boulder, CO). Mutant clones with the correct digestion pattern were further confirmed by sequencing and then subcloned into pGL3-basic.

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**Transient Transfection and Reporter Gene Activity Assay**—SH-SY5Y cells were transfected using the DOTAP (Boehringer Mannheim) lipofection method as described previously (18). Briefly, cells with approximately 40% confluency were transfected with an equimolar amount of test plasmid. The amount of DNA used was within the linear range of the relationship between the luciferase activity and the amount of DNA. Forty-eight h after transfection, cells grown to confluence were washed and lysed with lysis buffer (Promega). To control for differences in transfection efficiency from dish to dish, a 1/10 dilution of pCH110 plasmid (Amersham Pharmacia Biotech) containing the β-galactosidase gene driven by the SV40 promoter was included in each transfection and used for normalization. β-galactosidase SL2 cells were transfected with CellFECTIN™ (Life Technologies, Inc.), according to the manufacturer’s instructions. Briefly, for each transfection, 2 µg of test plasmid was mixed with CellFECTIN and incubated at room temperature for 30 min, before being added to SL2 cells. Forty-eight hours after transfection, cells were washed and lysed. Normalization among each sample in SL2 transient transfection followed the method described by Conn et al. (36). All transfection experiments were repeated three times or more with similar results, utilizing constructs that were independently prepared at least twice. The luciferase and β-galactosidase activities of each lysate were determined as described by the manufacturers (Promega and Tropix, respectively).
contains the important cis-acting elements for the basal activity. Accordingly, we analyzed the activity of constructs with deletions in this region. As shown in Fig. 1B, the pL340/300 (−340 to −300 bp) construct showed significant luciferase activity, whereas the pL400/340 construct (−400 to −340 bp) did not. In addition, reverse orientation of the −340 to −300 bp fragment (pL300/340 construct) resulted in background reporter activity. In summary, the sequence between nucleotides −340 and −300 plays an important role in proximal promoter of the mor gene, and it alone confers the promoter activity in an orientation-dependent manner.

Identification of Sp Transcription Factors Bound to the Minimum Promoter Region of the mor Gene—To elucidate the transcriptional mechanism of mor gene expression and, in particular, the contribution of the −340 to −300 bp region to the promoter activity, EMSAs were performed with the fragment of −340 to −300 bp as the probe and SH-SY5Y cell nuclear extract. As shown in Fig. 2A, the major (indicated by <, right) and some minor protein-DNA complexes (indicated by * and **) were observed (lane 2). The protein-DNA complex bands indicated in Fig. 2A by < and * (but not those indicated by **) were specific, because a 100-fold molar excess of unlabelled −340 to −300 fragment inhibited complex formation (data not shown).

Examination of the sequence from −340 to −300 bp revealed a continuous 26-bp poly pyrimidine/polypurine region, which contained the mor iGA motif and the 5′-adjacent sequence next to this motif has similar characteristics. Transcription factors, such as Sp1 (38), have been shown to bind to a similar motif. We therefore examined the possibility that Sp1 or a Sp1-like protein was the factor bound to this region. As shown in Fig. 2A, a consensus GC-rich Sp1 binding site oligonucleotide (5′- ATTCGATCCGGGGCGGGGGCAG-3′) effectively abolished the specific shift bands (indicated by < and *, lane 3) of these complexes containing Sp1 or Sp1-related proteins.

An antibody supershift assay was then performed to verify the involvement of Sp factors (23, 28, 29). As shown in Fig. 2, A and B, a supershift was observed using anti-Sp1 (indicated by the arrow in Fig. 2A, lane 4, and the arrow at right of Fig. 2B, lane 2), and also anti-Sp3 antibody (indicated by arrow in Fig. 2A, lane 6, and the arrow at right of Fig. 2B, lane 3). Incubation of the reaction with both Sp1 and Sp3 antibodies simultaneously led to an almost complete supershifting of the major protein-DNA complex (as indicated by <, Fig. 2B, lane 4).

