Identification of Four Novel Exon 5 Splice Variants of the Mouse μ-Opioid Receptor Gene: Functional Consequences of C-Terminal Splicing

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ABSTRACT

The rat μ-opioid receptor clone in which novel exon 5 was found in the place of exon 4 (MOR-1B) was one of the first MOR-1 variants described. We now have identified the mouse homolog of the rat MOR-1B as well as four additional variants derived from splicing from exon 3 into different sites within exon 5. The sequences of all of the variants were identical except for the intracellular tip of the C terminus encoded by exon 5, where each variant predicted a unique amino acid sequence ranging from 2 to 39 amino acids. All of the mMOR-1B variants were selective for μ-opioids in receptor-binding assays, as anticipated, because they have identical binding pockets defined by the transmembrane domains. However, the relative potency and efficacy of μ-agonists to each other varied from variant to variant in guanosine 5′-O-(3-[35S]thio)triphosphate-binding studies, as shown by morphine-6β-glucuronide, which was the most efficacious agent against mouse MOR-1B1 (mMOR-1B1) and the least efficacious agent against mMOR-1B2. mMOR-1B4 was quite unusual. Although mMOR-1B4 was μ-selective in receptor-binding studies and antagonists labeled mMOR-1B4 well, the binding affinities of most of the μ-agonists were far lower than those seen with mMOR-1, suggesting that the 39 amino acids at the C terminus of mMOR-1B4 influences the conformation of the receptor and its ligand recognition site itself either directly or through its interactions with other proteins. In conclusion, alterations in the amino acid sequence of the C terminus do not alter the μ-specificity of the receptor but they can influence the binding characteristics, efficacy, and potency of μ-opioids.

After the classification of the μ-, κ-, and δ-opioid receptors (Martin et al., 1976; Lord et al., 1977; Kosterlitz and Leslie, 1978), drugs were classified by their selectivity profiles in receptor-binding assays. Most clinical analogs are μ-selective, having little affinity for either κ- or δ-receptors, yet clinicians have long known that patient responses to μ-opioids can vary widely, both in terms of their relative analgesic activity and their side effects (Payne and Pasternak, 1992). Clinicians also have used incomplete cross-tolerance among μ-opioids to regain analgesic activity in patients highly tolerant to one μ-drug by switching them to a different μ-opioid, a technique termed opioid rotation (Cherny et al., 2001). These and other clinical observations raised the possibility that all of the μ-opioids might not be acting through identical receptor mechanisms.

The concept of multiple μ-opioid receptors was formally proposed almost 25 years ago (Wolozin and Pasternak, 1981) based on identification of a novel receptor-binding site (Pasternak and Snyder, 1975a; Lutz et al., 1984) and the actions of the novel antagonists naloxonazone and naloxonazine, agents that dissociated opioid analgesia from other opioid actions, including respiratory depression, the inhibition of gastrointestinal transit, and most signs of physical dependence (Pasternak et al., 1980; Ling et al., 1984, 1985; Heyman et al., 1988).

ABBREVIATIONS: MOR-1, μ-opioid receptor clone-1; mMOR-1, mouse MOR-1; rMOR-1, rat MOR-1; ANOVA, analysis of variance; DAMGO, [d-Ala₂,N-Me-Phe₆,Gly⁵-ol]-enkephalin; nor-BNI, norbinaltorphimine; [Dmt]DALDA, Dmt-d-Arg-Phe-Lys-NH₂; DPDPE, [d-Pen⁵,d-Pen⁶]enkephalin; CTAP, d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂; M6G, morphine-6β-glucuronide; U50,488H, trans-[(d)-3,4-dichloro-N-methyl-N-2-[1-pyrrolidinyl]cyclohexyl]benzeneacetamide; Gpp(NH)p, guanosine-5′-O[(β,γ)-imido]triphosphate; GDPβS, guanosine 5′-O-(thiotriphosphate); DAMLE, [d-Ala₂,d-Leu⁵]-enkephalin; CHO, Chinese hamster ovary; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s).
The cloning of the μ-opioid receptor MOR-1 opened the study of μ-opioid receptors at the molecular level (Chen et al., 1993; Eppler et al., 1993; Thompson et al., 1993; Wang et al., 1993). Soon after the initial reports, two MOR-1 splice variants were identified, providing the first appreciation of the complexity of the μ-opioid receptor gene, Oprm (Bare et al., 1994; Zimprich et al., 1995). MOR-1B, initially isolated from the rat, differed from MOR-1 with the replacement of exon 4 by exon 5. At the protein level, the predicted 12 amino acids at the C terminus of MOR-1 were replaced by a different sequence of five amino acids, which functionally made rat MOR-1B (rMOR-1B) less sensitive to agonist-induced desensitization. MOR-1 splicing has now been observed in a variety of species. A number of additional variants involving splicing at the C terminus have been identified in mice and rats (Pan et al., 1999, 2000, 2001; Narita et al., 2003; Kvam et al., 2004; Pasternak et al., 2004), and humans (Pan et al., 2003, 2005). Similar to rMOR-1B, these variants have alternative exons in place of exon 4; however, splicing in the mouse is even more complex. A series of additional variants derived from splicing at the 5′-end of the mouse Oprm gene also have been described previously (Pan et al., 2001). We now report the identification of the mouse homolog of rMOR-1B and a series of additional variants derived from alternative splicing within the mouse exon 5.

