0026-895X/99/020396-08$3.00/0

MOLECULAR PHARMACOLOGY, **56:**396–403 (1999).

Identification and Characterization of Three New Alternatively Spliced μ-Opioid Receptor Isoforms

YING-XIAN PAN, JIN XU, ELIZABETH BOLAN, CATHERINE ABBADIE, ALBERT CHANG, AMY ZUCKERMAN, GRACE ROSSI, and GAVRIL W. PASTERNAK

The Cotzias Laboratory of Neuro-Oncology, Memorial Sloan-Kettering Cancer Center, New York, New York

Received February 3, 1999; accepted April 13, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

We have identified four new μ-opiod receptor (*MOR*)-*1* exons, indicating that the gene now contains at least nine exons spanning more than 200 kilobases. Replacement of exon 4 by combinations of the new exons yields three new receptors. When expressed in Chinese hamster ovary cells, all three variants displayed high affinity for μ-opioid ligands, but κ and δ drugs were inactive. However, there were subtle, but significant, differences in the binding profiles of the three variants among themselves and from *MOR-1*. Immunohistochemically, the major variant, *MOR-1C*, displayed a regional distribution quite distinct from that of *MOR-1*. Region-specific processing also was seen at the mRNA level. Antisense mapping revealed that the four new exons were all involved in morphine analgesia. Together with two other variants generated from alternative splicing of exon 4, there are now six distinct *MOR-1* receptors.

The μ-opioid receptor (MOR) has a special place within the opioid receptor family. It mediates the actions of morphine and most clinical analgesic agents, as well as drugs of abuse such as heroin. In recent years, a number of μ receptor subtypes have been proposed. The first suggestion of μ_1 and μ_2 receptor subtypes came from a combination of binding and pharmacological studies based on the antagonists naloxonazine and naloxazone (Wolozin and Pasternak, 1981; Pasternak, 1993; Reisine and Pasternak, 1996). More recently, pharmacological and molecular differences between morphine and morphine-6β-glucuronide (M6G) suggested yet another μ receptor subtype (Pasternak and Standifer, 1995; Rossi et al., 1995a,b, 1996).

Soon after a μ receptor, *MOR-1*, was cloned (Chen et al., 1993; Wang et al., 1993), antisense approaches confirmed its involvement with morphine analgesia (Rossi et al., 1994, 1995a,b). Only a single μ receptor gene, *MOR-1*, has been identified (Min et al., 1994; Giros et al., 1995; Liang et al., 1995), yet antisense mapping studies against morphine and M6G analgesia revealed interesting patterns, with some exons implicated in the analgesic actions of one but not the other (Rossi et al., 1995a,b, 1997). Although the two analgesic agents acted through different receptors, the sensitivity of both agents to at least six different *MOR-1* antisense probes implied that both receptors were closely associated with *MOR-1*, raising the possibility of pharmacologically relevant *MOR-1* splice variants (Pasternak and Standifer, 1995; Rossi et al., 1995a,b). Alternative splicing has been observed with a number of G protein-coupled receptors, including somatostatin 2 (Vanetti et al., 1998), dopamine D_2 (Guiramand et al., 1995), prostaglandin EP_3 (Namba et al., 1993), serotonin receptor subtypes 5-hydroxytryptamine$_4$ and 5-hydroxytryptamine$_7$ (Lucas and Hen, 1995), and *MOR-1* (Bare et al., 1994; Zimprich et al., 1995). In view of the strong pharmacological evidence for distinct μ receptors, we have explored alternative splicing of the *MOR-1* gene. We report the identification of four new exons for the *MOR-1* gene that combine to yield three novel *MOR-1* splice variants.

Experimental Procedures

Materials. Male Crl:CD-1(ICR)BR mice were obtained from Charles River Laboratories (Wilmington, MA). [^{3}H][D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) was purchased from New England Nuclear Corp. (Boston, MA). Opiates and opioid peptides were the generous gift of the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). All other materials were obtained from the sources listed.