On the other hand, incubation with the anti-Sp2 (Fig. 2A, lane 5) or Sp4 (Fig. 2A, lane 7) antibodies did not result in any supershift, raising the question of whether these two factors are present in the cells. To address this question, Western blot analysis was carried out using the nuclear extract from the cells. Protein bands of approximately 100, 80, 120, and 130 kDa were observed when the blot was incubated with anti-Sp1, Sp2, Sp3, or Sp4 antibody, respectively (Fig. 2C). The molecular mass of each Sp factor is consistent with the published sizes (23, 28, 29). Thus, all four Sp transcription factors are present in the cells, but only Sp1 and Sp3 are the trans-acting factors bound to this minimum sequence of proximal promoter of the mor gene.

Mor iGA Motif Is Critical for Binding of Sp Transcription Factors—To localize the Sp1/Sp3 binding motif within the −340 to −300 bp region, three oligonucleotides spanning the entire region were synthesized and designated as fragments a, b, and c (Fig. 3A, fa, fb, and fc, respectively). These fragments were then tested for their ability to compete with the −340 to −300 bp oligonucleotide for the formation of major protein-DNA complexes in the EMSA. The formation of protein-DNA complexes (Fig. 3B, lane 2, indicated by < at right) was abolished by the addition of a 100-fold molar excess of fb (Fig. 3B, lane 8) or fragment c (Fig. 3B, lane 5). These data suggested that the binding of Sp proteins occurred in the fb (−333 to −308 bp) region.

To identify the Sp1 and Sp3 binding motif precisely, the fb region was further subdivided into fb-1 (devoid of mor iGA motif), fb-2 (containing the intact mor iGA motif), and fb-3 (containing partial mor iGA motif) (Fig. 3A). A competition assay was then performed (Fig. 3B). The formation of major protein-DNA complexes (Fig. 3B, indicated by <) was partially...
abolished by the addition of a 100-fold molar excess of fb-2 (lane 7) but not by fb-1 (lane 6) or fb-3 (lane 9), suggesting that the mor iGA motif is important for the binding of Sp factors. However, because the fb-2 containing this entire mor iGA motif was not as effective as the larger fb, sequences flanking the mor iGA motif appear to be important as well.

To provide further evidence for this conclusion, EMSAs were carried out using the 26-bp oligonucleotide fb as a probe and nuclear extracts from SH-SY5Y cells, as well as from mouse brain. As shown in Fig. 3C, the electrophoretic pattern (lane 2) was similar to that using the fragment of −340 to −300 bp as the probe. The GC-rich Sp1 binding site oligonucleotide effectively abolished the shift seen with the major band (Fig. 3C, indicated by <, lane 3). Moreover, the supershift was observed by using anti-Sp1 (lane 4, arrow) and Sp3 (lane 6, arrow) antibodies, but not anti-Sp2 (lane 5) or Sp4 (lane 7) antibodies. Using mouse brain nuclear extract, the binding of Sp1 and Sp3 was also detected (Fig. 3D). On the other hand, using the 15-bp fb-2 as a probe, the protein-DNA complex band was barely observed (data not shown). These results, corroborated with the previous data, demonstrated that binding of Sp1 and Sp3 required the mor iGA motif-containing region.

**Contribution of the mor iGA Motif to the Basal Proximal Promoter Activity**—To examine the contribution of mor iGA
to the functional activity of proximal promoter, the fb region of pL450 construct was mutated sequentially using a 10-bp linker bearing the XhoI restriction enzyme site. As shown in Fig. 4A, the mutant construct pLmb2, mutated at the region of fb-2, displayed less activity (49 ± 10%) than the wild type pL450 construct (100%); in contrast, the pLmb1 construct, mutated at the region of fb-1, showed no statistical difference in activity (82 ± 11%). These data suggested that the mor iGA motif is indeed important for the proximal promoter activity.