Materials and Methods

Isolation of Genomic BAC Subclones Containing Exon 5. To identify the mouse homolog of the rat exon 5 in mouse Oprm gene, the mouse BAC clone A (Pan et al., 1999, 2000, 2001) containing exons 1 to 4 was digested with HindIII. The HindIII-digested fragments were then subcloned into the HindIII site of Bluescript SK vector. After being transformed into JM109-competent cells, the transformants were plated on Luria broth plates containing 0.2 mM isopropyl 1-thio-β-D-galactopyranoside, 0.008% 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside, and 100 μg/ml ampicillin for colony lifting. A 280-bp cDNA fragment of the rat exon 5 was obtained by reverse transcription (RT)-polymerase chain reaction (PCR) with rat brain total RNA, and a pair of primers from rat MOR-1B (antisense primer, 5′-GGG GTT GGC ACC AGC-3′; sense primer, 5′-TTG CGG GGG GTG GGG GTG-3′) (Zimprich et al., 1995) was 32P-labeled and used as a probe for colony hybridization. The colony-lifting and hybridization procedures were performed using the protocols described previously (Pan et al., 1999, 2000, 2001). Six positive clones with identical −1-kb insertions were obtained. Sequence analysis of the fragment indicated that the clone contained the exon sequence, exon 5a, homologous to rat exon 5. The mouse exon 5a and rat exon 5 sequences shared 91% identity.

RT-PCR. Total RNA was extracted from mouse brain by the guanidinium thiocyanate-phenol-chloroform method (Pan et al., 1999, 2000, 2001) and reverse-transcribed with random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The first-strand cDNA was then used as template in PCR with a sense primer from exon 3 (5′-CCC AAC TTC TTC CAC AAT CGA A-3′) and antisense primer from exon 5a (5′-GTT CAT TGA GAG AAG CTT GCC CAG-3′), and antisense primer from exon 5b (5′-GTT CAT TGA GAG AAG CTT GCC CAG-3′), and sense primer from exon 5a (antisense A, 5′-CTT GCC CCA GAG AGC GAA TGA TGC AG-3′; sense primer B, 5′-GGG GTT GCC ACC AGC ATG TGG TAC TC-3′), two antisense primers from exon 5a (antisense A, 5′-CTT GCC CCA GAG AGC GAA TGA TGC AG-3′; antisense primer B, 5′-GGG GTT GCC ACC AGC ATG TGG TAC TC-3′), two antisense primers from exon 5b sequence (antisense primer C, 5′-GGT GTT CTT CTT TTC GGA TAT GGA ATG TGC AGT AGC at ASPET Journals on September 30, 2017 molpharm.aspetjournals.org Downloaded from monitored by ORCID iD
with G418 or hygromycin and screened in [3H]DAMGO or [3H]di-
prenorphine-binding assays (PerkinElmer Life and Analytical Sci-
ences).

Relative Semiquantitative RT-PCR. Total RNA obtained from
mouse brain as described above was treated with TURBO DNA-free
reagents (Ambion, Austin, TX) and reverse-transcribed with random
hexamers and Superscript II reverse transcriptase. Aliquots of the
first-strand cDNA were used as templates in PCRs with sets of
primers specific for each variant. The following PCR primers were
used: mMOR-1B1, exon 3 sense primer A (5'-GCT GCC ACC CAG
TTC TTT ATG CG-3') and an exon 5a antisense primer (5'-
GGG GTT GCC ACC AGC ATT AGG TAC TC-3'); mMOR-1B2, exon
3 sense primer B (5'-GCA TCC CAA CCT CCT CCA TCG
AAC-3') and an exon 5b antisense primer (5'-CCC CTT TGG TGC
CCT ATAT CAA GCC-3'); mMOR-1B3, exon 3 sense primer A
and an exon 5c antisense primer (5'-CTA GGT CTA GCT CAT GAA
TGC TCT TTG GTC GG-3'); mMOR-1B4, exon 3 sense primer A and
an exon 5d antisense primer (5'-GCA ACC TCC TAC CAA GTA GAT
GGG GTG GGC ACC AGC ATT AGG TAC TC-3'); mMOR-1B5, exon 3 sense primer A and an exon 5e
antisense primer (5'-TTG CGG GGG GTG TGC TCT TTG GTC GG-3'); and
mMOR-1, exon 3 sense primer A and an exon 4 antisense primer
(5'-GCA ACC TCC TAC CAA GTA GAT GCC AG-3'). PCRs were
performed in a thermal cycler (PTC-200; MJ Research (Watertown,
MA) using Platinum TaqDNA polymerase (Invitrogen) with an ini-
tial 2-min denaturing step at 94°C followed by 48 cycles, each con-
taining a 20-s denaturing step at 94°C, a 20-s annealing step at 65°C,
a 45-s of extension step at 72°C, and a final 3-min extension at 72°C.
PCR products were separated on a 1.5% agarose gel. The gel then
was stained with 0.02% ethidium bromide, and images were taken
with a FluorChem 8000 (Alpha Innotech, San Leandro, CA). Relative
band intensities were quantified with the AlphaEase FC software
(Alpha Innotech). All of the PCR products showed bands with correct
sizes predicted from the following primers: 295 bp for mMOR-1B1,
187 bp for mMOR-1B2, 362 bp for mMOR-1B3, 417 bp for mMOR-
1B4, 266 bp for mMOR-1B5, and 278 bp for mMOR-1. Each band was
purified, and the gel and its identity were confirmed by DNA se-
quencing. PCR controls without the template showed no visible
bands (data not shown).