Rapid Amplification of cDNA 3′ Ends (RACE) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). A Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) was used in 3′-RACE. A sense primer (sense primer A, 5′-CCCAACTTCCTCCACAATCGAA-3′), which is located at the 3′-end of the exon 3 and at position 1338–1359 of the mouse μ receptor; GenBank accession no.

This work was supported in part by the National Institute on Drug Abuse Grants DA02615, DA06241, and DA07242; Senior Scientist Award DA00220 (to G.W.P.) and Research Scientist Development Award DA00296 (to Y.-X.P.); and Core Grant CA08748 (to the Memorial Sloan-Kettering Cancer Center).

ABBREVIATIONS: MOR, μ-opioid receptor; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; M6G, morphine-6β-glucuronide; RT, reverse transcription; PCR, polymerase chain reaction; FISH, fluorescence in situ hybridization; BAC, bacterial artificial chromosome.

U26915) and an antisense primer, AP1 included in the kit, were used in PCR with a mouse brain marathon cDNA template. Multiple bands with different abundance were amplified. Each band was excised from agarose gel and amplified with the second set of nested primers: sense primer B (5′-GGGAACACCCCTCCACGGC-3′), which is at position 1394 to 1412 of the receptor, and an antisense primer, AP2. The PCR fragments were then subcloned into Bluescript plasmids and sequenced. Although most sequences were aligned with that of *MOR-1*, the sequence of one clone, 110222, of approximately 500 base pairs (bp) in length did not. It contained a partial sequence of the 3′-end of *MOR-1* exon 3 sequence, followed by a sequence that was totally different from that of *MOR-1* exon 4. The new sequence predicted seven amino acids beyond exon 3 (RNEEPSS), followed by a termination codon.

To obtain full-length cDNA clones of the variant, a sense primer based on the 5′ untranslated region of *MOR-1* at position 217 to 240 (5′-GGAACCCGAACACTCTTGAGTGCT-3′) and an antisense primer located at the 3′ untranslated region of the new sequence, antisense primer A (5′-CCACACTGCTCACCAGCTCATCCC-3′), were used in PCR with the first-strand cDNA reverse-transcribed from mouse brain RNA as template. Three fragments of approximately 1.3, 1.4, and 1.5 kilobases (kb) in length, respectively, were obtained; subcloned into pCRII-TOPO plasmid (InVitrogen, Carlsbad, CA); and sequenced in both directions with appropriate primers. Sequence analysis of the fragments revealed that all three clones contained coding exons 1, 2, and 3 from *MOR-1* but different sequences downstream from exon 3. The three clones were named *MOR-1C*, *MOR-1D*, and *MOR-1E*. *MOR-1D* has the same sequence seen with the 3′-RACE from clone 110222. *MOR-1C* contained a 89-bp insertion between exon 3 and the new sequence from clone 110222. Although *MOR-1C* contains the same new sequence found in *MOR-1D*, the 89-bp insertion results in a reading-frame shift. As a result, *MOR-1C* predicted 52 amino acids that did not include the amino acid sequence from *MOR-1D*. *MOR-1E* had a 209-bp insertion between exon 3 and the new sequence found in clone 110222, making it the longest novel sequence. The last 89 bp in this insertion were identical to those in *MOR-1C*; however, the *MOR-1E* sequence predicted only 15 amino acids.

Isolation and Characterization of Genomic Clones. Genomic clones containing the *MOR-1* exons and the new sequences were obtained from screening two mouse genomic bacterial artificial chromosome (BAC) libraries (GenomeSystems, Inc., St. Louis, MO, and Research Genetics, Huntsville, AL) and a mouse genomic P1 library (GenomeSystems, Inc.) by either PCR or hybridization methods. All the clones obtained were analyzed by restriction enzyme digestion, long PCR, Southern blotting, and sequencing with appropriate primers and fragments. Initially, BAC clone A, approximately 75 kb in length, was obtained from the GenomeSystems BAC library using *MOR-1* exon 4 primers in PCR. BAC clone A contained the *MOR-1* exons 1, 2, 3, and 4 but none of the new sequences. Because no positive clones were obtained by screening the BAC library with the new sequence in clone 110222, we screened the P1 library and obtained one P1 clone (P1 clone A) of approximately 100 kb in length, which contained the new sequence. However, it contained neither exon 4 nor the additional insertions seen in *MOR-1C* or MOR-E. To identify a clone containing these insertions and to fill the gap between the BAC clone A and the P1 clone A, another mouse BAC library (Research Genetics, Inc.) was screened by hybridization with the insertional sequence. Five positive clones of different lengths were identified. One of these, BAC clone B (~120 kb) contained exon 4 and the insertions present in *MOR-1C* and *MOR-1E*. The three BAC and P1 clones overlapped each other, predicting an *MOR-1* gene of approximately 230 kb, which is consistent with distance between BAC clone A and the P1 clone measured by fluorescence in situ hybridization (FISH) in interphase nuclei (see below).