To confirm the contribution of mor iGA motif to the promoter activity and to rule out the possibility of a linker-created artificial transcription factor binding site, point mutants were made and analyzed. As shown in Fig. 4A, the pLmGA construct, in which CCC in the mor iGA motif was changed to GGT, displayed less activity (52.6 ± 3.6%) than the wild type pL450 construct (100%). The pLmGA_{b1} construct, in which CC . . . C was changed to GG . . . G in the fb-1 region (Fig. 4A), displayed no statistically significant difference in activity (113 ± 5%) as compared with that (100%) of wild type pL450 construct. These results agreed with the previous linker mutant data.

Concomitantly, EMSA was used to determine whether the mutation of mor iGA motif (mGA oligonucleotide, as depicted in Fig. 4B) resulted in elimination of Sp transcription factor binding. As shown in Fig. 3B, using the fb oligonucleotides as a probe, the formation of the major protein-DNA complex (indicated by <) was inhibited by 100-fold molar excess of unlabeled fb (lane 3) or mGA_{b1} mutant oligonucleotide (lane 5) but not by the mGA mutant oligonucleotide (lane 4), in which the mor iGA motif was mutated. Taken together, the above data demonstrated that mutation of the mor iGA motif, which prevented binding of Sp, resulted in an approximately 50% decrease in promoter activity.

Verification of a canonical Sp1 binding site in the proximal promoter region—The 5'-deletion analysis (Fig. 1B) suggested that elements located in the −400 to −300 bp region contributed approximately 72% of the maximum proximal promoter activity; however, mutation of the mor iGA motif resulted in only a 50% decrease in activity (Fig. 4). This disparity would suggest the involvement of other cis-acting elements. DNA sequences analysis indicated a canonical Sp1 binding site, located 40 bp 5'-upstream of the mor iGA motif, also present in the −400 to −300 bp region (17). We therefore investigated the role of this canonical Sp1 binding site in the proximal promoter activity.

To verify whether Sp1 or a Sp1-related factor bound to the canonical Sp1 binding site, an EMSA was performed. The mosp oligonucleotide, 5'-GACTGAGGAGGCTGATTCTCAG-3' positioned within the −375 to −353 bp region, contains the canonical Sp1 site, indicated by the underlined area. Incubation of
the radiolabeled mosp with SH-SY5Y nuclear extract resulted in a major protein-DNA complex band (indicated by \(\ang\) in Fig. 6, lane 2). The formation of the major complex was abolished by the addition of a 100-fold molar excess of GC-rich Sp1 (lane 3), as well as by fb oligonucleotide (lane 4), suggesting that the \(\text{trans}^\text{-acting}\) factor bound to this fragment might be Sp1 or Sp1-like protein. This was addressed directly using the supershift assay. The major supershift was observed only using anti-Sp1 antibody (Fig. 6, lane 6, arrow). The anti-Sp3 (lane 7), Sp2, or Sp4 (data not shown) antibodies did not result in any supershift. The data therefore demonstrated that in addition to the mor iGA motif, Sp1 transcription factor can also bind to a canonical Sp1 binding site located in the \(-359\) to \(-367\) bp region. In contrast, the Sp3 transcription factor cannot bind to this canonical Sp1 site but only to the mor iGA motif.

**Interaction between Two Spatially Separated Sp Transcription Factors**—To determine the relationship between the canonical Sp1 binding and mor iGA motifs, we first examined the functional role of the canonical Sp1 binding site to the proximal promoter activity. The Sp1 binding site in the mosp oligonucleotide was mutated by TAT substitution of GGC, which resulted in the pmSp oligonucleotide. Mutation of these nucleotides drastically reduced binding of Sp1 to its binding site (Fig. 6, lane 5). The Sp1 binding site mutant construct (pLmSp) also displayed a slight decrease in promoter activity (approximately 20%) relative to the wild type pL450 construct (Fig. 7).

