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Transcriptional Regulation of μ Opioid Receptor Gene by cAMP Pathway

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ABSTRACT

The utility of morphine for the treatment of chronic pain is hindered by the development of tolerance. Fentanyl has been shown to be a potent analgesic with a lower propensity to produce tolerance and physical dependence in the clinical setting. Previous finding has shown that fentanyl induces μ opioid receptor gene expression in PC-12 cells (*Brain Res* **859:**217–223, 2000). In this report, we aim to identify the molecular mechanism of μ-opioid receptor (MOR) gene regulation by fentanyl. We demonstrated that the 4.7-kilobase MOR promoter could be induced by fentanyl in PC-12 cells, and we defined a partial cAMP response element (CRE) located at −106/−111 in 5′-untranslated region of the *MOR* gene. In electrophoretic mobility shift assay, cAMP response element-binding protein (CREB) was found in the protein-DNA complex formed on the CRE box. CREB was phosphorylated after forskolin induction, and both CREB and CREB-binding protein (CBP) binding to the endogenous MOR promoter was increased by forskolin in chromatin immunoprecipitation assay. The functional role of CREB in the induction of *MOR* gene was further elucidated by an experiment in which a dominant-negative mutant CREB, CREB-S133A, abolished the forskolin-mediated MOR induction. Moreover, we found that this CRE box is conserved in mouse, rat, and human *MOR* gene, implying physiological relevance in different species. Collectively, this study demonstrated that fentanyl-triggered *MOR* gene induction was mediated by the sequential activation of CREB and the binding of CREB and CBP to MOR promoter, thus provides direct evidence for lower propensity of fentanyl to produce tolerance.

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 (Kieffer, 1995). 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 (Wood and Iyengar, 1988).

The critical role of MOR in analgesia, as well as in the development of tolerance and dependence, has been further confirmed by the analysis of knockout mice in which this receptor has been eliminated (Matthes et al., 1996; Sora et al., 1997). Mice that lack the *MOR* gene are insensitive to morphine (Matthes et al., 1996; Sora et al., 1997; Tian et al., 1997; Loh et al., 1998) and are less sensitive to δ- and κ-agonists despite a normal expression of δ- and κ-opioid receptors in the brain (Matthes et al., 1996; Kitchen et al., 1997; Sora et al., 1997). Heterozygous mice with only one *MOR* allele have 50% less MOR protein than wild-type mice and show a reduced sensitivity to morphine (Sora et al., 1997; Loh et al., 1998). This suggests the amount of MOR affects the sensitivity to opioid analgesics.

MOR is mainly expressed in the central nervous system, with receptors varying in densities in different regions and perhaps playing different roles (Delfs et al., 1994; Mansour et al., 1995; Minami and Satoh, 1995). For example, MOR located in the periaqueductal gray has been suggested to mediate analgesia (Wood and Iyengar, 1988), 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; Hyman, 1996). The development of different roles for the MOR in different central nervous system areas must ultimately depend on how its expression is regulated in these areas. It is likely that this

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ABBREVIATIONS: MOR, μ-opioid receptor; UTR, untranslated region; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; CRE, cAMP responsive element; EMSA, electrophoresis mobility shift assay; PCR, polymerase chain reaction; SV40, simian virus 40; ATF, activating transcription factor; RT, reverse transcription; CREB, cAMP responsive element-binding protein; CBP, CREB binding protein; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; forskolin, 8-bromo-cyclic AMP.

expression is modified by fluctuating levels of various agents in certain brain regions (Delfs et al., 1994; Azaryan et al., 1996; Ronnekleiv et al., 1996), as well as in certain disease states (Ronnekleiv et al., 1996). 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.

The utility of morphine for the treatment of chronic pain is hindered by the development of tolerance to the analgesic effects of the drug. Fentanyl has been shown to be a potent analgesic with a lower propensity to produce tolerance and physical dependence in the clinical setting (Narita et al., 2002). Recent finding showing that fentanyl but not morphine triggers cAMP elevation and MOR mRNA induction (Yoshikawa et al., 2000), which may be important in compensating for the MOR reduction during long-term treatment of PC-12 cells with fentanyl. However, neither the downstream cAMP pathway nor responding *cis*-element on the *MOR* gene has been defined.

In this study, we aimed to identify the molecular mechanism of *MOR* gene regulation by fentanyl. We give direct evidence for the induction of *MOR* gene transcription by the stimulation of fentanyl in PC-12 cells, thus offering further evidence for the up-regulation of *MOR* gene as a mechanism of lessening of opiate tolerance.

Materials and Methods

Plasmid Construction. Mouse *MOR* gene was isolated and partially sequenced (Min et al., 1994), and MOR promoter was identified in the cell lines endogenously expressing MOR (Ko et al., 1997). Luciferase reporter plasmid pL4.7K was a generous gift from Dr. Jane Ko (Seton Hall University, South Orange, NJ). pCAT-MOR4.7/−253 was obtained by ligating *Kpn*I/*Xcm*I-filled in fragments into the *Kpn*I/*Sma*I site of pCAR3-basic (Promega, Madison, WI). To obtain 5′-UTR of mouse *MOR* gene, PCR primer pairs *Kpn*I-mMORF-527D, 5′-GGG GTA CCA TGA AAC AGG CTT CTT T, and RI-mMOR-ATG, 5′-GGA ATT CGC CGG TGC TGC TGT CCAT, were synthesized, and *Kpn*I/*Eco*RI PCR product was cloned into pcDNA3.1. P4.7/ATG was constructed by ligating *Kpn*I/*Bam*HI fragment of pL4.7K and *Bam*HI/*Nco*I fragment of 5′-UTR PCR product, which contains internal *Bam*HI/*Nco*I site, into pCAR3-basic (Promega). In pCAT-MOR4.7/ATG construct; ATG is relative to translational start site and is designated +1. pMOR4.7/ATG-Luc was constructed by inserting *Kpn*I/*Nco*I of pCAT-MOR4.7/ATG into pGL3-basic (Promega). The 5′-deletional (pCAT-1.3/ATG) and 3′-deletional (pCAT-4.7/−253, pCAT-4.7/Avr-97, pCAT-4.7/Pvu-93) constructs were generated by restriction digestion and filled in with *Xcm*I, *Avr*II, *Pvu*II, and the downstream *Nco*I site. The 4.7/Pvu-93 was obtained by partial digestion by *Pvu*II and *Nco*I because an extra *Pvu*II site existed in *CAT* gene. The sequences of restriction digestive clones were confirmed by sequencing. C-RII-P-Luc was constructed by ligating *Xho*I/*Sma*I fragment of pCAT4.7/Avr-97 into *Xho*I/*Sma*I site of pGL3-promoter (p-Luc in abbreviation; Promega). For mtCRE-1/ATG, primer sets mMOR-UTRF, 5′-GGCCCGGGATCCCTCA-CAGCCCATG; mt1R, 5′-CCTAGGCTCCGTTCGTTTCTTAC; R201, 5′-CATCAGGCGGGCAAGAATGTG; and mt1F, 5′-GTAAGAAAC-GAACGGAGCCTAGG were used in PCR-directed mutagenesis. The mutated sequence for the cAMP-responsive element (CRE) site is underlined. For mtCRE-2/ATG, primer sets mMOR-UTRF and mt2R, 5′-CCTAGGCTCATTCAGTTTCTTAC; R201 and mt2F, 5′-GTAAGAAACTGAATGAGCCTAGG were used. Three-piece ligation was conducted for pCAT-mtCRE-1/ATG and pCAT-mtCRE-1/ATG by using *Bam*HI/*Nco*I of mutated fragments isolated from mMOR-UTRF and R201 PCR, coupled with *Kpn*I/*Bam*HI fragment of CAT-MOR4.7/ATG and inserting into *Kpn*I/*Nco*I site of the pGL3-basic (Promega). pLuc-TATA was constructed by *Esp*I/*Nco*I of human GTP hydrolase I promoter into pCAR3-basic (Promega). C-RII-TATA-Luc was constructed by *Sma*I/*Xho*I fragment of PCR product (−300 to *Avr*II and −300 to *Pvu*II) into *Mlu*I-filled-in/*Xho*I site of pLuc-TATA; −300 to *Avr*II fragment was obtained by PCR using primer set mMOR-UTRF and Xho-Avr, 5′-CCGCTCGAG CTAGCTCC GT-CAGTTTCTTA, whereas −300 to *Pvu*II fragment was obtained by PCR using primer set mMOR-UTRF and Xho-Pvu, 5′-CCGCTCGAG CTGCCCTAGGCTCCGTCAGT. The nucleotide sequences of mutant clones were confirmed by sequencing.

