The 5'-flanking region of the mouse μ opioid receptor (MOR) gene has two promoters, referred to as distal and proximal, and the activities of each in the brain are quite different from each other. The 5'-distal promoter regulatory sequences (5'-DPRS), positioned between these two promoters, have strong inhibitory effects on the reporter gene expression driven by the MOR distal promoter. In our studies, detailed 3' deletion mapping of the 5'-DPRS narrowed down the negative cis-acting element to a 34-base pair (bp) segment (position −721 to −687). This 34-bp cis-acting element functions in both neuronal (NMB) and non-neuronal (CHO and RAW264.7) cultured cells. S1 nuclease protection assays indicated that this 34-bp cis-acting element suppresses distal promoter activity at the transcriptional level. Linker scanning mutagenesis demonstrated that nucleotides around position −721 and −689 in the 34-bp cis-acting element are essential for the regulation of distal promoter activity. Operational characterization of the 34-bp cis-acting element in the homologous MOR distal promoter and the heterologous SV40 promoter showed that its effects are position- and promoter-dependent while being orientation-independent in both promoters. Collectively, these data suggested that this 34-bp segment is a conditional transcriptional cis-acting element that blocks mouse MOR gene expression from the distal promoter.

Opioid receptors mediate the diverse functions of endogenous opioid peptides and the opioid alkaloid derivatives of morphine, including analgesia, reward, autonomic reflexes, and endocrine/immune regulation (1–3). Several opioid receptors have been discovered since their initial identification in the brain in 1973, and these receptors constitute three major types, referred to as μ, κ, and δ (4–6). All are members of the heptahelical transmembrane receptor superfamily and are coupled to their effectors by heterotrimeric GTP-binding proteins (G proteins) (7).

The μ opioid receptor (MOR),1 the major molecular target of morphine, has been identified in discrete brain regions with differential expression levels (8). A quantitative study of MOR mRNA in selected regions of the rat central nervous system showed that the medial thalamus has the highest levels while the cerebellum has the lowest (9). The expression of MOR gene is inducible in response to a variety of physiological and neuronal activities, including continuous chemical depolarization, which has been reported to down-regulate MOR gene expression (10, 11). Similarly, studies have shown that MOR gene expression can be regulated in vivo. Animals treated for 7 days with haloperidol, a treatment shown to alter striatal enkephalin mRNA levels, exhibit markedly reduced expression levels of MOR in the globus pallidus (12). Furthermore, it has been reported that interleukin-1β, in primary astrocyte-enriched cultures, produced a 55–75% increase in the MOR transcript, depending on the brain regions sampled (13). Taken together, these examples suggest that the expression of the MOR gene is temporally and spatially regulated. However, the detailed mechanisms governing MOR gene expression remain largely uninvestigated.

MOR genomic DNA has been cloned in mouse and rat, and its promoter region has been characterized (14, 15). In our laboratory, transcription initiation studies of the mouse MOR gene have shown that there is a major promoter domain (position −450 to −249) proximal to the translation start site (14, 16). A second promoter domain, distal to the translation start site, was identified at position −994 to −784 (17). Quantitative reverse transcriptase-PCR using adult mouse brain mRNA has shown that the proximal promoter is 20-fold more active than the distal promoter (16). Both promoters of the mouse MOR gene exhibit characteristics similar to housekeeping gene promoters, such as the lack of a TATA box, and multiple Sp1 binding sites (18). As these structural characteristics of MOR gene promoters predict, a recent anatomical study shows that MOR, which is presumed to be confined to the central nervous system, is also distributed in peripheral tissues (19).