Receptor-Binding Assays. Membranes were prepared from cells
stably transfected with the pcDNA3 constructs as described above.
[3H]DAMGO or [3H]diprenorphine saturation and competition-bind-
ing assays were performed at 25°C for 60 min in 50 mM potassium
phosphate buffer, pH 7.4, containing 5 mM magnesium sulfate (Pan
et al., 1999, 2000, 2001). Specific binding was defined as the differ-
ence between total binding and nonspecific binding, determined in
the presence of 10 M levallorphan. Protein concentrations were
determined as described previously using bovine serum albumin as

[35S]GTPγS-Binding Assay. [35S]GTPγS binding was performed
on membranes prepared from transfected cells in the presence and
absence of the indicated opioid for 60 min at 30°C in the assay buffer (50
mM Tris-HCl, pH 7.4, 3 mM MgCl2, 0.2 mM EGTA, and 10 mM NaCl)
containing 0.05 nM [35S]GTPγS and 30 μM GDP, as previously reported
(Pasternak et al., 2004; Pan et al., 2005). After the incubation, the
reaction was filtered through glass-fiber filters (Whatman Schleicher &
Schuell, Keene, NH) and washed three times with 3 ml of ice-cold 50

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Schematic of the mouse **Oprm** gene structure and alternative splicing. The schematic of the gene structure. Exons and introns are shown by boxes and horizontal lines, respectively. The transcriptional start points are indicated by arrows. The genomic BAC clones are shown by heavy horizontal lines on the top. The complete cDNA and deduced amino acid sequences of mMOR-1B1, mMOR-1B2, mMOR-1B3, mMOR-1B4, and mMOR-1B5 have been deposited in the GenBank database (accession numbers AF167566, AF167567, AF346813, AF346814, and AF346812, respectively).
Results

Cloning Exon 5 Splice Variants of the Mouse Oprm Gene. In the rat, rMOR-1B contains exon 5 in place of exon 4 (Fig. 1) (Zimprich et al., 1995). We obtained the mouse exon 5a sequence from the mouse genomic BAC clone A using a semiautomatic cell harvester. Filters were transferred into vials with 5 ml of Liquiscent (National Diagnostics, Atlanta, GA), and the radioactivity in vials was determined by scintillation spectroscopy in a Tri-Carb 2900TR counter (PerkinElmer Life and Analytical Sciences). Basal binding was determined in the presence of GDP and the absence of drug.

Exon 5a

```
C V * (mM-1B5)
AGTGGAGCGACAGCCTGCACTGCTGCAGAGGTTGACACCTGAGAATG
97
```

Exon 5d

```
A H Q P E C L K C R C L S L L T I L H F Q H
485
```

Exon 5b

```
AAACTCTTATGACTGAAAATAAGTTTCTTAACTCTAACAGGAAGAAACCACTTGTCTCTTTTTATGAA
582
```

Exon 5c

```
GTTTGTGATGCTACTAGAAGTGAATAGTTTCTTCTCCACACAGCAAGGCTTCTTCCACCTGTAAGTT
1843
```

Exon 5f

```
S L D N * (mM-1B2)
TGTTGTGATGCTACTAGAAGTGAATAGTTTCTTCTCCACACAGCAAGGCTTCTTCCACCTGTAAGTT
2716
```

Fig. 2. Genomic sequence and predicted amino acid sequences of the exon 5 splice variants. The exon sequences are shown in capital letters, and partial intron sequences are indicated in lowercase letters. The predicted amino acids are shown in boldface capital letters. Termination codons are indicated by asterisk. The threonine, a potential protein kinase C phosphorylation site, from exon 5b is shown as italic and underlined. The one mismatch in exon 5c and one more GTTT repeat in exon 5b are shown as italic and boldface. The sequence has been deposited in GenBank under accession number AY390763.
colony hybridization approach with a rat exon 5 probe. The mouse exon 5a sequence shared high homology (91% identity) with the rat exon 5. Subsequent RT-PCR with the primers from the mouse exons 3 and 5a revealed the anticipated splicing between exons 3 and 5a but also demonstrated a novel pattern of splicing upstream of exon 5a (Fig. 2). To generate full-length cDNAs, nested RT-PCR with primers from exons 1, 5a, and 5b led to the isolation of the full-length mMOR-1B, mMOR-1B3, mMOR-1B4, and mMOR-1B5 clones as well as mMOR-1B1, which was homologous to the rat MOR-1B. Thus, exon 5 in the mouse contains five different 3′-splice sites linked to exon 3.