Chromosomal Localization by FISH. Chromosomal localization of the P1 clone was carried out using FISH methods by GenomeSystems, Inc. In brief, the P1 clone A was labeled with digoxigenin dUTP and hybridized to metaphase chromosomes derived from a mouse embryo fibroblast cell line. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies, followed by counterstaining with 4′,6-diamidine-2′-phenylindole HCl. The initial experiment resulted in specific labeling of the proximal portion of a medium-sized chromosome, believed to be chromosome 10 on the basis of 4′,6-diamidine-2′-phenylindole HCl staining. Cohybridization of a specific probe for the telomeric region of chromosome 10 with the P1 clone A demonstrated that the P1 clone A was located immediately adjacent to the heterochromatic euchromatic boundary of chromosome 10, an area corresponding to band 10A2. A total of 80 metaphase cells were analyzed, with 68 exhibiting specific labeling.

Chromosomal Physical Distance Measurement by FISH. An interphase FISH analysis (van den Engh et al., 1992) was used to estimate the physical distance between the BAC clone A and the P1 clone A by GenomeSystems, Inc. In brief, the BAC clone A and P1 clone, labeled with biotin dATP or digoxigenin dUTP, respectively, were hybridized as differentially labeled pairs to interphase nuclei derived from mouse embryo fibroblasts. Specific hybridization signals were detected by fluorescein conjugate anti-digoxigenin antibodies and Texas red avidin. The mean distance between the two clones was calculated from measurements made from photographs of interphase cells exhibiting paired red and green signals and converted to the actual distance between the two clones in kilobase pairs. The estimated distance between the BAC clone A and the P1 clone A was approximately 250 kb, with a possible error of approximately 30%.

Northern Blot Analysis. To investigate the lengths of the transcripts encoding the new variants, Northern blot analysis was performed as described previously (Pan et al., 1994). In brief, total RNA was isolated from mouse brain by the guanidinium thiocyanate phenol-chloroform extract method. Then, 50 μg of total brain RNA/lane was loaded, separated on a 0.8% formaldehyde agarose gel, and transferred to GenePlus membrane. The membrane was hybridized with ^{32}P-labeled fragments of the new sequences generated by PCR with appropriated primers.

Expression of *MOR-1C*, *MOR-1D*, and *MOR-1E*. The cDNA fragments containing the full-length *MOR-1* or the *MOR-1* variants in pCRII-TOPO were subcloned into pcDNA3.1 (InVitrogen), a mammalian expression vector. The resulting plasmids, *MOR-1*/pcDNA3, *MOR-1C*/pcDNA3, *MOR-1D*/pcDNA3, and *MOR-1E*/pcDNA3, respectively, were used to transfect Chinese hamster ovary (CHO) cells by LipofectAMINE reagent (GIBCO, Gaithersburg, MD). Stable transformants were obtained 2 weeks after selection with G418 and screened with [^{3}H]DAMGO binding assay.

In Vitro Translation. *MOR-1*/pcDNA3, *MOR-1C*/pcDNA3, *MOR-1D*/pcDNA3, and *MOR-1E*/pcDNA3 plasmids were transcribed and translated in vitro with a TNT-coupled reticulocyte lysate kit (Promega, Madison, WI). Briefly, the plasmids were incubated with T7 RNA polymerase and reticulocyte lysate in the presence of 0.04 mCi of [^{35}S]methionine (>1000 Ci/mmol; DuPont-NEN, Boston, MA) at 30°C for 1 h. The translation products were separated by a 12.5% SDS-polyacrylamide gel, and the gel was treated with Amplify (Amersham Life Science), dried, and exposed to Kodak BioMax MR film.