Cell Culture. P19 and 293T cells were grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO_2 and 95% air at 37°C. PC-12 cells were generous gift from Dr. Chern Yijuang (Inst BioMed Science, Academia Sinica, Taiwan), and were grown in 10% horse serum and 5% fetal calf serum in an atmosphere of 5% CO_2 and 95% air at 37 C.

Transient Transfection and Reporter Gene Activity Assay. PC-12, 293T, and P19 cells were transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Briefly, cells at approximately 40% confluence were transfected with an equimolar amount of each test plasmid. The amount of DNA used was within the linear range of the relationship between the CAT activity and the amount of DNA. Forty-eight hours after transfection, cells grown to confluence were washed and lysed with lysis buffer (0.25 M Tris-HCl, pH 7.6). The luciferase activity was determined according to the manufacturer's instruction (Promega). To control for differences in transfection efficiency from dish to dish, a 1:5 molar ratio of pCH110 plasmid (Amersham Biosciences, Piscataway, NJ9) containing the β-galactosidase gene driven by the SV40 promoter was included in each transfection and used for normalization.

Reverse Transcription-PCR of Endogenous *MOR* gene. Cells were treated with hypotonic buffer (20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 0.5% Nonidet P-40) for 5 min on ice. After centrifugation, cytosolic RNA were extracted using mini-RNA extraction kit (Viogen Co., Taipei, Taiwan) and analyzed by reverse transcription-PCR (RT-PCR). Primers specific to total MOR mRNA are rMOR-F484, 5′-AGCGTGGACCGCTACATTGCTGTC, and rMOR-R839, 5′-CGGGTGATCCTGCGCAGATTCCTG, which span a 355-base pair coding region. Primer sets to rat β actin are β-actin-F, 5′-AA-GAGAGGC ATCCTGACCCT and β-actin-R, 5′-TACAGGCTGGGGT-GTTGAA. The signals were detected and semiquantified using a Kodak Image Software system (Eastman Kodak, Rochester, NY).

Nuclear Extract Preparation. Nuclear extracts were prepared from PC-12 cells using the method described by Schreiber et al. (1989). Briefly, cells were grown to confluence, harvested, and washed with phosphate-buffered saline. All of the steps were performed at 4°C. The cells were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). The lysate was microcentrifuged at 500*g* for 5 min to pellet the nuclei, which were washed with sucrose buffer without Nonidet P-40. The nuclei were resuspended in low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), followed by addition of high-salt buffer to extract the nuclei, with incubation for 20 min on a rotary platform. The sample was microcentrifuged at 13,690*g*. Aliquots of the supernatant (nuclear extract) were stored at −80°C.

Electrophoretic Mobility Shift Assay. EMSA was performed with ^{32}P-labeled double-stranded oligonucleotides that were incubated with nuclear extract in EMSA buffer [10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml poly(dI-dC)]. For oligonucleotide competition analysis, a 100- or 200-fold molar excess of competitor oligonucleotides were also added to the mixture. After incubation at 22°C for 20 min, the mixture was analyzed on 5% nondenaturing poly-

acrylamide gels. Oligonucleotide sequence for CRE in *FMR* gene is described elsewhere (Hwu et al., 1997). For antibody supershift assays, 1 μl of anti-CREB or ATF-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the mixture. The reaction was then incubated at room temperature for 15 min. Protein-DNA complexes and free DNA were fractionated on 5% polyacrylamide gels in Tris-glycine buffer (50 mM Tris, pH 8.3, 380 mM glycine, and 2 mM EDTA) at room temperature and were visualized by autoradiography. CREB recombinant protein was produced as detailed in Hwu et al. (1997).

Western Blot Analysis. Nuclear extracts (25 μg of total protein) from PC-12 and 293T cells were incubated with the treatment buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and boiled for 5 min. The treated extracts were electrophoresed through SDS-10% polyacrylamide gels. The gel was electroblotted onto nitrocellulose membrane Hybond C (Amersham Biosciences) in transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 10% methanol). The membrane was blocked in blocking solution (5% dry milk, 0.1% Tween 20 in Tris-buffered saline) overnight at 4°C. Western blotting with anti-phospho-CREB antibody (Transduction Lab) was performed and developed using ECL following the manufacturer's instructions (Amersham Biosciences).

Chromatin Immunoprecipitation Assay. Control PC-12 cells, or those treated with 10 μM forskolin for 15 min, were cross-linked with formaldehyde per the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). Cell lysates were subjected to immunoprecipitation overnight at 4°C using 2 μg of anti-CBP or anti-CREB antibody (Santa Cruz Biotechnology) or preimmune rabbit sera. After reversed cross-linking, DNA was precipitated and detected by PCR with pairs of primers specific to the MOR promoter region containing CRE (i.e., rMOR-F20, 5′-CAGGGAACACCAGCGACTGCTCAG, and rMOR-R214, 5′-TGA TGGTAATGGCTGTGACCATGG). The number of PCR cycles was determined empirically.