All of the mMOR-1B variants contained exons 1 to 3, differing only in their downstream sequence as a result of alternative splicing within exon 5 (Figs. 1 and 2). All of the splice junctions were consistent with the consensus splicing pattern. mMOR-1B1 had exon 5a as the fourth exon, which predicted the identical five amino acid sequence as reported in the rat rMOR-1B (Zimprich et al., 1995). mMOR-1B2 contained a 699-bp insertion (exon 5b) between exons 3 and 5a, which encoded 23 amino acids. In mMOR-1B3, splicing from exon 3 to exon 5c (108 bp) predicted six amino acids. Only two amino acids were predicted from exon 5e (362 bp) of mMOR-1B5 because of early termination of translation. On the other hand, exon 5d (362 bp) in mMOR-1B4 predicted 39 amino acids. Each of the amino acid sequences predicted from exon 5b to exon 5e was unique and differed from all of the previously identified C-terminal variants. The predicted amino acid sequence from exon 5b of mMOR-1B2 contained a protein kinase C phosphorylation site.

**Northern Blot Analysis of the Variant mRNA.** We next performed Northern blot analysis with various exon probes to estimate the relative size of the variant mRNAs (Fig. 3). All of the individual exon probes hybridized to a band of ~14 kb but with different intensities. The individual exon probes also revealed additional bands. For example, the exon 5b probe hybridized to a sharp strong band at approximately 11 kb and to a second diffuse band at 1.5 to 2.5 kb. A diffuse band in the 4- to 7-kb range was observed with all of the probes, although it was far more prominent with the exon 5c, 5d, and 5e probes. The different banding patterns illustrate the uniqueness of the various variant mRNAs and suggest that the individual exon probes associate with more than one transcript, as predicted from the structure.

**Relative Abundance of the Variants.** To estimate relative abundance of each variant mRNA, we performed semi-quantitative RT-PCR using mouse brain total RNA. The results showed that mRNAs of all of the mMOR-1B variants were significantly lower than that of mMOR-1B (p < 0.001), ranging from 18 to 39% mMOR-1 level (Fig. 4, a and b). However, it should be pointed out that the band amplified by the exons 3 and 4 primers to estimate mMOR-1 levels might also have amplified six other splice variants (mMOR-1G to mMOR-1L) generated from the exon 11 promoter that are...
also present but at lower expression levels than mMOR-1 itself (Pan et al., 2001). Thus, all of these relative values must be considered as only estimates of the expression of the variants.

**Receptor Binding of the Variants.** Earlier studies from our laboratory revealed that the mouse, rat, and human C-terminal splice variants of MOR-1 retained their μ-selectivity and affinity (Pan et al., 1999, 2000, 2001, 2005; Pasternak and Pan, 2004; Pasternak et al., 2004). To assess the affinity and activity (Pan et al., 1999, 2000, 2001, 2005; Pasternak et al., 2004), agonists were determined by nonlinear regression analysis using Prism (GraphPad Software Inc., San Diego, CA). Results are the mean values among the variants, but these differences were modest. DADLE showed the greatest differences, which were predominantly conformations. This is clearly shown when observing the $K_i$ values for opioids for mMOR-1 (Table 3). $K_i$ values with 

<table>
<thead>
<tr>
<th>Ligand</th>
<th>mMOR-1</th>
<th>mMOR-1B1</th>
<th>mMOR-1B2</th>
<th>mMOR-1B3</th>
<th>mMOR-1B5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphin</td>
<td>5.3 ± 2.0</td>
<td>5.3 ± 1.0</td>
<td>3.9 ± 0.4</td>
<td>1.5 ± 0.5</td>
<td>1.4 ± 0.6</td>
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<tr>
<td>M6G</td>
<td>5.2 ± 1.8</td>
<td>10.1 ± 1.6</td>
<td>8.4 ± 1.3</td>
<td>3.9 ± 1.3</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>DAMGO</td>
<td>1.8 ± 0.5</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>1.8 ± 0.9</td>
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</tr>
<tr>
<td>DADLE</td>
<td>2.1 ± 0.3</td>
<td>9.0 ± 1.1</td>
<td>4.9 ± 0.7</td>
<td>1.9 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>12.5 ± 3.6</td>
<td>8.9 ± 1.1</td>
<td>22.1 ± 1.5</td>
<td>8.7 ± 1.6</td>
<td>11.5 ± 5.2</td>
</tr>
<tr>
<td>Naloxone</td>
<td>4.2 ± 0.9</td>
<td>0.5 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.5</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>10.9 ± 0.5</td>
<td>14.6 ± 7.1</td>
<td>34.3 ± 18.4</td>
<td>8.7 ± 1.8</td>
<td>8.9 ± 2.3</td>
</tr>
<tr>
<td>β-Endorphin</td>
<td>10.6 ± 2.9</td>
<td>6.8 ± 3.2</td>
<td>4.9 ± 1.7</td>
<td>3.1 ± 1.4</td>
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</tr>
<tr>
<td>Endorphin 1</td>
<td>2.1 ± 0.8</td>
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<td>5.0 ± 1.8</td>
<td>3.2 ± 0.6</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>Endorphin 2</td>
<td>4.2 ± 1.6</td>
<td>12.4 ± 3.5</td>
<td>8.4 ± 1.1</td>
<td>3.2 ± 0.8</td>
<td>10.6 ± 1.8</td>
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<tr>
<td>U50,488H</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
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<tr>
<td>DPDPDE</td>
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Presumably because agonists label only the agonist receptor conformation, whereas antagonists bind to both agonist and antagonist conformations. This is clearly shown when observing the $B_{max}$ values for mMOR-1 using the agonist 