Studies have demonstrated that there is a strong inhibitory regulatory region (position −775 to position −444), referred to here as the 5'-distal promoter regulatory sequences (5'-DPRS), between the distal and proximal promoters of the mouse MOR gene (16). According to studies using deletional analysis and transfection assays, the 5'-DPRS strongly inhibited the activity of the luciferase reporter gene driven by the distal promoter in human neuroblastoma SH-SYSY (16). Because the 5'-DPRS has multiple translation start sites (ATG) and a potential folded structure, it has been proposed to have translational regulatory functions (17). In the present study, we identify the cis-acting element in the 5'-DPRS, comprised of a stretch of 34 nucleotides positioned between −721 and −687, which is responsible for the inhibition of distal promoter activity. This negative control element is functional in each cell line tested (NMB, CHO, and RAW264.7). The 34-bp segment exerts its positional effects in a promoter-specific fashion, while its ori-
presentation does not affect transcription in either promoter examined. Finally, our study suggests that the effects of this negative cis-acting element on the MOR distal promoter occur at the transcriptional level.

**EXPERIMENTAL PROCEDURES**

**Constructs for the Recombinant Luciferase Reporter Gene Plasmid—**

pL1.3K/444 and pLup constructs have been described previously (16). For deletional mutagenesis, 3' deleted fragments of the 5' flanking sequence of the mouse MOR promoters are illustrated. Vertical arrows indicate the potential upstream translation start sites (ATG) in the 5'-DPRS. DP, minimal functional domain of the mouse MOR distal promoter; PP, minimal functional domain of the mouse MOR proximal promoter; TIS, transcription initiation site.

**A**

**B**

**Relative Activity to Basic (fold)**

![Graph showing relative activity of luciferase reporter gene constructs](http://www.jbc.org/)

**Fig. 1.** Structural features of 5'-flanking sequences (nucleotides −1326 to +1) of the MOR gene. Nucleotide +1 corresponds to the translation start site (ATG). The putative cis-acting elements (Oct-1, PU.1, and Sp1) in the upstream sequences of the mouse MOR promoters are illustrated. Vertical arrows indicate the potential upstream translation start sites (ATG) in the 5'-DPRS. DP, minimal functional domain of the mouse MOR distal promoter; PP, minimal functional domain of the mouse MOR proximal promoter; and TIS, transcription initiation site.

**Fig. 2.** Identification of the 34-bp negative cis-acting element using 3' to 5' deletion analysis of the 5'-DPRS. A, schematic representation of a series of 3' deletion constructs of the 5'-flanking sequences (−1326 to −444) of the mouse MOR gene in the recombinant luciferase reporter gene constructs. B, summary of luciferase reporter assay results. Each deletion construct was transfected into the neuronal cell line NMB, which endogenously expresses MOR (dotted bar), and into non-neuronal cells, CHO (hatched bar) and RAW264.7 (closed bar), which have no MOR expression. The cells were harvested 24 h later, and a luciferase reporter assay was performed. pGL3-Basic (which contains no enhancer/promoter) and pGL3-Control (containing the SV40 enhancer and promoter) were used as negative and positive controls, respectively. Transfection efficiencies were normalized to the β-galactosidase activity from cotransfection with the internal control plasmid, pCH 110. 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 means ± S.D. of three independent experiments, with at least two different plasmid preparations. DP, minimal functional domain of the mouse MOR distal promoter; LUC, luciferase reporter gene.
probes were electrophoresed on a 5% polyacrylamide-8M urea gel, and overnight at 55 °C, followed by digestion with S1 nuclease. Protected probes were phosphorylated with T4 polynucleotide kinase and cloned at the Bfr1 site of pL1.3K/721, at the HindIII site of pGL3-promoter plasmid (Promega), and at the Sall/Bfr1 sites of pGL3-promoter vector, respectively. The 1.3K/721AS construct, in which the 34-bp cis-acting element is in antisense orientation in pL1.3K/721, was generated by PCR. The sequences of all constructs were verified by the dioxygenucleotide chain terminator sequencing method with the specific synthetic oligonucleotides as primers.