Results

Activation of Endogenous *MOR* Gene Expression by Fentanyl in PC-12 Cells. To explore the factors that regulate *MOR* gene expression, morphine analog fentanyl was used to treat PC-12 cells. To monitor the fluctuation of mRNA, we performed RT-PCR with 32 to 35 cycles to get a linear range (data not shown). The result revealed that the MOR mRNA level was elevated for 8 h in PC-12 cells after the treatment with fentanyl (Fig. 1A). The elevation of MOR mRNA continued for up to 24 h (Fig. 1D), which indicated a prolonged effect by fentanyl. When PC-12 cells were treated with morphine in our experiments, morphine did not induce the up-regulation of MOR mRNA (Fig. 1D). These results show that long-term treatments of fentanyl induce the up-regulation of MOR mRNA via an activation of MOR in PC-12

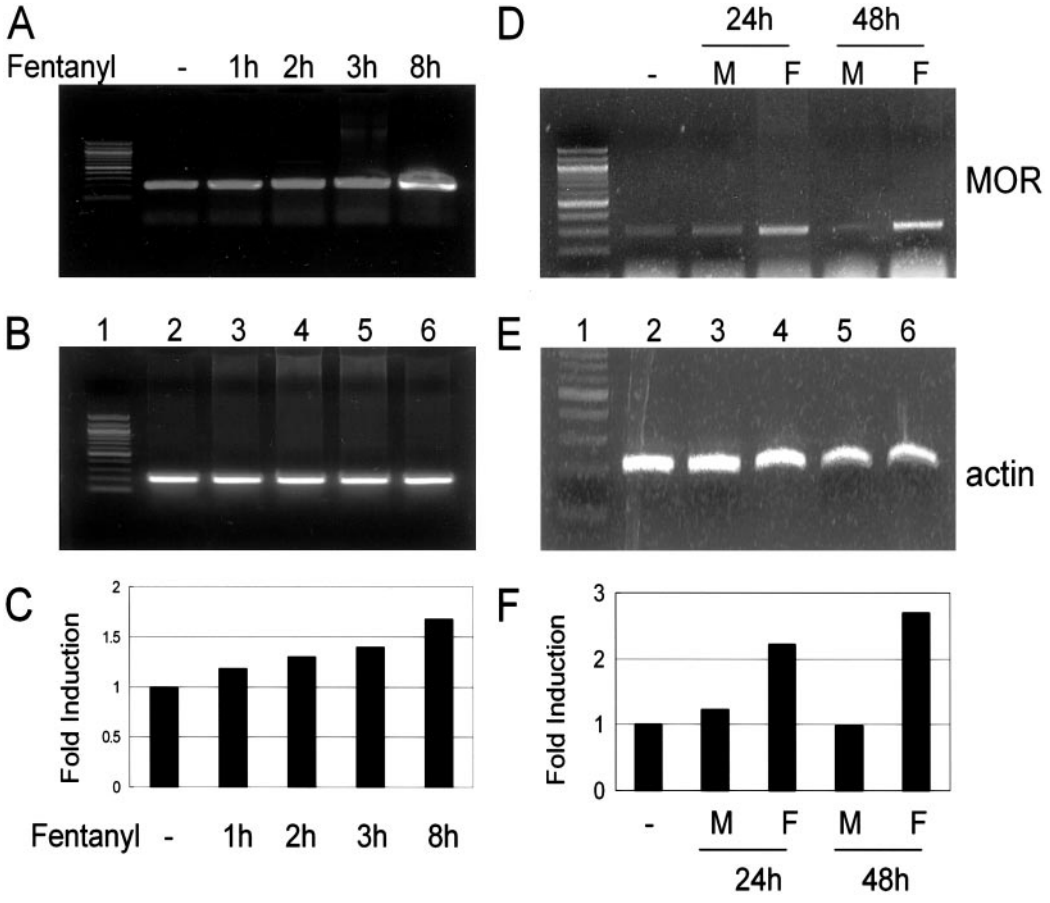

Fig. 1. Fentanyl induces endogenous *MOR* gene expression in PC-12 cells. A, MOR mRNA from PC-12 cells treated by 10 ng/ml fentanyl was reverse-transcribed and amplified by PCR (lanes 3–6). B, RT-PCR of actin mRNA treated as in A. C, quantification of the result. Fold induction is determined by dividing the intensity of MOR in fentanyl-treated samples by that of the untreated sample. D, MOR mRNA from PC-12 cells treated by 10 ng/ml fentanyl (lanes 4 and 6) or 1 μg/ml morphine (lanes 3 and 5) for 24 h (lanes 3 and 4) and 48 h (lanes 5 and 6) was reverse-transcribed and amplified by PCR. E, RT-PCR of actin mRNA treated as in D. F, quantification of the results from D and E. Fold induction is determined by dividing the intensity of MOR in fentanyl- or morphine-treated samples by that of the untreated sample after dividing the intensity of actin for each sample. We repeated the experiment three times with similar results, and we used one RT-PCR product image as a representative. The intensity of each lane is determined with the use of Kodak Image Software.

cells. This result is in agreement with previous finding (Yoshikawa et al., 2000).

To delineate the mechanism of fentanyl-mediated *MOR* gene induction, we used the PKA inhibitor H89 to treat PC-12 cells. As shown in Fig. 2, H89 effectively blocked the activation of MOR, which indicated that the activation was mediated by the cAMP-PKA dependent pathway (Fig. 2). The activation effect of fentanyl on the endogenous *MOR* gene expression and the *MOR* gene expression persisted over an extended period.

A CRE Element on MOR Promoter Is Responsible for Forskolin Induction. We next determined the regulatory region of *MOR* gene that mediated the induction. MOR reporter (pCAT-4.7/ATG), which contains an upstream 4.7-kilobase region and downstream to the translation start site DNA fragment was used in transfection experiments. By limiting less sensitive CAT reporter activity in PC-12 cells, we first examined the reporter in 293T cells to verify the inducibility of cAMP pathway on MOR promoter.

To determine the regulatory element for cAMP induction on MOR promoter, we used forskolin, which is an adenyl cyclase-enhancing agent and may increase cAMP and activate PKA as a result of the lack of MOR in 293T cells. When PC-12 cells were treated with forskolin, the MOR mRNA levels increased markedly, as fentanyl did, and the PKA inhibitor H89 prevented the cAMP-induced up-regulation of MOR in PC-12 cells (data not shown; Yoshikawa et al., 2000). We found that MOR reporter pCAT-4.7/ATG could be induced by forskolin in 293T cells (Fig. 3B). To identify the responsive element for induction, a serial deletion of the MOR promoter was done. As shown in Fig. 3B, 4.7/−253 lost the ability to be activated by forskolin, suggesting that the regulatory region resides within −253 to ATG. 4.7/Pvu-93 and 4.7/Avr-97 displayed a decrease in basal activities (Fig. 3B). However, forskolin inducibility was retained in 4.7/Avr-97. Therefore, a DNA fragment from −93 to −253 should contain the responsive sequence for forskolin induction. A search for transcription factor binding elements over this region was undertaken using the Signal Scan Program, and the results revealed a CRE (CTGACG) located at nucleotides −106 to −111 (Figs. 3A and 10), and three Sp1/GC boxes at nucleotides −235 to −230, − 204 to −210, and − 94 to −99, respectively. The fact that the inducibility of 4.7/Avr-97 was higher than that of 4.7/Pvu-93 implied a spatial hindrance by sp-1 (−94 to −99) for forskolin induction.

