Positive Transcriptional Regulation of the Human \(\mu\) Opioid Receptor Gene by Poly(ADP-ribose) Polymerase-1 and Increase of Its DNA Binding Affinity Based on Polymorphism of \(G^{-172} \rightarrow T^*\)

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\(\mu\) opioid receptor (MOR) agonists such as morphine are applied widely in clinical practice as pain therapy. The effects of morphine through MOR, such as analgesia and development of tolerance and dependence, are influenced by individual specificity. Recently, we analyzed single nucleotide polymorphisms on the human MOR gene to investigate the factors that contribute to individual specificity. In process of single nucleotide polymorphisms analysis, we found that specific nuclear proteins bound to \(G^{-172} \rightarrow T\) region in exon 1 in MOR gene, and its affinity to DNA was increased by base substitution from \(G^{-172}\) to \(T^{-172}\). The isolated protein was identified by mass spectrometry and was confirmed by Western blotting to be poly(ADP-ribose) polymerase-1 (PARP-1). The overexpressed PARP-1 bound to \(G^{-172} \rightarrow T\) and enhanced the transcription of reporter vectors containing \(G^{-172}\) and \(T^{-172}\). Furthermore, PARP-1 inhibitor (benzamide) decreased PARP-1 binding to \(G^{-172} \rightarrow T\) without affecting mRNA or protein expression level of PARP-1 and down-regulated the subsequent MOR gene expression in SH-SY5Y cells. Moreover, we found that tumor necrosis factor-\(\alpha\) enhanced MOR gene expression as well as increased PARP-1 binding to the \(G^{-172} \rightarrow T\) region and \(G^{-172} \rightarrow T\)-dependent transcription in SH-SY5Y cells. These effects were also inhibited by benzamide. In this study, our data suggest that PARP-1 positively regulates MOR gene transcription via \(G^{-172} \rightarrow T\), which might influence individual specificity in therapeutic opioid effects.

Opioids have potent analgesic effects, which are mediated by binding of agonists such as opioid alkaloids or opioid peptides to their endogenous receptors. Pharmacological and clinical studies have shown that the \(\mu\) opioid receptor (MOR) affords the greatest analgesic effect among all known opioid receptors.

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2 The abbreviations used are: MOR, \(\mu\) opioid receptor; PARP, poly(ADP-ribose) polymerase; SNP, single nucleotide polymorphism; BZD, benzamide; TNF, tumor necrosis factor; STAT, signal transducers and activators of transcription; BZA, sodium benzoate; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay(s); WB, Western blotting; shRNA, small hairpin RNA; RT, reverse transcription; CDTA, 1,2-cyclohexylenedinitrioltetraacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TK, thymidine kinase; mt, mutant type.
The affinity of NF-κB to A$^{-554}$ was lower than that to G$^{-554}$. Therefore, either the C$^{−995}$ → A or the G$^{-554}$ → A polymorphism has the possibility of influencing the MOR gene expression that interleukin-4 or TNF-α causes through respective transcriptional factors (10, 11). CXBK mice, a cross-breed between C57BL/6By and BALB/cBy mice (12), are known as MOR knockdown mice. It was reported that the base substitution at C$^{−202}$ → A detected in CXBK mice decreased the SP1 binding affinity to the MOR gene (13).

Poly(ADP-ribose) polymerase-1 (PARP-1) is a 116-kDa nuclear protein known to have DNA binding activity and enzymatic activity of ADP-ribosylation (14). PARP-1 catalyzes the nuclear protein known to have DNA binding activity and enzymatic activity of ADP-ribosylation (14). PARP-1 catalyzes the reaction that adds the ADP-ribose unit of NAD$^+$ to several nuclear proteins, including PARP-1 itself (15). Initial study of PARP-1 implicated many biological functions, including DNA repair, recombination, apoptosis, and tumor genesis (15, 16). However, recent studies demonstrated that PARP-1 also contributed to gene transcription in several ways. It was reported that PARP-1 could act as a transcription activator (17–19), but data from other studies showed that PARP-1 might repress transcription (14, 20, 21). Furthermore, PARP-1 modified histones to alter chromatin structure or bound to other DNA-binding factors as coactivators (22, 23).