Linker Scanning Analysis—For linker scanning mutagenesis, two fragments from the 5′-flanking sequences (−1105 to −678) were generated by PCR; for one fragment (Bfr1-Sall), with the sense primer bearing a Bfr1 restriction site (−1105) and with all of the different positioned antisense primers (5′-TGTGCGACA-3′) bearing a Sall site (5′-GTCGAC-3′) flanked 3′ and 5′ with A and T, respectively, and for another fragment (Sall-NcoI), with all of the different positioned sense primers (TGTGCGACA-3′) bearing a Sall site flanked 3′ and 5′ with A and T, respectively, and antisense primers bearing a NcoI site (−678) and essential Kozak sequences. These fragments with the overlapping 8-bp Sall linker were cut with restriction enzymes and cloned into the pL1.3K/444 plasmid digested with Bfr1 and NcoI. The location of the substitution of the 8-bp linker is specified in Fig. 4A. Sequencing was carried out in both directions by the dioxygenucleotide chain terminator.

Cell Culture, Transfection, and Reporter Gene Assay—Human neuroblastoma NMB and murine macrophage-like cell line RAW264.7 were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO2 and 95% air at 37 °C. Fibroblasts of Chinese hamster ovary cells (CHO) were maintained in Dulbecco’s modified Eagle’s medium. For transfection, cells were plated 24 h prior to transfection at a density of 1 × 105 cells per plate in 6-well plates. Two transfection methods were performed in different experiments, and methods used in each experiment will be specified in the figure legend. Briefly, for the calcium phosphate-cotransfection precipitations, a DNA-CaCl2 mixture (1.5 pmol of MOR-luc reporter plasmid in 100 μl of modified Eagle’s medium. For transfection, cells were plated 24 h prior to transfection at a density of 1 × 105 cells per plate in 6-well plates. Two transfection methods were performed in different experiments, and methods used in each experiment will be specified in the figure legend. Briefly, for the calcium phosphate-cotransfection precipitations, a DNA-CaCl2 mixture (1.5 pmol of MOR-luc reporter plasmid in 100 μl of 2.5 M NaCl was added dropwise to 100 μl of a transfection mixture (250 mM Heps, 250 mM NaCl, 1.5 mM Na2HPO4, adjusted to pH 7.1). The precipitates were mixed by pipetting and applied dropwise directly to the cells and incubated at 37 °C. Twenty-four h post-transfection, cells were washed with phosphate-buffered saline and lysed with lysis buffer (Promega). The luciferase and β-galactosidase activities of each lysate were determined by a luminometer as described by the manufacturers (Promega and Tropix, respectively). In the second method, cells were transfected with construct plasmids using the SuperFect Transfection reagent (Qiagen) as described by the manufacturer. All transfection experiments were repeated four or more times with similar results, utilizing plasmids that were independently prepared at least twice. To correct the differences in transfection efficiency, one-fifth molar ratio pCH 110 (Amersham Pharmacia Biotech) containing β-galactosidase gene under the SV40 promoter was included in each transfection and used for normalization.

S1 Nuclease Protection Assay—Cells were harvested 24 h after transfection of CHO cells on a 10-cm dish with 1.5 pmol of pL1.3K/444, pL1.3K/722, and pGL3-control. Total RNA was purified using RNeasy kit (Ambion). Another set of transfected cells was used simultaneously for a luciferase assay. For S1 nuclease protection assay, synthesized luciferase probe (71 nucleotides) and β-galactosidase probe (42 nucleotides) were purchased from Life Technologies, Inc. To quantitate luciferase mRNA, 25 μg of total RNA from cells transfected with pL1.3K/444, pL1.3K/722, and pGL3-control was hybridized with 3 × 106 cpm of luciferase and β-galactosidase probe in hybridization solution (3 mM NaCl, 0.5 mM Heps, pH 7.5, and 1 mM EDTA) for 10 min at 80 °C and overnight at 55 °C, followed by digestion with S1 nuclease. Protected probes were electrophoresed on a 5% polyacrylamide-8 M urea gel, and the dried gel was scanned and quantitated by PhosphorImager (Molecular Dynamics).