Transactivation of Heterologous Promoter. Although the 5′-UTR is involved in post-transcriptional control, this region could also influence transcriptional activity (Choe et al., 1998). Because the CRE element at −106 to −111 is located within 5′-UTR of *MOR* gene, the forskolin-triggered transcription activation of MOR was further tested on a heterologous promoter pLuc, a luciferase reporter driven by

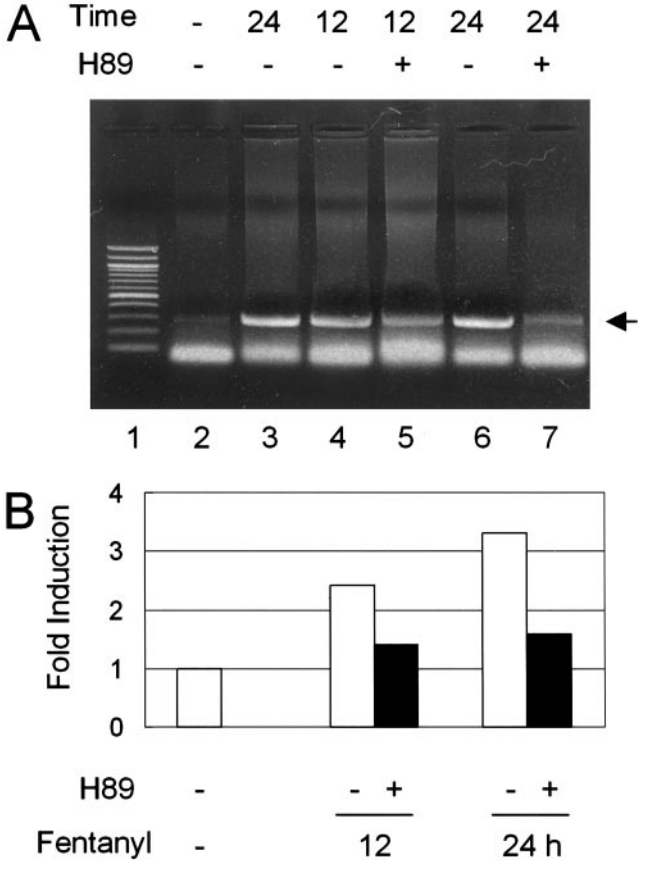

Fig. 2. PKA inhibitor H89 inhibits the fentanyl-induced MOR expression in PC-12 cells. A, MOR mRNA was reversed-transcribed and amplified by PCR. PC-12 cells were treated with 20 μM H89 (lanes 5 and 7) 1 h before fentanyl treatment (lanes 3–7). B, quantification of the result. Fold induction is determined by dividing the intensity of MOR in fentanyl-treated samples by that of the untreated sample.

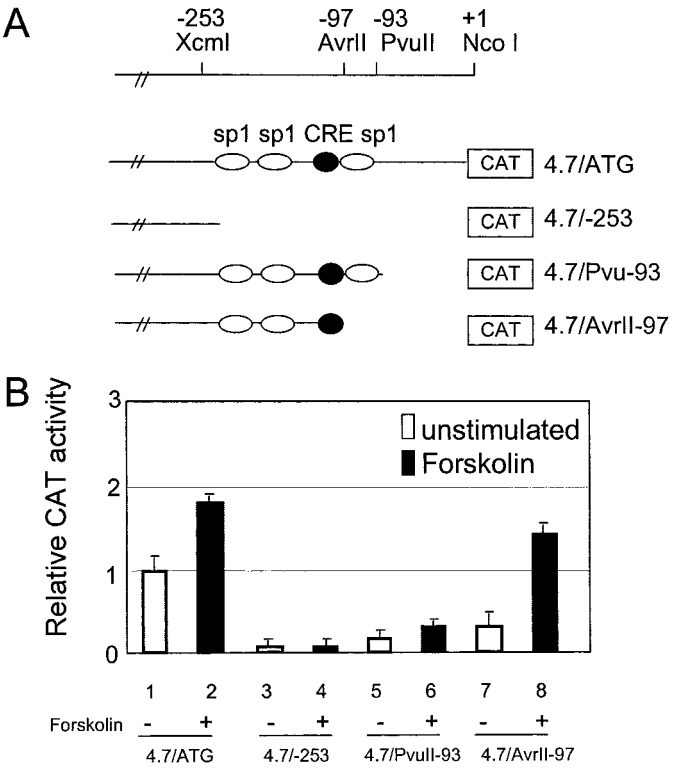

Fig. 3. Effect of forskolin on the MOR promoter. A, schematic diagrams representing the 5′-regulatory region of mouse μ-opioid receptor gene from upstream −4.7kb to the translation start site (ATG) and the deletion clones. B, 293T cells transiently transfected with MOR reporter gene constructs and lacZ internal control. After 24 h of transfection, cells were treated with 10 μM forskolin for 18 h. The promoter activity of each construct was expressed as the activity relative to the uninduced pCAT-MOR4.7/ATG reporter. The histograms represent the mean values of relative activity from four independent experiments. The error bars indicate 1 S.D.

SV40 promoter. The results revealed that when the −300 to −97 MOR 5′-UTR fragment was inserted in the pLuc reporter plasmid, C-RII-R-P-Luc, a 1.8-fold induction by forskolin was still demonstrated (Fig. 4A). Therefore, the CRE element may be involved in transcriptional regulation by forskolin despite its localization on 5′-UTR. To anticipate whether the 5′-UTR bears transactivating activity, we introduced the 5′-UTR into a basal TATA-luciferase reporter. By using the higher sensitivity of the luciferase assay, we went back to PC-12 cells to observe the forskolin induction. A 1.6- to 2.8-fold induction was observed for C-RI and C-RII (Fig. 4B). C-RI has 5-fold higher activity than TATA-Luc, which indicates that 5′-UTR indeed plays a role in transcription activity for MOR expression. Again, the inducibility of C-RII was higher than C-RI as equivalent to 4.7/Avr-97 and 4.7/Pvu-93 in Fig. 3. To get insight into whether the forskolin induction of MOR promoter was tissue specific, we constructed a MOR promoter with luciferase reporter and performed the cAMP induction experiment in 293T, PC-12, and P19 cells. The result was similar in three cells (Fig. 4C).