Recently, we analyzed SNPs on the MOR gene in the Japanese population and found the novel linkage of SNPs (G$^{−1748}$ → A and G$^{−172}$ → T) (24). In these analyses, we found that specific nuclear proteins bound to the G$^{−172}$ → T region, and its affinity was increased by base substitution of G$^{−172}$ → T. In this study, we demonstrated that the identified PARP-1 bound to the G$^{−172}$ → T region in the MOR gene, preferentially to T$^{−172}$, and positively regulated MOR gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Reagents**—The human neuroblastoma cell line SH-SY5Y and the human embryonic kidney cell line HEK293T were cultivated in Dulbecco’s modified Eagle’s medium (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Invitrogen).

Benzamide (BZD; WAKO Pure Chemical Industries, Ltd.) and sodium benzoate (BZA; WAKO Pure Chemical Industries, Ltd.) were dissolved in dimethyl sulfoxide (DMSO; WAKO Pure Chemical Industries, Ltd.). TNF-α was purchased from WAKO Pure Chemical Industries, Ltd. In all experiments, the same amount of DMSO was added to the control samples.

**Nuclear Extract Preparation**—Nuclear extracts were prepared from HEK293T or SH-SY5Y cells. All of the steps were performed at 4 °C. The cells were resuspended in lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin). The lysate was centrifuged at 500 × g for 3 min to pellet the nuclei, which were washed with lysis buffer.

The nuclei were incubated in elution buffer (50 mM HEPES, 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 20% glycerol, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin) for 1 h. The samples were centrifuged at 14,000 × g for 15 min. The supernatant was used for nuclear extracts. Protein concentration of nuclear extracts was determined using the Coomassie protein assay reagent kit (Pierce).

**Electrophoretic Mobility Shift Assays (EMSA)**—All of the oligonucleotides were synthesized by Operon Biotechnologies Inc. (Tokyo, Japan). To obtain double-stranded DNA, equimolar amounts of consensus oligonucleotides were heated to 85 °C for 15 min with 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 50 mM NaCl. After heating, each sample was allowed to cool down to room temperature slowly.

Oligonucleotides used for EMSA were 5′ end-labeled with T4 kinase (Toyobo, Ltd., Osaka, Japan) and [γ-32P]ATP (PerkinElmer Life Sciences). After the reaction, labeled oligonucleotides were separated with MicroSpin™ G-25 Columns (GE Healthcare) and diluted to 100 μl. Oligonucleotide sequences are shown as follows: G$^{−172}$ probe, 5′-AGAGGAGAATTGTCAGATGCTCAAGCTCGGTCCCCTCCGCCTGA-3′; and T$^{−172}$ probe, 5′-AGAGGAGAATGTGCTAGCTCGGTCCCCTCCGCCTGA-3′. Nuclear extracts were preincubated for 15 min at room temperature in 10 μl of binding reaction mixture contained 1 μg of nuclear extracts and 2 μl of 5× gel shift binding buffer (Promega Co., San Luis, CA). Subsequently, 35 fmol of labeled probe was added, and incubation was continued for 30 min at room temperature. For competition assay, unlabeled probe was added to the binding reaction mixture before the addition of the labeled probe. For supershift assay, anti-PARP-1 antibody (sc-8007; Santa Cruz Biotechnology, Santa Cruz, CA) was dialyzed with 0.1× phosphate-buffered saline for 2 days. Subsequently, dialyzed antibody and buffer were concentrated on 8-fold by lyophilization. Two microliters of anti-PARP-1 antibody, concentrated dialyzed buffer, or anti-SP1 antibody (sc-59X; Santa Cruz Biotechnology) was added to the binding reaction and incubated for 1 h at 4 °C before the addition of the labeled probe.