\[ \text{Luciferase} \rightarrow (71 \text{ nt}) \]

\[ \beta\text{-gal.} \rightarrow (42 \text{ nt}) \]

Fig. 3. Comparison of luciferase reporter mRNA levels derived from pL1.3K/444 and pL1.3K/721 in CHO cells. The luciferase mRNA levels derived from CHO cells transfected with pL1.3K/444 (lane 1), pL1.3K/721 (lane 2), and pGL3-Control (lane 3) or from un-transfected CHO cells (lane 4) were quantitated by an S1 nuclease protection assay. Transfection efficiencies were normalized to the β-galactosidase band density from cotransfection of the internal control plasmid, pCH 110. The expression levels of luciferase reporter mRNA and β-galactosidase mRNA were quantitated by PhosphorImager. This experiment was performed twice.

**RESULTS**

3′ Deletional Mutagenesis for Further Mapping of the 5′ Distal Promoter Regulatory Sequences (5′-DPRS)—Previous studies have shown that the 5′-flanking region of the mouse μ opioid receptor (MOR) gene has two major promoters, referred to as proximal and distal (14, 16, 17, 20) (Fig. 1). Previously, we showed that the sequences (−775/−444) in the 5′-flanking region suppress the expression of the pL1.3K/444 reporter construct in neuroblastoma SH-SY5Y cells, in which the distal promoter (−1326 to −444) is cloned into the luciferase reporter vector (16). These sequences (−775/−444) are referred to here as the 5′-distal promoter regulatory sequences (5′-DPRS) (Fig. 1).

To define specific sequences in the 5′-DPRSs that inhibit the expression of the pL1.3K/444 reporter construct, the 5′-DPRS was subjected to detailed 3′ deletional mutagenesis. The 3′-deleted fragments of the 5′-flanking sequences (−1326 to −444) were generated by PCR and were cloned into pL1.3K/444 digested with NcoI and Bfr1 (Fig. 2A). The resulting luciferase reporter constructs were transiently transfected into the neuronal cell line NMB, which contains a proportion of endogenous MOR (15%) in addition to two other opioid receptors δ (60%) and κ (25%) (22), since the low amount of MOR is speculated to be due to the suppression of the distal promoter. Parallel experiments were also carried out in non-neuronal cells RAW264.7 and CHO to determine whether the distal promoter is regulated in a tissue-specific pattern.

Compared with pL1.3K/444, in which the entire 5′-DPRS segment is intact, constructs pL1.3K/508, pL1.3K/586, L1.3K/626, and pL1.3K/687 are not active in these cells (Fig. 2). The lack of a significant change in the luciferase activity of these constructs suggests that the segment between −687 and −444 in the 5′-DPRS does not have the negative cis-acting elements regulating the expression of the luciferase reporter gene. With progressive fine 3′ deletions to position −721, constructs pL1.3K/694, pL1.3K/702, pL1.3K/717, and pL1.3K/721 show significant increases in luciferase reporter activity, reaching a maximum of about 25-fold relative to pGL3-Basic in the NMB cell line. These findings suggest that the critical negative cis-acting element in the 5′-DPRS is a 34-bp segment located between −721 and −687. Further 3′ deletions from position...
2775 to 2722 resulted in decreased luciferase activity to a maximum of a 70% decrease in activity in NMB cells (pL1.3K/728 and pLup). One interpretation of this biphasic response would be that this 54-bp region has a positive regulatory function, although this remains to be tested.

Taken together, these results suggest that the 5'-DPRS has a very strong negative cis-acting element, located between position −2721 and position −687, with an apparent positive cis-acting element at positions −775 to −721. In addition, these results indicate that this negative cis-acting element does not appear to be tissue-specific, as its deletion elicits the same effect on the luciferase activity in neuronal (NMB) and non-neuronal (CHO and RAW264.7) cells as shown in pL1.3K/728 construct.