Regional Expression of *MOR-1C*, *MOR-1D*, and *MOR-1E* mRNA. Total RNA was extracted from different mouse brain regions as described and reverse-transcribed with SuperScript II Reverse Transcriptase (GIBCO) in the presence of random hexamers. For the new variants, the first-strand cDNAs were amplified with a nested PCR strategy. The first-round PCR using the sense primer A (see above), which was designed from exon 3, and an antisense primer (5′-GAAAGGCATCTTCCCTCTCGCTGT-3′), which was derived from exon 9 did not yield visible bands on agarose gel. We then used the first PCR products in the second-round PCR with a pair of nested primers (sense primer B and antisense primer A, see above). Three major bands were amplified. RNA loading was estimated by from a parallel PCR with β_2-microglobulin primers (Clontech). The agarose gel was stained with ethidium bromide and photographed with

Kodak DC120 Digital Camera and Imagine System. The predicted sizes of the PCR products for *MOR-1C*, *MOR-1D*, and *MOR-1E* are 246, 157, and 366 bp, respectively. Each band was extracted from agarose gel, subcloned into pCRII-TOPO plasmid, and sequenced. The sequences showed that they all correspond to respective variants. For *MOR-1*, PCR was performed using a sense primer (5′-GCATCCCAACTTCCTCCACAATCG-3′) and an antisense primer (5′-CCAGGAAACCAGAGCCTCCCACAA-3′).

Binding Assays. Membranes were prepared from stable transfectants with the pcDNA3.1 constructs as previously described (Pan et al., 1994, 1996). [^{3}H]DAMGO binding was performed at 25°C for 60 min in 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM magnesium sulfate. Specific binding was defined as the difference between total binding and nonspecific binding, defined by levallorphan (1 μM). K_D and K_i values were calculated by nonlinear regression analysis (Prism; GraphPAD Software, San Diego, CA). Protein concentration were determined as described by Lowry et al. (1951) using BSA as the standard.

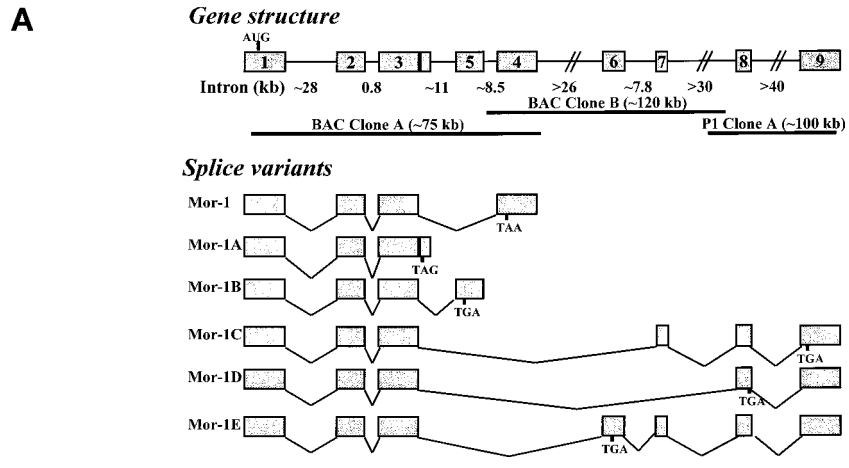
Immunohistochemistry. After an injection of sodium pentobarbital (100 mg/kg i.p.), mice received an intracardiac perfusion of PBS 0.1 M (50 and 20 ml, respectively) followed by 4% formaldehyde in 0.1 M phosphate buffer (300 or 50 ml, respectively). After the perfusion, the brain and spinal cord were removed, postfixed for 4 h in the same fixative, and then cryoprotected overnight in 30% sucrose in 0.1 M PBS. Immunostaining was performed on 40-μm sections cut in the coronal plane on a freezing microtome. Immunostaining was performed according to the avidin-biotin peroxidase method of Hsu et al. (1981). Sections were incubated with a solution of 0.1 M PBS with 3% normal goat serum and 0.3% Triton-X. The blocking solution was removed from the tissue, and the sections were incubated overnight at room temperature in the primary antiserum. The sections were washed and then incubated in biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex (Vector Labs). To localize the horseradish peroxidase immunoreaction product, we used a nickel-intensified diaminobenzidine protocol with glucose oxidase adapted from Llewellyn-Smith and Minson (1992). Finally, the sections were washed in phosphate buffer, mounted on gelatin-coated slides, dried, and coverslipped with DPX (Aldrich, Milwaukee, WI). For immunohistochemical controls, the primary antibody was either omitted, replaced by preimmune sera, or adsorbed with several concentrations of the synthesized peptide.