Samples were loaded on a 6% polyacrylamide gel in 0.5× TBE buffer. After running, the gel was dewatered, exposed to the imaging plate and analyzed with Typhoon9410 (GE Healthcare).

**Affinity Purification of DNA-binding Protein**—For blocking nonspecific protein binding, 10 μl of streptavidin-agarose beads (Merck) were preincubated in 2% bovine serum albumin and washed twice in the buffered solution (10 mM Tris-HCl, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, 0.5 mM dithiothreitol, and 50 mM NaCl). Biotin-labeled oligonucleotides (90 pmol) were added to nuclear extracts of 100 μg and incubated for 30 min at room temperature. Next, streptavidin-agarose beads pre-treated with bovine serum were added to the oligo-DNA-protein complex and incubated for 1 h at 4 °C. Bead-oligo-DNA-protein complexes were washed twice in the buffered solution and then collected by centrifugation at 1,400 × g for 5 min. The sample for SDS-PAGE was prepared by heating precipitated complex with 100 μl of Laemmli buffer (62.5 mM Tris-HCl, pH 6.5, 2% SDS, 10% glycerol, and 0.00125% bromphenol blue) containing 5% β-mercaptoethanol.

**PARP-1 Expression Vector**—The PARP-1 coding sequence was cloned from SH-SY5Y cells using PCR amplification; PCR was performed using KOD plus (Toyobo, Ltd.) and the follow-
ing set of primers: forward, 5'-TATCTCGAGATGGCGGAGTCTT-3'; and reverse, 5'-TGCCCTGAGTACCCTTCT-3'. Following sequencing, the fragments were digested with Xhol (Takara Bio Inc., Shiga, Japan) and inserted to expression vector digested with same enzyme. DNA fragments were purified using the Wizard PCR Preps DNA purification system (Promega Co.), and plasmid DNA was purified using the Genopure plasmid maxi kit (Roche Applied Science).

Western Blotting (WB)—Nuclear extracts containing 2 μg of protein were diluted to 100 μl with Laemml buffer contained of 5% β-mercaptoethanol and heated for 5 min at 95 °C. The samples were loaded on 8% polyacrylamide gel and transferred to polyvinylidene fluoride membrane. The PARP-1 protein was detected by using mouse monoclonal antibody that recognizes the C terminus of PARP-1 (sc-8007), anti-mouse horseradish peroxidase secondary antibodies, and ECL reagent. SP1 protein for loading control was detected by using rabbit polyclonal antibody (sc-59), anti-rabbit horseradish peroxidase secondary antibodies, and ECL reagent.

RNA Interference against PARP-1—PARP-1 RNA interference was carried out by using shRNA expression vector. All of the shRNA expression vectors were based on the pGÊNE™tRNA Neo/KpnI/SacI (iGENE Therapeutics, Inc., Tokyo, Japan). Construction of shPARP-1 vectors were accomplished by inserting oligonucleotides directed against human PARP-1. Target area was decided according to the research of Kameoka et al. (25).

The oligonucleotide sequences were as follows: SH-1 (human PARP-1 2401–2421 bp), 5'-AAGCCCTGGCCTCCTGGAAACATCTCTCTGTCAATTGTTCAGGAGCGGAGGCTT-3'; and SH-2 (human PARP-1 2671–2691 bp), 5'-AAGATAGAGCGTGAAGGCGAACTTCGTTCATTCGCTTTCCCGCCACTC-3'. Each shRNA expression vector was transiently transfected into SH-SY5Y cells by using FuGENE 6 (Roche Applied Sciences). Seventy-two hours after transfection, total RNA was prepared and used for RT-PCR and real time RT-PCR analyses.