The 34-bp cis-Acting Element as Transcriptional Negative Regulatory Sequences—Although the 5'-untranslated region (5'-UTR) of mRNA is predominantly involved in translational control (23–25), this region can also influence transcriptional activity (26). Since the negative cis-acting element in the 5'-DPRS is located in the 5'-UTR, we speculated that its inhibitory effect on luciferase reporter activity shown in Fig. 2 may occur at the transcriptional level. To test this hypothesis, we tried to measure the amount of luciferase reporter transcript generated. Total RNAs were extracted from CHO cells that were transfected with pL1.3K/444 and pL1.3K/721. An S1 nuclease protection assay was run using the antisense oligonucleotide probes of luciferase (71 nucleotides) and β-galactosidase (42 nucleotides). As Fig. 3 shows, the transcript of pL1.3K/721 is 2-fold that of pL1.3K/444, suggesting that the essential 34-bp nucleotides in 5'-UTR identified in Fig. 2 constitute a transcriptional cis-acting element. Compared with the difference in luciferase reporter activity (more than 30-fold) between pL1.3K/444 and pL1.3K/721 in Fig. 2, the 2-fold difference in transcript level between them is relatively small. This result may be due to the rapid degradation of luciferase reporter mRNA or to the fact that the luciferase activity is not within the linear range of the relationship between the luciferase protein concentration and the amount of transcript.

Fig. 4. Systematic analysis of the 34-bp cis-acting element. A, schematic representation showing the relative locations of linker scanning mutation to the 34-bp negative cis-acting element. Each rectangle (□) represents the position of a clustered region of 8 nucleotide substitutions. B, eight sets of these 8-bp clustered mutations were then introduced into, and around, the 34-bp negative cis-acting element by PCR. Each of the eight sets of mutations was incorporated into the recombinant MOR distal promoter fragment (−1326 to −678)-luciferase plasmid, and the resulting constructs (Linker 1–Linker 8) were transfected into NMB cells. Transfection efficiencies were normalized according to β-galactosidase activity from cotransfection of the internal control plasmid, pCH 110. 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 means ± S.D. of five independent experiments with at least two different plasmid preparations. DP, minimal functional domain of the mouse MOR distal promoter; NCAE, 34-bp negative cis-acting element.

−775 to −722 resulted in decreased luciferase activity to a maximum of a 70% decrease in activity in NMB cells (pL1.3K/728 and pLup). One interpretation of this biphasic response would be that this 54-bp region has a positive regulatory function, although this remains to be tested.

Taken together, these results suggest that the 5'-DPRS has a very strong negative cis-acting element, located between position −721 and position −687, with an apparent positive cis-acting element at positions −775 to −721. In addition, these results indicate that this negative cis-acting element does not appear to be tissue-specific, as its deletion elicits the same effect on the luciferase activity in neuronal (NMB) and non-neuronal (CHO and RAW264.7) cells as shown in pL1.3K/728 construct.

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Sequences around −721 Are Essential for the Regulation of the Distal Promoter—To further define the negative 34-bp cis-
acting element (−721/−687), we utilized linker scanning mutagenesis. Linker oligonucleotides (5'-TGTCGACA-3') containing the SalI site (5'-GTCGAC-3') flanked with T and A were introduced to the MOR distal promoter fragment (−741 to −678) by PCR. These mutated fragments were subcloned into pL1.3K/444, and the resulting linker constructs were then transfected into NMB cells. The luciferase activities were expressed as fold activity over that of the promoterless plasmid (pGL3-Basic) (Fig. 4B).