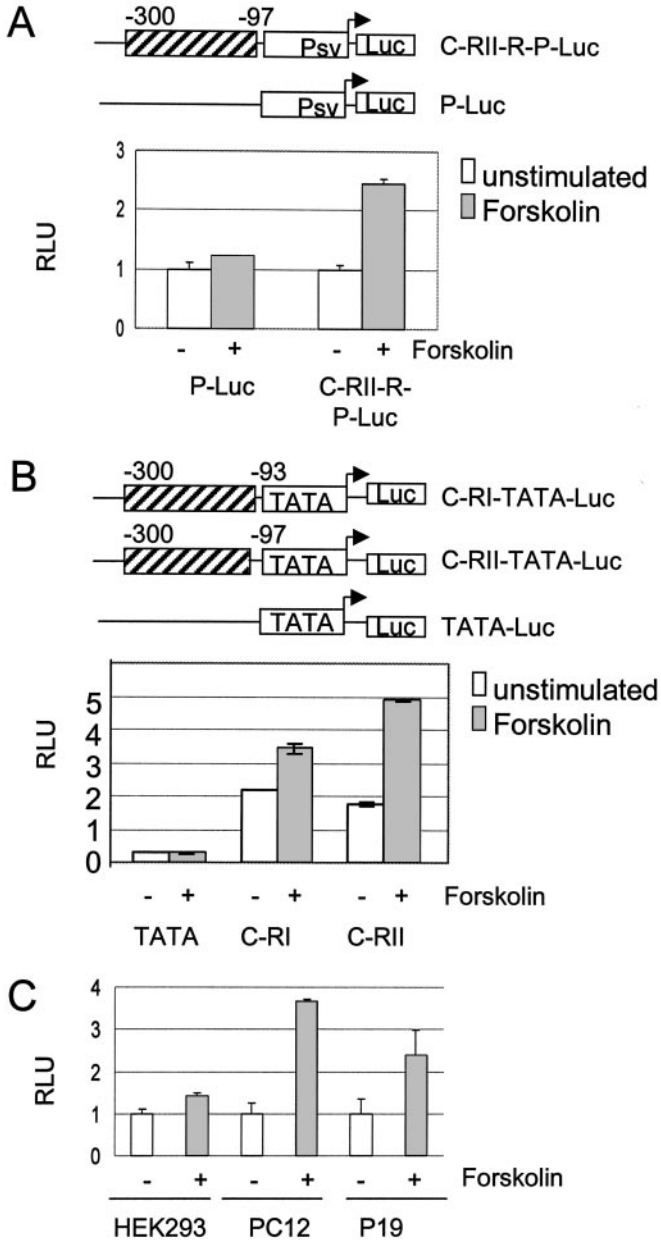

Fig. 4. Forskolin induces MOR 5′-UTR in heterologous promoter. A, −300 to −97 region of the MOR 5′-UTR was inserted upstream to the SV40 promoter in p-Luc (abbreviation for pGL3; Promega). Luciferase activity relative to the uninduced p-Luc vector after transient transfection in 293T cells. B, −300 to −93 (C-RI), and −300 to −97 (C-RII) region of the MOR 5′-UTR was inserted upstream to basic TATA-promoter in p-TATA-Luc. Luciferase activity relative to the uninduced pLuc-TATA vector after transient transfection in PC-12 cells. C, transient transfection using 4.7/ATG-Luc reporter was performed in 293T, PC-12, and P19 cells. The error bars represent 1 S.D. from three independent experiments.

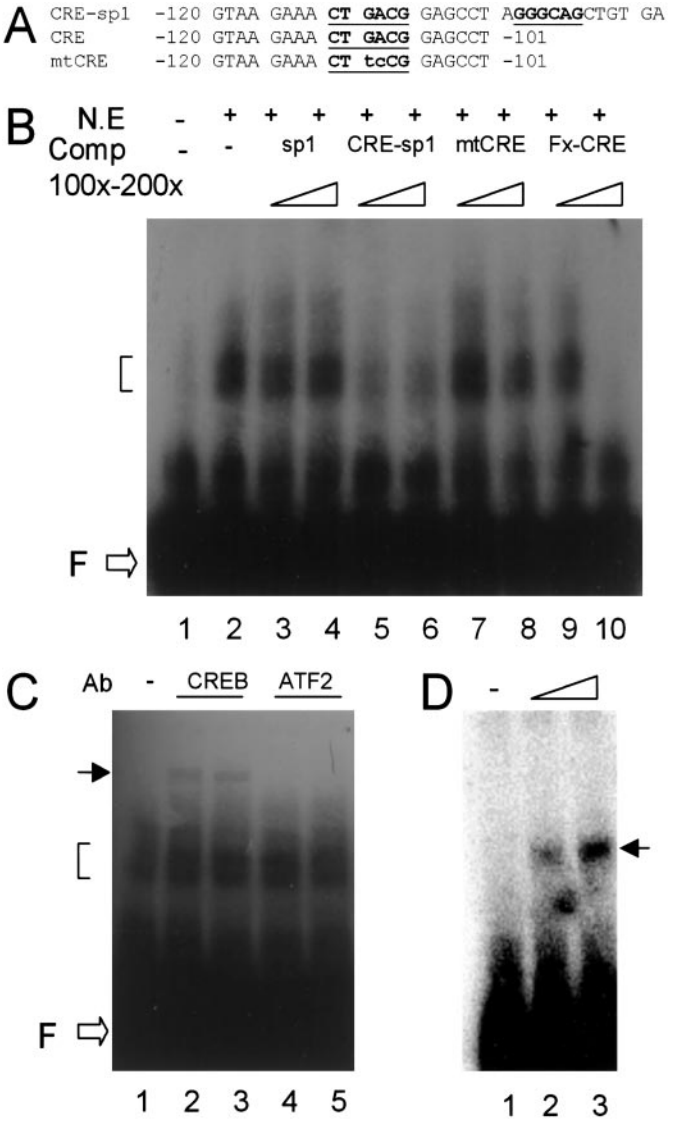

Fig. 5. CREB binds to the CRE element of MOR 5′-UTR. A, competing double-stranded oligonucleotides with consensus sites underlined and mutations in lower case. B, EMSA. PC-12 nuclear extracts were incubated with α-^{32}P–labeled probe of CRE-sp1 (lanes 1–10) in the presence of 100- and 200-fold excesses of cold sp-1 (lanes 3 and 4), CRE-sp1 (lanes 5–6), mtCRE (lanes 7–8), or Fx-CRE (lanes 9–10) oligonucleotides for 30 min at 4°C. Competing oligonucleotides were added before the probe. The bracket indicates a shifted band. F indicates the free probe. C, Antibody supershift assay. Nuclear extracts (2 μg) were incubated with ^{32}P-labeled CRE-sp1 before the addition of anti-CREB (lanes 2–3) or anti-ATF2 (lanes 4–5). The supershifted band was indicated by an arrow. D, EMSA. 0, 200 ng, and 300 ng (lanes 1, 2 and 3, respectively) CREB recombinant protein were incubated with α-^{32}P–labeled probe of CRE-sp1. Arrow indicates the shifted band.