**TABLE 1**

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<thead>
<tr>
<th>Clone</th>
<th>$K_i$</th>
<th>$B_{max}$</th>
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<tr>
<td>nM</td>
<td>pmol/mg protein</td>
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<tr>
<td>mMOR-1</td>
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<td>1.20 ± 0.33</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>mMOR-1B2</td>
<td>1.99 ± 0.29</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>mMOR-1B3</td>
<td>0.77 ± 0.11</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>mMOR-1B4</td>
<td>1.60 ± 0.41</td>
<td>0.11 ± 0.02</td>
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**TABLE 2**

Competition of [3H]DAMGO binding in cells stably expressing mMOR-1 variants. 

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</table>

N.S., not significant; DSLET, [D-Ser²,Leu⁵]enkephalin-Thr⁶.
the \([^3H]\)diprenorphine competition studies in mMOR-1- and MOR-1B4-expressing cells (Table 3). The binding to the MOR-1B4 cells was \(\mu\)-selective, as shown by the poor affinity of the \(\kappa\)- and \(\delta\)-agonists nor-BNI and naltrindole. However, the affinity of many agonists in the mMOR-1B4 cells was far lower than in mMOR-1-expressing cells, with \(K_i\) values for most over 100 nM. The poor affinity of DAMGO in the competition studies explains why attempts to demonstrate \([^3H]\)DAMGO binding directly were not successful. Several agonists stood out from the others. Etorphine, etonitazene, [DMT\(^3\)]DALDA, and buprenorphine all retained high affinity in the mMOR-1B4 cells, with \(K_i\) values under 15 nM. Indeed, these ultrapotent analgesics bound to the mMOR-1B4 cell membranes with higher affinity than some of the antagonists. From a behavioral standpoint, these drugs are notable for their extremely high analgesic potency, yet not all of the highly potent \(\mu\)-opioids retained high affinity, as illustrated by fentanyl, with a \(K_i\) value higher than 100 nM.

To assess whether the mMOR-1B4 sites labeled by \([^3H]\)diprenorphine represented antagonist conformations of the receptor, we explored the effects of the stabilized GTP analog guanosine Gpp(NH)p on the binding in both mMOR-1 and mMOR-1B4 cells (Table 4). GTP and its analogs selectively diminish the affinity of agonists (Childers and Snyder, 1978). In the current study, we saw agonist shifts but they remained relatively modest. The antagonists naloxone and CTAP showed no shift in the mMOR-1 cells. However, they did reveal a small shift with mMOR-1B4. Although this shift is very modest, it raises the possibility that they may be partial agonists at this variant. Overall, the small shifts for the agonists is consistent with the earlier studies, implying that the vast majority of the sites in both the mMOR-1 and mMOR-1B4 cells are in an antagonist conformation.

**Characterization of the Variants by \([^{35}S]GTP\gamma S\)-Binding Assay.** Prior studies have documented functional differences among a number of MOR-1 variants (Bolan et al., 2004; Pasternak et al., 2004; Pan et al., 2005). Therefore, we examined the ability of the mMOR-1B variants to activate G-proteins using a \([^{35}S]GTP\gamma S\)-binding assay (Table 5). The range of the EC_{50} values differed from one variant to another. Some showed little difference among the drugs, whereas mMOR-1B1 revealed a greater range. For most of the drugs, their relative EC_{50} values in the \([^{35}S]GTP\gamma S\) stimulation assay did not correlate well with their relative receptor-binding affinities. Although the \(K_i\) and EC_{50} values for M6G (\(r^2 = 0.72\)) and for endomorphin 2 (\(r^2 = 0.73\)) showed a modest correlation, the correlations for the remainder of the drugs were very poor, as was the correlation for all of the drugs together (\(r^2 = 0.16\)).

The EC_{50}/\(K_i\) ratio provides an indication of the ability of the drug to activate the receptor relative to its receptor occupancy (Table 5). Because the conditions used in the receptor binding and \([^{35}S]GTP\gamma S\)-binding assays were not identical, this ratio should not be considered a direct indication of intrinsic activity, but it still is useful in evaluating the drugs. The ratios within a given variant ranged up to 10-fold, and the rank order of the ratios of the drugs to each other varied among the splice variants. For example, endomorphin 2 had the lowest ratio for mMOR-1B5 and the highest ratio for mMOR-1B3. M6G was among the lowest for mMOR-1B5 and the highest against mMOR-1B1.