Antisense Mapping. Groups of mice ($n \geq 20$) received the indicated oligodeoxynucleotide (10 μg i.c.v.) daily for 5 days, after which analgesia was assessed in the radiant heat tail-flick assay (Rossi et al., 1995a,b, 1996). In brief, the tails of mice were exposed to a light source and a baseline latency determined, which was typically between 2 and 3 s. Analgesia was defined as a doubling or greater of the baseline latency. Significance between groups was assessed using Fisher's exact test.

Results

Cloning New Splice Variants of *MOR-1* Gene and Its Structure. Using 3′-RACE, we identified a novel sequence downstream from exon 3 in *MOR-1* that replaced the sequence of exon 4, subsequently identified as exons 8 and 9. Reverse transcription (PT)-PCR with an upstream primer in the 5′-untranslated region of *MOR-1* along with a downstream primer from the new sequence yielded three different full-length cDNA clones: *MOR-1C*, *MOR-1D*, and *MOR-1E* (Figs. 1 and 2). All three contained exons 1, 2, and 3 as originally described in *MOR-1*, and the nucleotide sequence first identified using 3′-RACE. *MOR-1C* had an additional 89-bp insertion, whereas the insertion in *MOR-1E* was longer (209 bp).

We next established the gene structure of *MOR-1* using genomic clones (Fig. 1). BAC clone A (~75 kb) contained all four original *MOR-1* exons as well as the mouse homolog of the rat exon 5, which encodes the splice variant MOR-1B (Zimprich et al., 1995) (Figs. 1 and 2). However, BAC clone A



B

Exon 6

--gtctgtgcttctgtgtctcagAAGAAAAAGCTGGACTCCCAGAGAGGGTGTGTACAGCATCCAGTGTGACCTGTCCCTTGTCTTTGAGCCTGGGGGCCATCTTCTTTCA
CAGCATACCATTTCCTTGTATCCTCTCTGAAGgtacttttcaaatatttaat---

Exon 7

--tccctttccttttttcttgtacagCCAACCCTGGCAGTCAGCGTGGCCCAGATCTTTACAGGATATCCTTCTCCGACTCATGTTGAAAAACCCTGCAAGAGTTGCATGGACAGg
tgagtgtgacccggactca---

Exon 8

atctgtgtcgtagAGGAATGAGGAACCTTCTTCCTGATGATGGCCCAAGACAGGAATCCGGGGAAGGCCAGCTTGGCAGgtaaccttaaactctggtta--

Exon 9

tgtgcctttgtgtcttcgagGTGAATGTCATCCGAACACAGGGATGAGCTGGTGAGCAGTGTGGAGGTGCAGGCTCTGCTAGAAGACAGCGAGAGGGAAGATGCCTTTCACTCAT
AAAGGTTAGGAGTTGTAAGTGTAATGTCTAATGTATTGTCTGGTTTGCCGTATTGCGTGTTTTCATACGAATGACTTGGCAATGTAGAGACAGCCTGTTAAACATGGCAATGTGG
CTTCTGTGGACACCGCTCTCATGCCACTGTGTGCAAATAAACAAGGGACTTCCTTAGGAGCAGCCTAGCCACCTTACATGAAGAGTTTCCTAATGTCACAGAAGAGGAAGAAAGC
CAGTATTTGTTATGACACTGTTTCTTCTAAATAATGTAATTAATAAAATAAAGAAAAACATGG