In these linker constructs, changing nucleotides −725 to −718 (Linker 3) and −693 to −686 (Linker 7) restored much of the distal promoter activity, suggesting that these two regions are essential for the negative effect of the 34-bp cis-acting element on the distal promoter (Fig. 4B). Comparison of the deletion constructs pL1.3K/687 and pL1.3K/694 in Fig. 2 suggested that nucleotides at positions −687 to −694 are not important. Contrary to this proposal, mutation of these sequences in Linker 7 indicates that they act as a negative cis-acting element, suggesting that the regulatory function of these sequences depends on the DNA context. Taken together with the 3' deletion studies (Fig. 2), the mutational analysis in Linker 3 confirmed that the sequences around nucleotide −721 are functionally important in mediating the effect of the 34-bp cis-acting element on the distal promoter.

The 34-bp cis-Acting Element Is Position-, but Not Orientation-, dependent—To further understand the configuration under which the 34-bp cis-acting element negatively exerts its effect on the promoters, this fragment was subcloned downstream of the distal promoter of pL1.3K/721 in the antisense orientation (1.3K/721AS). The resulting construct was transfected into NMB cells. As shown in Fig. 5, the expression level of 1.3K/721AS is comparable with that of pL1.3K/687. This implies that the effect of this segment in the 5'-DPRS is independent of orientation.

In addition, the promoter specificity of the negative cis-acting element was examined for its effect on the heterologous simian virus 40 (SV40) promoter and the homologous MOR distal promoter when placed in different positions. To do this, these sequences were subcloned upstream (SS1.3K/721 in the sense orientation and AS1.3K/721 in the antisense orientation) of the homologous MOR distal promoter and upstream (SS-SV40) and downstream (SV40SS in the sense orientation and
SV40AS in the antisense orientation) of the heterologous SV40 promoter. The resulting constructs were transfected into the neuronal cell line NMB, which expresses endogenous MOR, and into non-neuronal cells RAW264.7 and CHO (Fig. 5). Regardless of its orientation in the cell lines tested, when positioned upstream of the distal promoter, the 34-bp cis-acting element did not effect distant promoter activity (SS1.3K/721 and SS1.3K/721), implying that it is position-dependent. In contrast with the homologous MOR distant promoter, the effects of the cis-acting element on the heterologous SV40 promoter were more noteworthy. When this segment is fused upstream of the SV40 promoter (SS-SV40), it exerts an inhibitory effect, whereas there was no inhibitory effect when located downstream of the SV40 promoter (SV40AS and SV40SS), regardless of its orientation in the cell lines tested.

Taken together, these results indicate that the 34-bp negative cis-acting element in the 5'-DPRS of MOR gene is position- and promoter-dependent while being orientation-independent in both promoters.

**DISCUSSION**

Previous studies have shown that the 5'-DPRS (-775 to -444) exerts negative control over the mouse MOR distal promoter (16, 20). Here we present a detailed analysis of 5'-DPRS as a first step toward understanding the mechanism of the regulation of the mouse MOR distal promoter. Our data indicate that the activity of the MOR distal promoter is controlled by the 34-bp cis-acting element (-721 to -687) at the transcriptional level.

Transient transfection, using 3'-deleted mutations of pL1.3K/444, into the MOR expressing neuronal cell line NMB and non-expressing non-neuronal cells, CHO and RAW264.7, shows that the 34-bp cis-acting element has a very strong inhibitory effect on the expression of the luciferase reporter gene driven by the basal distal promoter in all three cell lines tested. In addition, since the basal distal promoter domain is active in all three cell lines it suggests that the 34-bp cis-acting element is not involved in regulating tissue-specific expression of the MOR gene, although a comprehensive survey of other cell lines remains to be performed. Similar results suggesting that the basal distal promoter is active in different cell lines, SK-N-SH, Neuro 2A, and HeLa, have been reported (20).

In some mRNAs the presence of multiple upstream translation start sites (ATG) has been reported to interfere with translation (21). Considering this, the putative six translation start sites in the MOR 5'-DPRS (Fig. 1) were expected to be responsible for the inhibitory effect on the reporter gene expression at the translational level (20). Unexpectedly, our results show that deletion of the six translation start sites had no significant effect on the luciferase reporter gene activity, as shown by the expression level of the pL1.3K/687 construct (Fig. 2). This may be due to the fact that none of these six sites has the typical Kozak consensus sequences, which have been demonstrated to be important for the initiation of translation (20).