RT-PCR—Total RNA was prepared using TRIzol (Invitrogen), and reverse transcription was performed in a final volume of 20 μl using 1 μg of RNA, 1 μl of oligo(dT)15 primer (Promega), and 50 units of Rever Tra Ace (Toyobo, Ltd.). The reaction conditions were as follows: 70 °C for 10 min, 4 °C for 5 min, 42 °C for 60 min, and 99 °C for 5 min. PCR was carried out using an ABI PRISM 7000 system (Applied Biosystems, Carlsbad, CA). The reactions were performed in a final volume of 25 μl using 5 μl of diluted cDNA, 12.5 pmoI of each primer, and 0.625 units of Taq polymerase (Qiagen). Amplification conditions were as follows: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min. Primer sequences used are as follows: MOR forward, 5'-CTGCCTGAGATGGCGGAGTCTT-3'; and reverse, 5'-GCCACCAACACCAGGACACCACC-3'; glyceraldehyde-3-phosphate dehydrogenase forward, 5'-GAACATCATCCCTGCTTACT-3'; and reverse, 5'-CTTCCTCTTGTGCGTTCGTG-3'; and dopamine D2 receptor (DRD2) forward, 5'-ATCCCCACCCAGGACACCACCAAG-3'; and reverse, 5'-CACGCGGAGCCCCACAAAGAG-3'.

Real Time RT-PCR—Real time RT-PCR was carried out using a My iQ™ single-color real time PCR detection system (Bio-Rad). A total reaction volume of 50 μl was prepared containing 4 μl of template cDNA, 25 μl of iQ SYBR Green Supermix (Bio-Rad), and 250 nM each of the forward and reverse PCR primers. Amplification conditions were as follows: 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. To amplify the MOR mRNA, a forward primer (5'-CTGGGTCAACCTGTCCC-3') and reverse primer (5'-CTGAGTACGGGCGGACTG-3') were used for amplification of a 146-bp fragment. As an internal control, a 169-bp fragment of the L19 ribosomal protein gene was amplified with a forward primer (5'-CTAGTGTCCTCCGCGTG-3') and reverse primer (5'-AAGGTTGTTTTCCCGCCATC-3'). These primers sequences and the calculation method of relative MOR mRNA level were referred to Bedini's report (26). A nontemplate control was included in each experiment. Melting curve analysis and agarose electrophoresis were carried out to validate the specificity of the amplification products.

Reporter Gene Plasmids—All of the reporter plasmids were based on the pGL3 basic vector (Promega Co.). Construction of pGL3 TK G-172 or T-172 was accomplished by inserting oligonucleotides used for EMSA into the pGL3 basic vector digested with Smal (Takara Bio Inc.). Next, thymidine kinase promoter sequence was obtained from the pRL TK vector (Promega) by digesting with BgIII (Takara Bio, Inc.) and HindIII (Takara Bio, Inc.) and then inserting in all pGL3 vectors. Construction of pGL3G-172 or T-172 was accomplished by inserting the MOR promoter ranged from translation initiation site to 3 kilobase pairs upstream into the pGL3 basic vector at Xhol and HindIII sites. The inserted G-172 fragment was amplified from genmic DNA of the G-172 → G genotype with PCR; PCR was performed using KOD plus (Toyobo, Ltd.) and the following set of primers: forward, 5'-ATCTCGAGATGGCGGAGTCTT-3'; and reverse, 5'-TGCCCTGAGTACCCTTCTCCCATCAGC-3'. The T-172 fragment was amplified from the G-172 fragment by mutagenesis that employed PCR. DNA fragments were purified using the Wizard PCR Prep DNA purification system (Promega), and plasmid DNA was purified using the Genopure plasmid maxi kit.

Transfection—Plasmid DNA transfection to HEK293T cells was performed in a 24-well plate by using NP-OH transfection reagent (27). Plasmid DNA (0.5 μg) and 2.4 μl of NP-OH transfection reagent were added to 12.5 μl of NaCl (40 mM). This mixture was incubated at room temperature for 20 min and used for each well. SH-SY5Y cells were transfected by using FuGENE 6.