For many of the variants, the maximal stimulation varied

---

**TABLE 3**

Receptor binding of \([^3H]\)DAMGO and \([^3H]\)diprenorphine binding in mMOR-1 and mMOR-1B4

<table>
<thead>
<tr>
<th>Ligand</th>
<th>mMOR-1</th>
<th>([^3H])DAMGO</th>
<th>([^3H])Diprenorphine</th>
<th>mMOR-1B4</th>
<th>([^3H])DAMGO</th>
<th>([^3H])Diprenorphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>5.3 ± 2.0</td>
<td>94.9 ± 18.3</td>
<td>349 ± 79</td>
<td>5.7 ± 2.0</td>
<td>94.9 ± 18.3</td>
<td>349 ± 79</td>
</tr>
<tr>
<td>M6G</td>
<td>5.2 ± 1.8</td>
<td>609.3 ± 110</td>
<td>&gt;1000</td>
<td>5.5 ± 2.0</td>
<td>609.3 ± 110</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>DAMGO</td>
<td>1.8 ± 0.5</td>
<td>50.2 ± 25</td>
<td>208 ± 23</td>
<td>2.0 ± 0.5</td>
<td>50.2 ± 25</td>
<td>208 ± 23</td>
</tr>
<tr>
<td>DADLE</td>
<td>2.1 ± 0.3</td>
<td>133 ± 13</td>
<td>600 ± 48</td>
<td>2.3 ± 0.4</td>
<td>133 ± 13</td>
<td>600 ± 48</td>
</tr>
<tr>
<td>Endomorphin 1</td>
<td>2.1 ± 0.8</td>
<td>56.4 ± 7.4</td>
<td>&gt;1000</td>
<td>2.4 ± 0.9</td>
<td>56.4 ± 7.4</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Etorphine</td>
<td>0.7 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td></td>
<td>0.8 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Fentanyl</td>
<td>48.5 ± 6.0</td>
<td>137 ± 22</td>
<td></td>
<td>50.5 ± 6.0</td>
<td>137 ± 22</td>
<td></td>
</tr>
<tr>
<td>Etonezatene</td>
<td>3.0 ± 0.7</td>
<td>6.6 ± 0.5</td>
<td></td>
<td>3.2 ± 0.7</td>
<td>6.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>(\beta)-Endorphin</td>
<td>10.8 ± 2.9</td>
<td>34.6 ± 5.5</td>
<td>873 ± 116</td>
<td>11.0 ± 2.9</td>
<td>34.6 ± 5.5</td>
<td>873 ± 116</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>10.9 ± 0.5</td>
<td>127 ± 29</td>
<td>&gt;1000</td>
<td>11.2 ± 0.5</td>
<td>127 ± 29</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>NalBzOH</td>
<td>1.9 ± 0.3</td>
<td>5.4 ± 2.0</td>
<td></td>
<td>2.1 ± 0.4</td>
<td>5.4 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>[Dmt(^3)]DALDA</td>
<td>2.1 ± 0.4</td>
<td>13.6 ± 5.3</td>
<td></td>
<td>2.4 ± 0.5</td>
<td>13.6 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>Nalorphine</td>
<td>27.4 ± 6.0</td>
<td>80 ± 31</td>
<td></td>
<td>30.0 ± 6.0</td>
<td>80 ± 31</td>
<td></td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>0.4 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td></td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Naloxone</td>
<td>4.3 ± 0.9</td>
<td>8.2 ± 1.0</td>
<td>17.6 ± 1.8</td>
<td>4.6 ± 0.9</td>
<td>8.2 ± 1.0</td>
<td>17.6 ± 1.8</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>1.0 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td></td>
<td>1.2 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Naltrazexone</td>
<td>1.4 ± 0.1</td>
<td>6.1 ± 0.4</td>
<td></td>
<td>1.6 ± 0.1</td>
<td>6.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CTAP</td>
<td>1.9 ± 0.2</td>
<td>8.0 ± 1.3</td>
<td></td>
<td>2.1 ± 0.3</td>
<td>8.0 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>CTOP</td>
<td>3.5 ± 0.3</td>
<td>18.8 ± 4.3</td>
<td></td>
<td>3.8 ± 0.4</td>
<td>18.8 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Nor-BNI</td>
<td>188 ± 28</td>
<td>267 ± 34</td>
<td></td>
<td>200 ± 30</td>
<td>267 ± 34</td>
<td></td>
</tr>
<tr>
<td>Naltrindole</td>
<td>66.7 ± 3.3</td>
<td>198 ± 33</td>
<td></td>
<td>70.0 ± 3.5</td>
<td>198 ± 33</td>
<td></td>
</tr>
<tr>
<td>U50,488H</td>
<td>&gt;500</td>
<td>1000</td>
<td></td>
<td>&gt;500</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>DPBPE</td>
<td>&gt;500</td>
<td>1000</td>
<td></td>
<td>&gt;500</td>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>

CTOP, d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2; NalBzOH, naloxone benzylhydrazine.

**TABLE 4**

Effect of guanosine 5'-\(\beta,\gamma\)-imido)triphosphate on \([^3H]\)diprenorphine binding in mMOR-1 and mMOR-1B4

<table>
<thead>
<tr>
<th>Ligand</th>
<th>mMOR-1</th>
<th>([^3H])Diprenorphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>104.3 ± 2.2</td>
<td>147.8 ± 8.1*</td>
</tr>
<tr>
<td>DAMGO</td>
<td>55.5 ± 1.2</td>
<td>82.8 ± 10.8</td>
</tr>
<tr>
<td>Etorphine</td>
<td>0.49 ± 0.06</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>53.6 ± 4.0</td>
<td>89.3 ± 7.4</td>
</tr>
<tr>
<td>Naloxone</td>
<td>15.4 ± 1.6</td>
<td>14.1 ± 0.7</td>
</tr>
<tr>
<td>CTAP</td>
<td>3.7 ± 0.5</td>
<td>2.1 ± 0.4**</td>
</tr>
</tbody>
</table>

\(nM\)

\(*\) \(P < 0.05\).