CREB Binds to the CRE of MOR Promoter. To determine the transcription factors involved in the CRE binding on MOR promoter, we performed EMSA with α-^{32}P–labeled DNA probes spanning the CRE element. A major band was revealed in EMSA using nuclear extracts prepared from PC-12 cells (Fig. 5). This band could be efficiently competed out by nonradiolabeled DNA fragments for the CRE and a CRE element on the FMR1 promoter (Fx-CRE), but not by sp-1 or a mutated CRE (Fig. 5). Furthermore, this band was supershifted by CREB antibody (Fig. 5C, lanes 2 and 3) but not by ATF-2 antibody (Fig. 5C, lanes 4 and 5). To confirm the CREB binding to this element, CREB recombinant protein was used in EMSA. Figure 5D showed that CREB binds well to this element. Therefore, CREB is the transcription factor responsible for the cAMP-dependent *MOR* gene activation.

Fentanyl Induces CREB Phosphorylation. Because CREB is phosphorylated and activated by PKA pathway (Vitolo et al., 2002) and fentanyl-induced MOR expression was inhibited by PKA inhibitor H89 (Fig. 2), the phosphorylation state of CREB was determined. The result revealed that fentanyl treatment for 15 min induced CREB phosphorylation in PC-12 cells (Fig. 6A), and the phosphorylation was inhibited by H89 (Fig. 6B). The protein levels of CREB were not changed.

CREB Is Responsible for Forskolin-Induced *MOR* Gene Transcription. Although the binding consensus sequences for CREB (ATGACGTCAT) and AP-1 (ATGACTCAT) are very close, the CG dinucleotide underlined is crucial for CRE. The two sequences should both allow the binding by AP-1. To show the specificity of CREB binding to the CRE, and to exclude the involvement of AP-1, we mutated the CG dinucleotide of CRE box (CTGACG) into AT (CTGAAT). We also mutated the TG dinucleotide (CTGACG) into GA (CGAACG), which will not affect CREB binding. The result revealed that forskolin induction is abolished for mtCRE-2/ATG but not mtCRE-1/ATG (Fig. 7). Thus, cAMP induction of MOR is dependent on CREB, but not AP-1.

To find an in vivo evidence for the role of CREB in forskolin-mediated *MOR* gene activation, we cotransfected a dominant-negative mutant CREB-S133A with pCAT-MOR4.7/ATG. It turned out that the forskolin-mediated MOR activation is abolished in cells transfected with CREB-S133A (Fig. 8).

CREB and CBP Recruitments on Endogenous MOR Promoter by Forskolin. It has been shown that CREB is able to recruit coactivators including histone acetyltransferases to induce an open chromatin conformation for gene expression (Ngo et al., 2002). To examine whether CREB binding and coactivator CBP recruitment to the endogenous MOR promoter could be triggered by forskolin treatment in PC-12 cells, chromatin immunoprecipitation assays were performed. As shown in Fig. 9, forskolin increased CREB binding to the CRE sequence of the endogenous *MOR* gene (lanes 2 and 3). The amount of CBP in this DNA region also increased (lanes 4 and 5). This provided a direct in vivo evidence that forskolin indeed increased CREB occupancy and coactivator recruitment on the regulatory region of the endogenous *MOR* gene. Therefore, we concluded that cAMP activated *MOR* gene transcription in PC-12 cells through a signaling cascade of CREB phosphorylation, formation of CREB-DNA complexes, coactivator CBP recruitment and possibly alteration in chromatin structure.

Discussion

In this study, we defined the regulatory mechanism underlying the fentanyl-induced expression of the *MOR* gene in PC-12 cells. We identified a partial CRE, TGACG, located at −106/−111 in 5′-UTR of *MOR* gene, that accounted for the

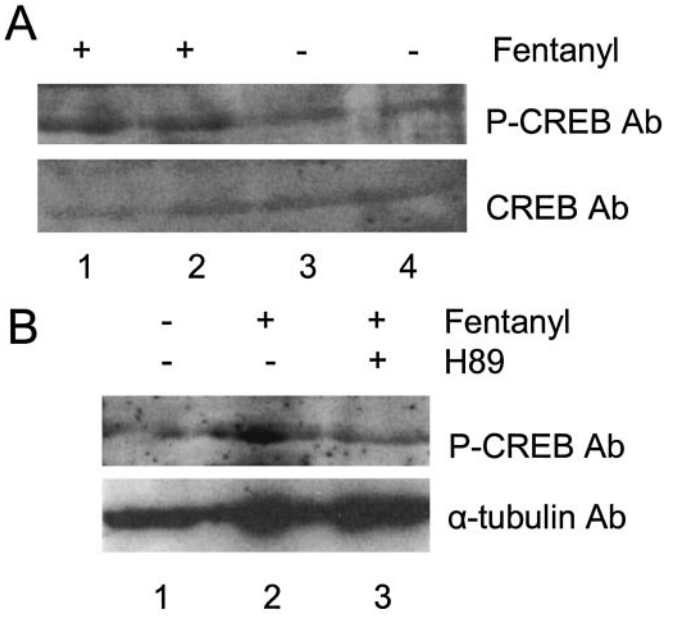

Fig. 6. Fentanyl-induced CREB phosphorylation. A, Western blot. PC-12 cells were serum starved overnight and then treated with 10 ng/ml fentanyl for 15 min (lanes 1, 2) or not (lanes 3, 4). Whole lysate (30 μg) was immunoblotted with anti-phospho-CREB (top) or anti-CREB antibodies. B, PC-12 cells were treated with H89 for 1 h (lane 3) before the addition of fentanyl (lanes 2 and 3). Western blot was performed using anti-phospho-CREB or anti-tubulin antibodies.