Luciferase Assay—HEK293T cells and SH-SY5Y cells were cultivated in 24-well plates at a density of 5 × 104/well for 24 h before transfection. Transient transfection was performed using 0.25 μg of the pGL3 vector, 0.125 μg of the pEF-bos β-galactosidase vector, and 0.125 μg of the pcDNA3-myc vector. The pEF-bos β-galactosidase vector was used for β-galactosidase assay as an internal control of transfection efficacy. After incubation for 24 h, the cells were lysed in 200 μl of lysis solution (25 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 2 mM CDTA, 10% glycerol, 1% Triton X-100, 4 mM MgCl2, and 4 mM EGTA). Each lysate (40 μl) was used for luciferase assay and for
RESULTS

Increase in Specific Nuclear Protein Binding by the Base Substitution of G−172 to T−172—To determine whether specific nuclear proteins bind to the G−172 → T region, we performed EMSA using nuclear proteins extracted from SH-SY5Y or HEK293T cells and 32P-labeled oligonucleotide probes containing the G−172 → T region. SH-SY5Y or HEK293T cells were respectively employed as MOR-expressing cells or as advantageous cells for gene transfer analysis. In each cell, specific nuclear protein binding to both the G−172 and T−172 probes were observed as doublet bands. More proteins displayed binding to the T−172 probe than to the G−172 probe (Fig. 1A). To compare the binding affinity of specific nuclear proteins to the G−172 or to the T−172 probe, a cross-competition assay was carried out using respective nonlabeled probes as a competitor (Fig. 1B). Nonlabeled T−172 probe dose-dependently interfered with specific nuclear protein binding to the G−172 probe (Fig. 1B, lanes 5–7). However, inhibitory effects by the nonlabeled G−172 probe to the T−172 probe were comparatively weak (Fig. 1B, lanes 9–11). These results were similar for both SH-SY5Y and HEK293T cells.

Transcriptional Activity of G−172 → T Region and the Influence of the Base Substitution in MOR Promoter—The reporter vector of G−172 → T TK or of G−172 → T was used for the luciferase assay to monitor transcriptional activity of the MOR gene in SH-SY5Y or HEK293T cells. Oligonucleotide containing the G−172 or T−172 probe was cloned upstream of the thymidine kinase promoter sequence in the pGL3 TK vector. Luciferase activity of pGL3G−172 TK or of pGL3T−172 TK was indicated against that of pGL3 TK (A), and that of pGL3T−172 was expressed against that of pGL3G−172 (B). Activities of coexpressed β-galactosidase were utilized for the correction of transfection efficiency in respective samples. The asterisks indicate significantly difference (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

β-galactosidase assay. Luciferase activity was quantified in MiceoLumat Plus LB96Y (Berthold Technologies, Bad Wildbad, Germany) by measurements based on the luciferase assay using nonlabeled probe for investigation of relative protein binding affinity to G−172 or T−172. Lanes 1–7, radiolabeled G−172 probe; lanes 8–14, radiolabeled T−172 probe; lanes 1 and 8, noncompetition; lanes 2–4 and 9–11, 5–20-fold molar excess of G−172; lanes 5–7 and 12–14, 5–20-fold molar excess of T−172. All of the data were repeated three times in independent experiments or more.

Statistical Analysis—For statistical evaluation of the experiments, one-way analysis of variance was performed. The asterisks indicate significantly difference (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
PARP-1-regulated MOR Gene Transcription

Purification of Specific Binding Protein on G<sup>−172</sup> → T Region Using Biotin-labeled T<sup>−172</sup> Probe, and Identification of the Protein by MALDI-TOF Mass Spectrometry and Immunoblotting with Specific Antibody—For identification of binding proteins in the G<sup>−172</sup> → T region, the biotin-labeled T<sup>−172</sup> probe and streptavidin-agarose beads were applied to nuclear protein purification. Protein separated by SDS-PAGE was stained with Coomassie brilliant blue and observed as a single band at 120 kDa (Fig. 3A, lane 5). MALDI-TOF mass spectrometry revealed that the 120-kDa protein separated from acrylamide gel was PARP-1, which was confirmed by WB with anti-PARP-1 antibody in both SH-SY5Y and HEK293T cells (Fig. 3B).