\(**\) \(P < 0.01\).

Gpp(NH)p, guanosine 5'-\(\beta,\gamma\)-imido)triphosphate.
little among the drugs (Table 5). mMOR-1B1 had the greatest variation, with responses ranging from 117% DAMGO to only 68% endomorphin 1. DAMGO was the most efficacious among all of the mMOR-1B variants, with the exception of mMOR-1B1. The most intriguing observation was that the relative efficacy of the drugs to each other varied from one variant to another. Among the mMOR-1B variants, M6G was the most efficacious with mMOR-1B1 and the least efficacious against mMOR-1B2. In contrast, endomorphin 1 was the lowest against mMOR-1B1 and among the highest with mMOR-1B3. Thus, the C-terminal differences of these variants were associated with varying effects on efficacy and potency of the \( \mu \)-drugs.

As noted earlier, mMOR-1B4 proved to be a very unique variant. Although \( \mu \)-opioid antagonists retained high affinity for this variant, the affinity of agonists in the binding was far lower (Table 3). Yet a number of the compounds were active in \( [35S] \)GTP\( \gamma \)S-binding assays, although their level of stimulation was lower than that seen with the other variants (Table 6). Of the active drugs, dynorphin A stimulated binding the most followed by etonitazene, fentanyl, and etorphine. As with the other variants, the maximal stimulation induced by the agonists did not correlate with their affinity for the receptors. Dynorphin A displayed very poor affinity in the binding assays, yet it was the most effective in the \( [35S] \)GTP\( \gamma \)S-binding study. Likewise, fentanyl displayed an affinity 20-fold lower than either etorphine or etonitazene but stimulated \( [35S] \)GTP\( \gamma \)S binding as well.

To determine whether or not the stimulation was opioid-specific, we examined the sensitivity of both etorphine and etonitazene to several opioid antagonists (Fig. 5). The maximal stimulation of both agents at 1 \( \mu \)M was modest but similar to values seen at 25 \( \mu \)M. Etorphine stimulated binding by only 27 ± 3% and etonitazene by only 35 ± 6%. The stimulated binding of both opioids was reversed by the opioid-selective antagonists, naloxone, naltrexone, and diprenorphine, confirming its specificity.

### Discussion

The rMOR-1B variant was the first variant identified in the rat (Zimprich et al., 1995) and was recently reported in the mouse (Narita et al., 2003). Other splice variants of the mouse \( Opmr \) gene have also been reported, many of which are alternatively spliced downstream of exon 3 (Pan et al., 1999, 2000, 2001, 2005; Kvam et al., 2004; Pasternak and

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Stimulation of ([35S]GTP\gamma S) Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>16.7 ± 2.8</td>
</tr>
<tr>
<td>DAMGO</td>
<td>25.9 ± 2.6</td>
</tr>
<tr>
<td>Etorphine</td>
<td>27.6 ± 7.0</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>31.7 ± 12.2</td>
</tr>
<tr>
<td>Etorphine</td>
<td>34.6 ± 5.8</td>
</tr>
<tr>
<td>( \beta )-Endorphin</td>
<td>17.5 ± 2.6</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>38.5 ± 10.4</td>
</tr>
</tbody>
</table>
variants differed in both the relative potency and efficacy of
Pasternak et al., 2004; Pan et al., 2005), the mMOR-1B splice
Similar to the other full-length variants (Bolan et al., 2004;
amino acid sequence differences at the tip of the C terminus.

defined by exons 1, 2, and 3, which are identical in all of the
Variants previously isolated, there are now 12 full-length and
functional C-terminal variants of the mouse Oprm gene.

Similar to the other full-length MOR-1 variants, all of the
full-length MOR-1B variants were μ-selective in binding as-
says, which was anticipated because the binding pocket was
defined by exons 1, 2, and 3, which are identical in all of the
Variants. Therefore, functional differences must be due to the
amino acid sequence differences at the tip of the C terminus.

Similar to the other full-length variants (Bolan et al., 2004;
Pasternak et al., 2004; Pan et al., 2005), the mMOR-1B splice
variants differed in both the relative potency and efficacy of

a number of μ-opioids in stimulating [35S]GTPγS binding, a
measure of G-protein activation. Differences in the relative
efficacy of the drugs to each other and in their relative po-
tency from variant to variant raise interesting questions. It is
presumed that drugs administered in vivo will interact with
all of the variants. However, the relative activation of one
splice variant to another will probably differ from one drug to
the next, leading to differences in their overall activation
profiles. This may help explain the subtle but potentially
significant behavioral differences among these agents.