In addition to the multiple translation start sites mentioned above, it has been demonstrated that the level of folded RNA structure in the 5'-UTR is also an important element in mRNA translation efficiency (27, 28). We sought to determine whether the transcript of the 5'-DPRS has a folded RNA structure, which would result in suppression of luciferase reporter gene expression at the translational level. Computer analysis was used to test this possibility and indicated the lack of any stable stem-loop secondary structure in the 34-bp cis-acting element or the rest of the 5'-DPRS of the MOR gene (data not shown). This indirect evidence suggested that the 5'-DPRS inhibits luciferase reporter gene expression at the level of transcription. Results of the S1 nuclease protection assay, in which the level of the pL1.3K/721 transcript was 2-fold that of pL1.3K/444 (Fig. 3), confirm this conclusion. However, it may still be possible that trans-acting factor/DNA interactions between position -687 and position -444, if any, are masking the effect of the 34-bp negative cis-acting element. Additional studies would be required to check this possibility. Taken together, our results are compatible with a model in which these 34 nucleotides function as a transcriptional cis-acting element, although mRNA stability has not been determined and the 2-fold difference in transcript level between pL1.3K/444 and pL1.3K/721 is relatively small.

Our study of operational properties of the 34-bp negative cis-acting element in the homologous MOR distal promoter and the heterologous SV40 promoter showed that it is position- and promoter-dependent while being orientation-independent in both promoters (Fig. 5). The varying position effects of the 34-bp cis-acting element in both promoters suggest that it may be conditional regulatory sequences that require an appropriate promoter context for the inhibitory effect on the promoter.

The 34-bp cis-acting element is highly homologous between rat, mouse, and human (Fig. 6). Such conservation of these sequences strongly supports the transient transfection results that the 34-bp cis-acting element is functionally important (Fig. 2), since the functional sequences in the 5'-flanking region of genes are generally homologous between species. However, the question remains as to how this negative cis-acting element in the 5'-UTR exerts its inhibitory effect on the distal promoter. Because of its location, one possible explanation for its suppressive activity is that it utilizes an attenuation mechanism similar to that by which eukaryotic RNA polymerase II can terminate transcription around these sequences (29). On the other hand, unless the action of the 34-bp cis-acting element is influenced by the context of DNA, this mechanism is not plausible since the 34-bp cis-acting element is still functionally active upstream of the SV40 promoter (SS-SV40) (Fig. 5).

Another reasonable explanation is that this region could mediate its inhibitory effect on transcription initiation by a trans-acting factors. Therefore, we were interested in whether
the 34-bp cis-acting element contains consensus DNA sequences for some of the known transcription factors. Analysis of this element revealed highly conserved sequences (TTTGAGGAACCTG) between −703 and −695 homologous to eE3 PU-box (5’TATTGAGGAACCTG-3’) of immunoglobulin κ light chain which binds to a nuclear factor PU.1, an Ets family member (30). Although the substitution of those putative cis-acting sequences (Linker 5 and Linker 6) by linker scanning mutation did not show any effect on the distal promoter activity (Fig. 4), it is necessary to determine whether this putative cis-acting element is important for the mouse MOR gene expression. Furthermore, research of the sequences (−741 to −687) on transcription data bases resulted in no findings of any trans-acting factor except PU.1. Currently we are experimenting to identify whether there is a novel trans-acting factor(s) that binds to the 34-bp cis-acting element.

In conclusion, we have identified the negative 34-bp cis-acting element which is functional in all cell lines of different tissue origin examined, in a position-dependent and orientation-independent fashion. Future experiments on the regulatory processes that control the inhibitory effect of the 34-bp cis-acting element on the MOR distal promoter will contribute to the elucidation of the mechanism regulating MOR gene expression.

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