Enhancement of Protein Binding to T<sup>−172</sup> and of Transcriptional Activity by Overexpressed PARP-1—PARP-1 overexpression enhanced protein binding to the T<sup>−172</sup> probe and was detected as doublet bands in EMSA (Fig. 4B); it also increased transcriptional activity of pGL3<sup>T−172 TK</sup> or of pGL3<sup>T−172</sup> (Fig. 4C). Similarly, the increase in transcriptional activities was also observed in pGL3G<sup>−172 TK</sup> or in pGL3G<sup>−172</sup> (data not shown). Furthermore, in supershift analysis, anti-PARP-1 antibody specifically diminished PARP-1-delivered doublet bands (Fig. 5, lanes 3 and 7). SP1 was initially predicted by computer program to bind G<sup>−172</sup> → T; however, anti-SP1 antibody did not influence protein-binding signals in T<sup>−172</sup> (Fig. 5, lanes 4 and 8). These results clearly indicate that PARP-1 binds to the G<sup>−172</sup> → T region and activates transcription of MOR, which is influenced by base substitution.

Detection of PARP-1 Binding Sequence in T<sup>−172</sup> Region—For detection of a PARP-1-binding sequence in the G<sup>−172</sup> → T region, a competition assay was performed using shortened or mutated T<sup>−172</sup> probes. In the analysis using shortened probes, mt 1 competed to T<sup>−172</sup> probe and diminished PARP-1 signals, but mt 2 did not have this effect. Among mutated probes of mt 1, mt 3 completely diminished PARP-1 signals, but inhibition by mt 4, mt 5, or mt 6 was partial (Fig. 6A). These results indicate that TGTCAGATG was the center of PARP-1 binding in the MOR gene, and the sequence CTCAT beside the T<sup>−172</sup> also related to its binding.
SH-SY5Y cells are known to express MOR constitutively. MOR mRNA expression detected by RT-PCR was suppressed by BZD in a dose-dependent manner. However, BZA, as a negative control for BZD, did not influence the tested mRNA expressions, including MOR (Fig. 7A). Real time RT-PCR analysis showed that BZD resulted in a 0.7-fold depletion of MOR mRNA expression (Fig. 7B).

Moreover, PARP-1 expression was knocked down by two kinds of PARP-1 shRNA vector in SH-SY5Y cells, which was investigated for the PARP-1-dependent regulation of MOR expression. For correction of the nonspecific effects by transfection, SH-SY5Y cells were also transfected with a control shRNA. The MOR mRNA expression was analyzed by RT-PCR or real time RT-PCR 72 h after transfection. The MOR mRNA levels were decreased by depletion of PARP-1 (Fig. 7A). Real time RT-PCR analysis showed that PARP-1 knockdown resulted in a 0.6-fold depletion of MOR mRNA expression (Fig. 7B). The increase in DRD2 mRNA by BZD or by PARP-1 knockdown indicated that the inhibition of MOR mRNA expression by both treatments was not merely caused by their nonspecific toxic effects.

**TNF-α Increased PARP-1 Binding to G−172 → T Region and MOR Promoter Activity—**Next, we searched for factors that increase PARP-1-binding to the G−172 → T probe. We found that TNF-α increased the binding of PARP-1 1–6 h after the addition of TNF-α in SH-SY5Y cells (Fig. 8A). Phorbol 12-myristate 13-acetate, prostaglandin E2, quinpirole (dopamine D2 receptor agonist), trichostatin A, and [d-Ala2,N-Me-Phe4,Gly5-ol]enkephalin also up-regulated MOR mRNA expression, but all of which did not activate PARP-1 binding to T−172 probe in SH-SY5Y cells. Only TNF-α up-regulated both MOR expression and PARP-1 binding to G−172 → T region. Moreover, the signals of PARP-1 binding to the T−172 probe were higher than those of G−172 probe with or without stimulation by TNF-α (Fig. 8A). Corresponding to the results of PARP-1 binding to the G−172 → T probe, TNF-α preferentially increased transcri-}