Of the exon 5-containing variants, mMOR-1B4 clearly
stands apart from the rest. Although its μ-selectivity was
clearly demonstrated by the [3H]diprenorphine competition
studies, direct binding studies with [3H]DAMGO were unsuc-
sessful, presumably because of its low affinity. Most agonists
displayed poor affinities against [3H]diprenorphine binding
in both mMOR-1- and mMOR-1B4-expressing cells, presum-
ably because of the predominance of sites labeled by the
radiolabeled agonist in an antagonist conformation. The
nearly 20-fold affinity shift of morphine, the 100-fold shift for
M6G, and the 25-fold shift for DAMGO in mMOR-1 cells were
anticipated. Yet the affinities of most of the agonists were
even lower in the mMOR-1B4 cells. It is noteworthy that
several agonists stood out from the others. Etorphine, etoni-
tazene, [DMT1]DALDA, and buprenorphine all retained high
affinity for both variants. From a pharmacological stand-
point, all of these drugs are very potent analgesics (Blane et
al., 1967; Schiller et al., 2000; Neilan et al., 2001), raising the
interesting possibility that their enhanced analgesic activity
might result from a unique ability to interact with receptors
in both agonist and antagonist conformations.

Why mMOR-1B4 displayed this unique binding profile re-
mains unclear. It has 39 amino acids at the tip of the C
terminus instead of the 12 amino acids encoded by exon 4.
Yet the increased length of the tail alone cannot be respon-
sible for its unusual characteristics because several other
variants with even longer sequences, mMOR-1C with 52
amino acids and mMOR-1F with 58, have typical binding
affinities for the μ-opioids (Pan et al., 1999, 2000). Thus, the
distinct properties of mMOR-1B4 must reside in the se-
quence of the 39 amino acids. Because the binding pocket,
which is defined by the transmembrane domains, is identical
to that of the other full-length mMOR-1 variants, the binding
and pharmacological differences may reflect the presence or
absence of interactions of the C terminus with other mem-
brane and/or cellular proteins. The presence of four cysteine
residues within the 39 amino acid C terminus of mMOR-1B4
raises additional possibilities regarding its structure and
interactions with other proteins.

A number of proteins associated with G-protein coupled
receptors can modulate binding and function, including G-
proteins, receptor (calcitonin) activity modifying proteins
(Morais et al., 2003), or regulators of G-protein signaling (De
Vries et al., 2000; Sierra et al., 2002; Zachariou et al., 2003).
Receptor dimerization also influences binding and function
(Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998;
Jordon and Devi, 1999; George et al., 2000; Pan et al., 2002).
Could the absence of a needed G-protein be responsible for
the inability of mMOR-1B4 to show high affinity agonist
binding? The repertoire of G-proteins varies among cell lines.
CHO cells used in the current study reportedly lack G111, but
transfection of G111 into the cells expressing mMOR-1B4 did
not change any of the binding parameters (data not shown).
We also expressed mMOR-1B4 in human embryonic kidney
293 cells to determine whether different cellular environ-
ments might restore agonist binding but without success
(data not shown). Overexpression alone seems unlikely to
explain our findings, because the expression levels of
mMOR-1 were even greater than mMOR-1B4. Other recep-
tors also can modulate function. For example, some receptors
lack function unless coexpressed with a second one, as seen
with the GABA type B (GABAB) receptors (Jones et al., 1998;
Kaupmann et al., 1998; White et al., 1998). In other situa-
tions, heterodimerization may change the pharmacology of
the complexed receptors compared with the individual recep-
tors expressed alone, as shown for several pairs of opioid
receptors (Jordon and Devi, 1999; George et al., 2000; Pan et
al., 2002). All of these possibilities must be considered.

MOR-1 splice variants also display regional distributions
distinct from those of MOR-1 at the regional, cellular, and
ultracellular levels (Abbadie et al., 2000a,b, 2001). Likewise,
the regional distribution of mMOR-1B4 was unlike any oth-
ers, including rMOR-1B (Y. Zhang, X. Y. Pan, and G. W.
Pasternak, manuscript in preparation). Within the spinal
cord, it weakly labeled the dorsal horn, robustly labeled
Onuf's nucleus at the L5 and L6 levels, and diffusely labeled
the anterior horn. Within the brain, the most intense labeling
was observed within the Purkinje cells of the cerebellum,
with some labeling in the olfactory bulb. In the rat, rMOR-1B
has an unusual immunohistochemical labeling pattern within the brain, with high levels of labeling restricted to the olfactory bulb and little labeling in areas typically associated with opioid modulation of pain (Schulz et al., 1998).

These MOR-1 splice variants may help explain many of the clinical and behavioral observations seen μ-opioids. Both clinical and preclinical studies reveal subtle differences among the μ-opioids. Except for mOR-B14, μ-opioids label all of the full-length variants with similar affinities. However, the overall pharmacological response of a drug reflects the summation of the activation of all of the MOR-1 variants bound. Because both the efficacy and potency of each drug varies from variant to variant and does not correlate with binding affinity, the overall pharmacological activation profiles of each μ-drug would be expected to differ. This ability to dissociate efficacy and potency from binding also opens the possibility of novel drugs. Selectivity among the MOR-1 variants is not likely to be achieved based on the binding affinities alone, because their binding pockets are all structurally identical. However, it may be possible to obtain selectivity of action by independently modulating the efficacy and/or potency of the compounds at the different variants.

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