To examine the possible interaction between these two spatially separate Sp1 binding sites, the mor iGA and a canonical Sp1 site, a double mutation was constructed. As shown in Fig. 7, the double mutation construct pLmGA/Sp displayed a more extensive decrease (approximately 65%) in activity than that of either the pLmSp (approximately 20%) or the pLmGA (approximately 50%) construct, suggesting that the two Sp binding sites can cooperate in an additive manner.

**FIG. 6.** Verification of a canonical Sp1 binding site in the proximal promoter. EMSAs were performed with nuclear extracts from SH-SY5Y cells and the fragment of \(-359\) to \(-367\) bp (mosp) as the probe. Lane 1, probe alone; lanes 2–7, 5 \(\mu\)g of nuclear extract; lane 3, 100-fold molar excess of GC-rich Sp1 competitor; lane 4, 100-fold molar excess of fb competitor; lane 5, 100-fold molar excess of mutant oligonucleotides pmSp; lanes 6 and 7, in the presence of 1 \(\mu\)g of anti-Sp1 (lane 6) or anti-Sp3 (lane 7) antibodies (Ab). The major protein-DNA complexes are indicated by \(\ang\). The anti-Sp1 antibody supershift band is indicated by the arrow.

**FIG. 7.** Transfection analysis of mutants of the mor iGA and the canonical Sp1 binding motifs. SH-SY5Y cells were transfected with various luciferase reporters driven by either \(\mu\)-opiod receptor wild type proximal promoter (pL450) or with reporter constructs containing mutated iGA motif (pLmGA) (\(\bigcirc\)), mutated canonical Sp1 binding motif (pLmSp) (\(\square\)), or both (pLmGA/Sp). Relative luciferase activities were obtained compared to the wild type promoter activity, arbitrarily defined as 100%. Error bars represent the S.E. from four different experiments.

**Sp Factors trans-Activate the Proximal Promoter via Direct Interaction with mor iGA and/or Canonical Sp Binding Motifs**—We have shown the contribution of both mor iGA motif and canonical Sp1 binding site to the proximal promoter activity and the binding of transcription factor Sp1 and/or Sp3 to these two sites (above). These results suggested that Sp factors were able to trans-activate the promoter upon binding to these motifs. To determine this directly, we performed co-transfection assays in Drosophila SL2 cells, which do not express endogenous Sp1 and Sp1-like proteins.

As shown in Fig. 8A, Sp1 can specifically trans-activate the promoter activity of the pL450 construct in a dose-dependent manner. Mutation of the mor iGA motif, canonical Sp1 binding site, or both sites reduced trans-activation by Sp1. The rank order of reduction of Sp1 trans-activation is pLmGA/pSp (double mutation) > pLmGA > pLmSp construct (Fig. 8A). Activation by co-expressed Sp1, however, was not completely abolished by double mutations, which may reflect the binding of overexpressed Sp1 to low affinity sites within the promoter.

In addition to Sp1 factor, Sp3 was also found to bind to the mor iGA motif. Because Sp3 is a bifunctional transcription factor that can activate or repress transcription (39, 40), we examined the role of Sp3 on the proximal promoter using co-transfection assays in SL2 cells. As shown in Fig. 8B, Sp3 alone can trans-activate the promoter activity of the pL450 construct, although Sp3 at the same concentration has a much smaller effect than Sp1. Mutation of the mor iGA motif (pLmGA) reduced the trans-activation by Sp3. Upon co-transfection of a constant amount of pPacSp1 (100 or 200 ng) and different quantities of pPacSp3, together with wild type construct (pL450), Sp1 and Sp3 were able to trans-activate the promoter in an additive manner (Fig. 8B).

Overall, these results demonstrated that mor iGA and canonical Sp1 motifs can mediate trans-activation by direct interaction with Sp1. Both Sp1 and Sp3 can bind to the mor iGA motif and exhibit an additive effect toward the promoter activity.