Fig. 1. Schematic of *MOR-1* gene structure and alternative splicing. A, the gene structure of *MOR-1*. Exons and introns are indicated by boxes and horizontal lines, respectively. Translational start codon and termination codon are indicated by AUG and TAA or TAG or TGA. The exons are numbered according to when they were reported. Exon 5 corresponds to the exon associated with MOR-1B (Zimprich et al., 1995). Overlapping genomic clones covering the entire *MOR-1* gene are showed by heavy horizontal lines on the top. B, the nucleotide sequences of exons 6, 7, 8, and 9 of *MOR-1* gene. The exon sequences are shown in capital letters, and partial intron sequences are presented in lowercase letters. The 3′RACE sequence and the complete cDNA sequences of *MOR-1C*, *MOR-1D*, and *MOR-1E* are present in GenBank accession no. AF062752, AF062753, AF074973, and AF074974, respectively. The poly(A)$^+$ signal is underlined.

did not contain the new sequence we identified using 3′-RACE (exons 8 and 9). We then isolated P1 clone A (~100 kb), which contained the sequence found with the original 3′-RACE (exons 8 and 9), but this clone did not overlap with BAC clone A. Furthermore, it also did not contain the additional sequences found in either *MOR-1C* or *MOR-1E*. The use of FISH localized the P1 clone to band 10A2 of chromosome 10, the same region to which *MOR-1* was localized (Giros et al., 1995; Kaufman et al., 1995). Interphase FISH analysis revealed a distance of ~250 kb between BAC clone A, which contained the original *MOR-1* gene, and the P1 clone. This distance was confirmed after we finally obtained BAC clone B, which overlapped with the original *MOR-1* exon 4 and the P1 clone (Fig. 1).

Using these overlapping clones, we then mapped the new sequences. The three new variants were generated from combinations of four separate exons located downstream from the original exon 4 (Fig. 1). Exon 6 was 120 bp, whereas exon 7 was only 89 bp. Exon 8 was the shortest, only 66 bp, whereas exon 9 was the longest, 388 bp. The cDNA sequences obtained in the original cloning were identical with the gene sequences. The original sequence obtained in the 3′-RACE corresponded to exons 8 and 9. The 89-base insertion in *MOR-1C* was exon 7, whereas the insertion in *MOR-1E* was exons 6 and 7. All four new exons had flanking sequences consistent with consensus splice junctions (Fig. 1B). Thus, the *MOR-1* gene consists of nine exons spanning at least 200 kb.

The predicted amino acid sequences for these new exons differed from *MOR-1* and from each other. Exon 4 of *MOR-1* has 12 predicted amino acids. *MOR-1C* had the longest predicted sequence, 52 amino acids, whereas *MOR-1D* had only 7. Although exon 8 is translated in both *MOR-1C* and *MOR-1D*, the presence of exon 7 with its 89 bp in *MOR-1C* produced a reading frame shift in exon 8. The termination codon in *MOR-1E*, which has 15 amino acids, is in exon 6, so that exons 7, 8, and 9 are not translated. These sequences differed from *MOR-1* in other respects as well. For example, *MOR-1E* is quite basic and contains a protein kinase C phosphorylation site not present in *MOR-1*. *MOR-1C* contains two putative casein kinase II phosphorylation sites.

Analysis of *MOR-1* mRNA. We next examined the expression of the mRNA encoding the variants. A probe containing exons 7, 8, and 9 hybridized to a diffuse band, ranging in size from ~6 to 9 kb. This was easily distinguished from an exon 4 probe from the original *MOR-1*, which revealed a single transcript of ~12 kb (Fig. 3). An exon 7 probe, which would detect only *MOR-1C* and *MOR-1E*, revealed a weaker band of similar size as the combined exon 7/8/9 probe (data not shown). We were unable to identify *MOR-1E* in this assay using an exon 6 probe.