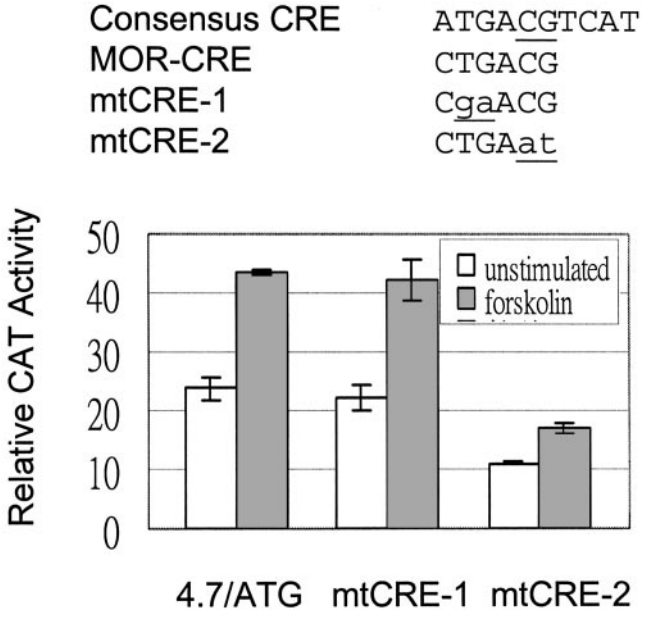

Fig. 7. CRE mutant loses forskolin inducibility. Consensus CRE and MOR CRE sequences are listed; the mutated sequence for mtCRE-1 and mtCRE-2 is underlined in lowercase. Transient transfection using 4.7/ATG and mtCRE-2/ATG reporter was performed in 293T cells. Forskolin (10 μM) was added 24 h after transfection. The error bars indicate 1 S.D. from three experiments in duplicate.

induction by cAMP. Gel shift mobility assay, chromatin immunoprecipitation experiment and function assays all indicated that CREB acted on this CRE. The experiments in which CRE and CREB mutants reduced *MOR* gene expression (Fig. 7) provide a functional link for the involvement of CREB in *MOR* gene expression. Although 5′-UTR sequences diverged during evolution, this CRE is conserved among mouse, rat, and human (Fig. 10), suggesting that cAMP-CREB for *MOR* gene expression shares a common regulation in different species. This study is the first to locate the regulatory element for cAMP-CREB pathway for *MOR* gene expression.

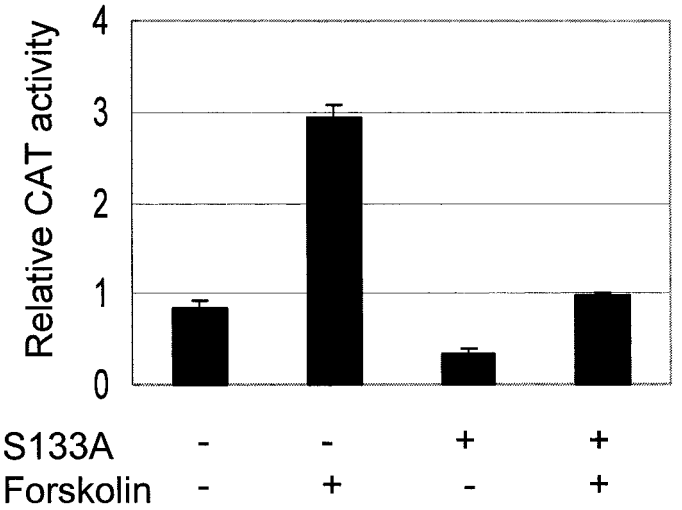

Fig. 8. Dominant-negative mutant CREB-S133A abolishes forskolin-induced activation of *MOR* gene. Transient transfection using pCAT-MOR4.7/ATG reporter was performed in 293T cells. Dominant-negative CREB-S133A was cotransfected (lanes 3 and 4). Forskolin (10 μM) was added (samples 2 and 4) 24 h after transfection.

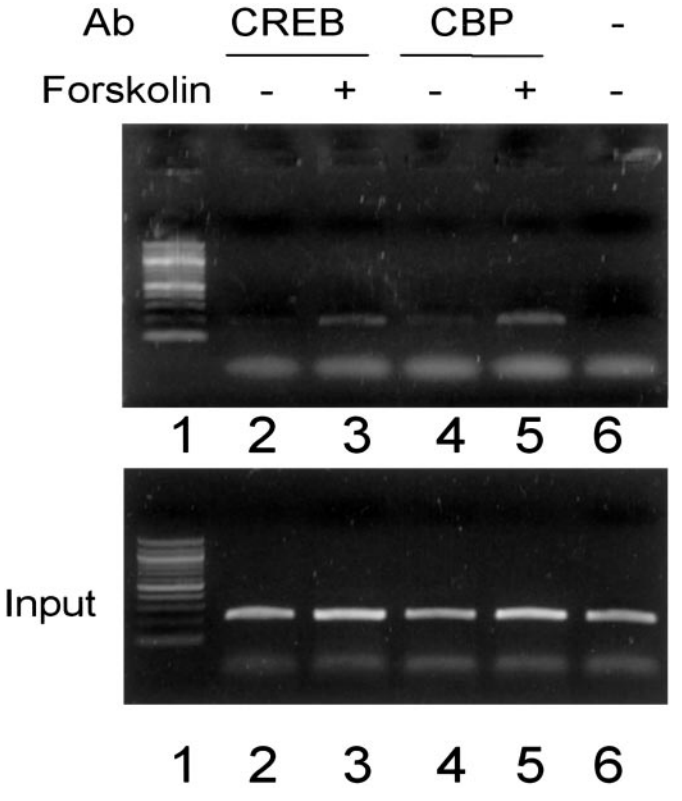

Fig. 9. Forskolin enhances in vivo binding of CREB and CBP to MOR promoter. Top, chromatin immunoprecipitation assays were performed as described under *Materials and Methods*. Cells were treated for 15 min with forskolin (lanes 3 and 5) before the experiments. Bottom, input. A rabbit preimmune serum was used as a control.

Different kinds of opioids are not equal in their tendencies to induce tolerance. Morphine and fentanyl are used extensively in the management of pain. Long-term morphine injections to rats induced tolerance to the analgesic responses of both morphine and fentanyl, but long-term administration of an equieffective dose of fentanyl did not produce tolerance (Paronis and Holtzman, 1992). Similarly, patients with cancer-related pain refractory to morphine do not exhibit tolerance to fentanyl or sufentanil (Paix et al., 1995). These data suggest that although morphine and fentanyl interact with MOR, they may induce distinct biochemical cellular adaptations.

Morphine treatment induces a decrease in cellular cAMP level, but long-term morphine administration could lead to the superactivation of cAMP signaling pathway (Avidor-Reiss et al., 1996; Chieng and Williams, 1998; Nestler, 2001). In a PC-12 cell model, fentanyl leads to cAMP reduction within the first 5 min, followed by a cAMP increase, implying a long-term effect of fentanyl (Yoshikawa et al., 2000), and cAMP elevation is catalyzed by adenyl cyclase, which can be activated by forskolin. However, the functional impact of the difference on the transcriptional activity of CREB during long-term morphine and fentanyl treatment is unclear.