**DISCUSSION**

In this report, we have identified two spatially separated DNA sequences that bind Sp1 and/or Sp3 factors and are necessary for the maintenance of the proximal promoter activity of mouse mor gene (Fig. 9). One of these, a cis-acting element (mor iGA motif), is positioned adjacent to the transcriptional start sites. Transient transfection analysis of deletional and mutation constructs indicate that the mor iGA motif plays an important role in the proximal promoter. Mutation of the mor iGA motif reduced Sp1 trans-activation in Drosophila SL2 cells, demonstrating that the mor iGA motif is clearly capable of mediating trans-activation by Sp1, whereas a chimera of the mor iGA motif placed upstream of the heterologous SV40 promoter was also able to activate this promoter.

Using EMSA with fb as probe, we detected the presence of
a major complex that was specifically reduced in intensity by unlabeled Sp1 and that was supershifted with antibody against Sp1 and Sp3. These data suggest that the major complex contains both Sp1 and Sp3. Because Sp1 is known to be posttranslationally modified (41), the other minor protein-DNA complexes may contain either modified forms of proteins present in major complexes or distinct other factors (42), or they may simply consist of degradation products.

In regard to Sp3, although it is a bifunctional transcription factor (39, 40), Drosophila SL2 cell co-transfection experiments indicated that the forced expression of Sp3 can trans-activate the proximal promoter of mor gene via binding to the mor iGA motif. However, Sp3 displays a much smaller trans-activation effect of the mor gene than Sp1 does at the same concentration. Furthermore, Sp1 and Sp3 can trans-activate the mor gene promoter in an additive manner.

Our results indicate that the second DNA element (the canonical Sp1 binding site), located 40 bp upstream of the mor iGA motif, binds only Sp1. Unlike the mor iGA motif, which is found in rat and human as well as mouse, the canonical Sp1 binding site may be unique to the mouse mor gene, because the same consensus Sp1 sequence is not present in rat (43) or human mor promoter. However, whether the Sp1 factor can actually bind to rat and human mor promoter needs to be tested experimentally. Our data clearly show that both transcription binding sites are required for full proximal promoter activity, although the mor iGA motif contributes a larger effect by itself than the canonical site. Moreover, we cannot rule out the possibility that other closely positioned positive and/or negative elements went undetected by our set of deletions.

Sp1 is expressed in most if not all tissue or cell types, including brain (30), and therefore it seems to be a reasonable candidate for a factor activating constitutive basal expression of the mor gene proximal promoter. Furthermore, the ratio of Sp1 and Sp3 molecules in a cell may contribute to the transcriptional regulation of a gene with appropriate Sp binding sites by modulation of their degree of activation under certain conditions, such as during differentiation or in certain stages of the cell cycle. Tissue- or cell-specific regulatory factors (31–35, 44) presumably modulate the ability of Sp factors and of perhaps additional, as yet unidentified, factors to regulate the promoter activity. Further analysis will be required to determine the role of these elements for tissue-specific expression of mor in vivo and also the position of any additional cis element outside the region.

Comparison of mor gene promoters with those of several other G-protein coupled receptor genes (42, 45, 46), as well as with neuronally restricted genes (47–49), reveals several similarities, such as multiple transcription initiation sites, lack of
a TATA box, and the presence of Sp1 binding sites. Although a direct interaction of Sp1 with these promoters has been demonstrated for a few G-protein coupled receptors, including the serotonin 1a receptor (42), this finding, together with our results, indicates that these genes are controlled by constitutive promoters, activity of which must be modulated by sequence-specific enhancer and/or silencer binding proteins (49) to produce restricted patterns of expression in the nervous system. It has indeed been demonstrated that a large number of neuron-specific genes (49) are regulated by a silencer protein, NRSF/REST. Based on the structural features of the mor promoter, it is expected that additional cis elements influence promoter activity to restrict its in vivo pattern of transcription.

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