The regional mRNA levels of the different new variants was particularly intriguing. Using RT-PCR, we examined the mRNA expression of the different variants in a number of brain regions (Fig. 4). *MOR-1* was expressed in all regions (Fig. 4B). Of the new variants, *MOR-1C* was the predominant isoform in all the regions, but the relative expression of the other variants varied widely (Fig. 4A). This was particularly evident in the thalamus, a region noted for high levels of μ-opioid receptor binding (Pert et al., 1976; Atweh and Kuhar, 1977a,b,c; Goodman and Pasternak, 1985), where there was little evidence for either *MOR-1D* or *MOR-1E* expression. The spinal cord also contained predominantly *MOR-1C* mRNA, with low levels of *MOR-1E* and no observable *MOR-1D*. In contrast, the periaqueductal gray and striatum displayed all three variants, with highest levels of *MOR-1C*, followed by *MOR-1E* and then *MOR-1D*. In contrast, the cortex had higher levels of expression of *MOR-1D* than *MOR-1E*, as did the cerebellum and brainstem.

Characterization of Expressed Variants. In vitro translation of the three full-length cDNA clones revealed that *MOR-1D* and *MOR-1E* had molecular weights similar to that of *MOR-1*, whereas the size of *MOR-1C* was larger than the others, as expected based on the predicted sequence (Fig. 5). We then stably transfected CHO cells with the three clones and examined opioid binding. In saturation studies, [^{3}H]DAMGO displayed high affinity for all the variants (Ta-

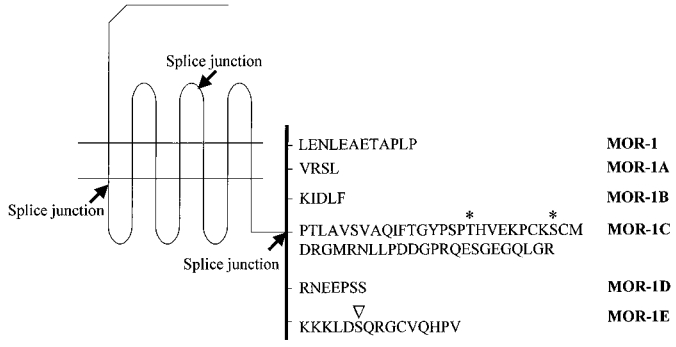


Fig. 2. Amino acid sequence of *MOR-1* splice variants predicted from the cDNA clones. All represent murine variants, with the exceptions of MOR-1A (Bare et al., 1994) and MOR-1B (Zimprich et al., 1995), which are from humans and rat, respectively.

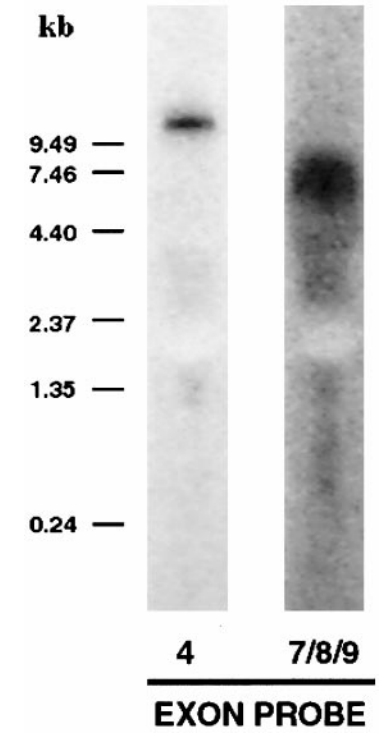


Fig. 3. Northern blots were performed on mouse brain using an exon 4 probe and a probe including exons 7/8/9, as described in the text.

ble 1). Indeed, the new variants bound [^{3}H]DAMGO with higher affinities than *MOR-1*, but the differences did not achieve statistical significance (Table 1).