**FIGURE 5.** Super shift analysis with anti-PARP-1 antibody for reconfirma-
* SH-SY5Y
  * HEK293T
  * CON
  * Dialysate
  * Anti PARP-1
  * Anti Sp1

**FIGURE 6.** Analysis of PARP-1 binding sequence in the T−172 probe by

**DISCUSSION**

The G−172 → T polymorphism in the MOR gene promoter is caused by base substitution from guanine to thymine, which is located 172 bp upstream of the translation initiation site. In previous studies, no functional differences were found between G−172 and T−172 on MOR promoter activity (28). However, we
definitely detected such differences, i.e. the T\(^{-172}\) probe displayed higher binding affinity to unknown nuclear protein and higher transcriptional activity than the G\(^{-172}\), thereby implying that this protein regulates MOR transcription at G\(^{-172}\) → T (Figs. 1 and 2). Therefore, we employed the computer program TFSEARCH to predict known transcription factors bindable to neighborhood of G\(^{-172}\) → T region, which assessed that the sterol regulatory element binding protein, SP1, or GATA-1 possibly bound to G\(^{-172}\) → T region. On the basis of the anticipated results, we attempted to compete protein binding to T\(^{-172}\) probe with oligo-DNA-probes containing the consensus binding sequences for the anticipated transcription factors, but the binding was not influenced by these competitions (data not shown). Consequently, we purified the specific nuclear protein bound to the T\(^{-172}\) probe and identified PARP-1. WB using anti-PARP-1 antibody confirmed that this nuclear protein was definitely PARP-1 (Fig. 3B). The binding of overexpressed PARP-1 to the G\(^{-172}\) → T probe was also detected as doublet bands in EMSA (Fig. 4B), and anti-PARP-1 antibody diminished both bands in supershift analysis (Fig. 5). Although we can only speculate about the details, PARP-1 might have two kinds of binding forms to the G\(^{-172}\) → T probe.

In the relationship between PARP-1-related transcriptional regulation and gene polymorphisms, it has been reported that G\(^{-228}\) → T at the transcriptional region in the MARCB1 gene significantly increased PARP-1 binding affinity and reporter activity; furthermore, this SNP altered the level of SMARCB1 mRNA and protein expression in human acute lymphoblastic leukemia cell lines (29). It was also reported that PARP-1 bound to NACP-Rep1 in microsatellite repeats located in the SNCA gene, which exhibited different transcriptional activities resulting from the varying individual length of NACP-Rep1 sequences (30, 31).

Previously, many reports have shown the PARP-1-binding sequence, but some of these results are controversial, e.g. those for the murine inducible nitric-oxide synthase gene (5'-AAT-TATATAATT-T-3'), the rat Reg gene (5'-TGGCCCTCCCAT-3'), the mouse Tcirg1 gene (5'-TTCCCAAGC-3'), the human SNCA gene [5'-(TC)\(_{10}\)(T)\(_2\)(TC)\(_{10}\)(TA)\(_9\)(CA)\(_{11}\)-3'], and the human SMARCB1 gene (5'-CTTCTTGTGGCATGCCTGCCGCGCCGCGCTC-3') (29, 31–34). We speculate from these reports that PARP-1 tends to bind to the CCCC sequence like GC-box, but it is difficult to forecast the appropriate PARP-1-binding region from DNA sequences, as with other transcription factors. In many cases in previous studies, the PARP-1-binding region has been revealed only after the DNA protein binding to a specific DNA region was identified as PARP-1 by mass spectrometry. Such is also the case in our study.