Some studies have demonstrated that treatment of opioids leads to the decrease of opioid receptor (Jordan and Devi, 1998), but others showed no significant change in opioid receptor number (De Vries et al., 1993). Although the mechanisms mediating morphine tolerance remain controversial, Yoshikawa et al. (2000) have suggested that the cAMP-mediated *MOR* gene induction would be a mechanism compensating for the development of tolerance for fentanyl and [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (Yoshikawa et al., 2000; this study). In this study, we raised further functional evidence for this mechanism in reducing the tolerance to fentanyl. Nevertheless, long-term morphine treatment would also lead to cAMP superactivation; it is intriguing that *MOR* gene induction was not observed (Yoshikawa et al., 2000). It is possible that more complex biochemical cellular adaptations, including cAMP signaling and other cellular factors, may have evolved that differ between fentanyl and morphine treatment to control the transcriptional regulation of the *MOR* gene. Two lines of study have evolved. Opioid receptors may undergo a conformational change that switches their coupling from Gi to Gs, as proposed by Crain and Shen (2000). The coupling has been shown to be interconverted rapidly after physiological alteration in the concentration of a specific cAMP-PKA–dependent glycolipid in the neuronal cell membrane. This switch in Gi or Gs coupling may underlie the different effects of fentanyl on cellular cAMP levels.

Another intriguing feature in the difference of MOR signaling is the differential receptor trafficking and desensitization properties after activation by distinct agonists. Although etorphine and morphine activate MOR, etorphine but not morphine elicits MOR phosphorylation (Zhang et al., 1998). Phosphorylation of MOR by G protein-coupled receptor kinase and subsequent β-arrestin binding and receptor desensitization or endocytosis may be related to morphine

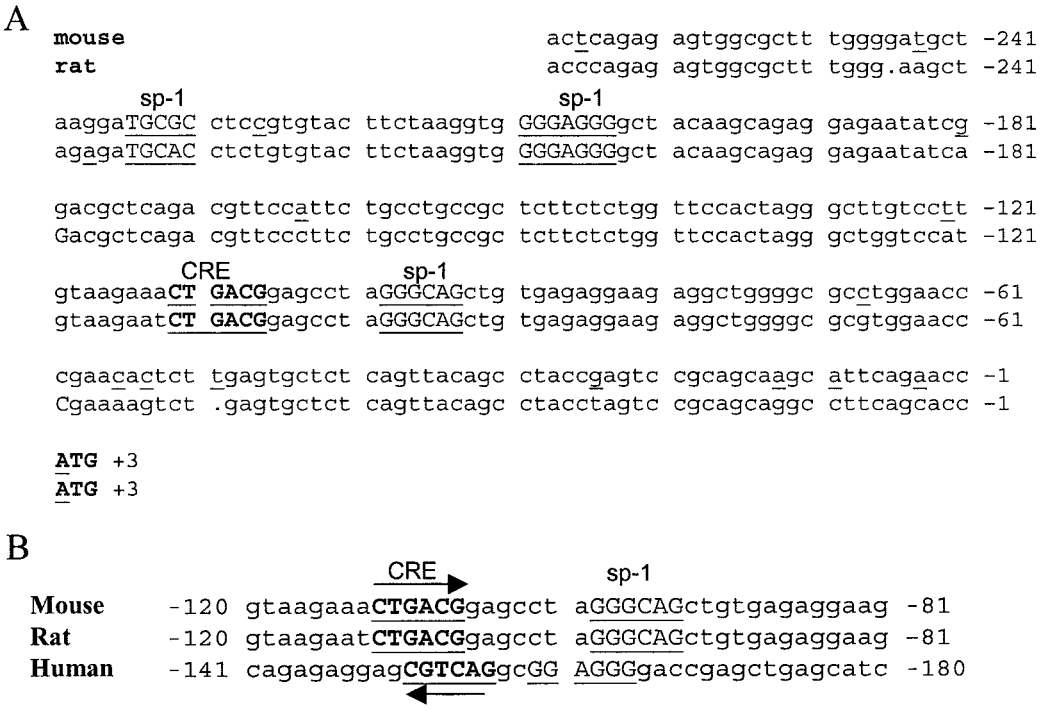

Fig. 10. Sequence conservation of MOR 5′-UTR among mouse, rat, and human. A, the similarity of MOR 5′-UTR between mouse and rat is 95% (255/268 identity). Three sp1 sites and one CRE element are marked on the sequence. B, the CRE element is conserved among mouse, rat, and human (reversed).

tolerance (Zhang et al., 1998). Because morphine is unique among opiates in that it activates the MOR without promoting its desensitization and endocytosis, endocytosis of MOR can reduce the development of tolerance (Li et al., 2002). β-Arrestin would involve morphine-triggered signaling, the observation that β-arrestin knockout mice show reduced analgesic tolerance (Bohn et al., 2000) also suggests that receptor desensitization may contribute directly to morphine tolerance, perhaps by serving as a first step toward receptor down-regulation. Receptor internalization would not be the only method of determining the differences triggered by fentanyl and morphine, even though two drugs caused cAMP elevation. Our finding that fentanyl but not morphine induces *MOR* gene expression in PC-12 cells provides evidence that distinct agonists may induce distinct cellular adaptations.

Receptor internalization by β-arrestin would trigger ASK1 and Raf kinase signaling pathways, which activate MAPK pathway thereafter. CREB is a converged transcription factor for the MAPK and PKA pathways, and serine 133 is phosphorylated by both MAPK and PKA, which leads to CBP recruitment (reviewed by Pierce and Lefkowitz, 2001). In this report, we demonstrated the recruitment of acetyltransferase CBP protein on the chromatin of the MOR promoter region. CBP was originally defined as a protein that binds to the CREB (Chrivia et al., 1993), serving as a bridge to connect sequence-specific transcription factors to transcription apparatus, providing a protein scaffold to build multicomponent transcriptional regulatory complex, and bearing histone acetyl transferase activity to modulate nucleosomal histones (Chan and La Thangue, 2001). The recruitment of CBP suggests that histone acetylation and changes in chromatin structure may be the final step for the activation of *MOR* gene.

Activity-regulated transcription has been implicated in adaptive plasticity in the central nervous system. This plasticity depends upon the transcription factor CREB. CREB plays an important role in many neuronal genes and alters their transcription when it is phosphorylated (Maldonado et al., 1996; Shaywitz and Greenberg, 1999). We demonstrated in this study that fentanyl triggered the phosphorylation of CREB. It is possible that the phosphorylated CREB triggered by fentanyl would exert other cellular functions; conversely, activation of CREB by other stimuli may alter the expression of *MOR* gene. It has been clear that opioids possess immunomodulatory effects, and the cytokine-triggered cAMP-PKA pathway is also elevated in inflammation. Recently, interleukin-4 was shown to induce MOR expression (Kraus et al., 2001). The identification of the CRE box on MOR promoter does support the interplay between pain, neuromodulation, inflammation, and other putative cytokine pathways.

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