In a report concerning the mouse MOR gene, PARP-1 bound to the poly(C) sequences (5'-CTTCTGCTCCCCCCTACCC-3'; \(-430\) to \(-407\) bp) and repressed MOR gene expression in mouse neuroblastoma NS20Y cells (35). In contrast to...
that report, our results show that PARP-1 increases human MOR gene transcriptional activity (Fig. 4C), BZD, an inhibitor of PARP-1, or PARP-1 shRNA decreases MOR gene expression in human neuroblastoma SH-SY5Y cells (Fig. 7, A and B). When an unknown protein binding to the T-172 probe was revealed as PARP-1, we expected that PARP-1 bound to the CCCC sequence of these probes located 162–165 bp upstream from PARP-1, we expected that PARP-1 bound to the CCCC sequence of these probes located 162–165 bp upstream from the translation start site of the MOR gene (Fig. 6B). However, the results in Fig. 6 clearly show that PARP-1 binds to TGTCA-GATG within the T-172 probe (5’-AGAGGAGAGATGCAGATGCTCA (G/T) CTCGGCCCTCCGCCTGA-3’; −194 to −153 bp) but not to CCCC. Thus, human MOR gene transcription regulated by PARP-1 might be in different mechanism from that of the mouse because the human MOR regulatory region including the G-172 → T region did not have a region similar to the poly(C) sequences in the mouse MOR gene.

We explored the inducing factors for MOR expression, as well as those for PARP-1 binding to the G-172 → T region, and we found that TNF-α increased MOR expression and PARP-1 binding to T-172 probe. TNF-α was reported as the inducer of MOR transcription in human immune effector cells through NF-κB (10). In our study, the increase of MOR gene expression by TNF-α (Fig. 9, A and B) was suppressed by BZD in SH-SY5Y cells (Fig. 9, E and F). These results suggest that TNF-α induces MOR transcription in SH-SY5Y cells not only through NF-κB but also through PARP-1. Both the increase of PARP-1 binding to T-172 probe by TNF-α and decreasing of PARP-1 binding by BZD did not involve changing the amount of PARP-1 protein level. Furthermore, anti-PARP-1 antibody that recognized the PARP-1 C terminus containing the catalytic domain completely diminished bands of PARP-1 binding to the G-172 → T region in EMSA but did not shift it upward. Considering that BZD inhibits PARP-1 catalytic activity and that anti-PARP-1
antibody recognizes the PARP-1 C terminus containing the catalytic domain, the catalytic activity of PARP-1 might be crucial for its own binding to the G$^{-172} \rightarrow T$ region, as well as for MOR gene transcription.

In this study, TNF-α remarkably increased PARP-1 binding to the T$^{-172}$ probe, but details of the mechanism for this binding are still not known; it might result from post-translational modification of PARP-1 through auto-PARylation, acetylation, or phosphorylation (36–39). PARP-1-dependent transcription was reported to be regulated by cellular signaling pathways. Actually, direct phosphorylation of PARP-1 by ERK1/2 or phosphorylation (36–39). PARP-1-dependent transcription studies reported that DNA binding activity of PARP-1 was altered because of its catalytic activity by NAD$^+$ in in vitro experiments (35). In our preliminary study, NAD$^+$ stimulated PARP-1 binding to T$^{-172}$ probe; furthermore, TNF-α stimulated PARP-1 catalytic activity (data not shown). Therefore, the regulation of nuclear NAD$^+$ synthesis could be critical for PARP-1-dependent gene regulation including MOR expression.

In summary, our study demonstrated that PARP-1 binds to the G$^{-172} \rightarrow T$ region and up-regulates MOR gene transcription. PARP-1 binding affinity to T$^{-172}$ sequence was higher than that of G$^{-172}$, and the transcriptional activity with or without TNF-α was also enhanced by T$^{-172}$ substitution, which was considered to be related to relate to own catalytic activity. In conclusion, PARP-1 positively regulates MOR gene transcription via G$^{-172} \rightarrow T$, which possibly influences individual specificity in the therapeutic opioid effect, furthermore, which might provide useful information for inflammatory pain therapy.

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