Competition studies established that the variants all belonged within the μ-opiod receptor family (Table 2). The μ ligands such as morphine, DAMGO, and M6G and the endomorphins all competed binding quite potently, whereas the κ_1-opioid U50,488H and the δ-opiod ligand [D-Pen^2,D-Pen^5]enkephalin were ineffective. However, the binding selectivity profiles among the variants were significantly different. For example, morphine competed binding to the *MOR-1D* variant over 3-fold more potently than against *MOR-1* itself ($p < .05$). Similarly, the opioid peptide [D-Ser^2,Leu^5]enkephalin-Thr was twice as potent against binding to the *MOR-1D* variant than *MOR-1* ($p < .05$). However, the most dramatic differences in potency were seen with the endogenous opioids dynorphin A ($p < .0001$) and β-endorphin ($p < .0003$). The *MOR-1D* variant had the highest affinity for both dynorphin A and β-endorphin. *MOR-1E* also had a significantly higher affinity for β-endorphin than *MOR-1*. Dynorphin A had significantly higher affinity for *MOR-1C* and *MOR-1D* than either *MOR-1* or *MOR-1E*.

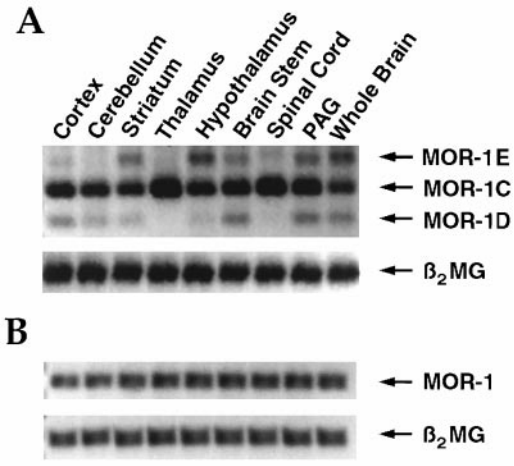


Fig. 4. Regional distribution of the *MOR-1C*, *MOR-1D*, and *MOR-1E* mRNA. a, RT-PCR was performed on the indicated brain regions using primers designed to identify *MOR-1C*, *MOR-1D*, and *MOR-1E*, as described in the text. β-Microglobulin was used an internal control. b, RT-PCR was performed on the indicated brain regions using primers designed to identify *MOR-1*. β-Microglobulin was used as an internal standard.

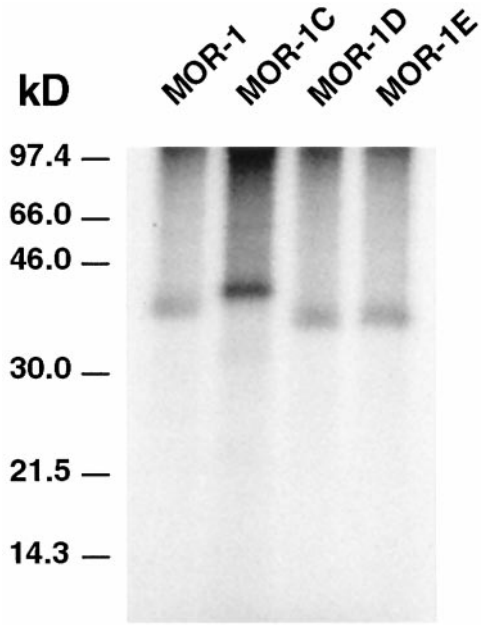


Fig. 5. In vitro translation of *MOR-1*, *MOR-1C*, *MOR-1D*, and *MOR-1E* was performed as described in the text.

Regional Expression of *MOR-1C*. We explored the regional distribution of *MOR-1C*, the most abundant of the three variants, using a polyclonal antibody generated against a unique amino acid sequence in this variant (Abbadie et al., in press). The polyclonal antibody recognized *MOR-1C* but not *MOR-1* in transfected cells in Western blots (data not shown)

Sections through the striatum (Fig. 6, A and B) demonstrated marked differences between *MOR-1* and *MOR-1C*. The labeling of *MOR-1* corresponded closely to regions previously reported to contain high levels of μ binding autoradiographically. *MOR-1* immunolabeling was observed in patches in the striatum, as well is in the subcallosal streak. Dense areas of labeling also were seen in the nucleus accumbens. In contrast, the *MOR-1C* antiserum failed to label these areas. There also was *MOR-1C* immunoreactivity in regions of the lateral septum that had minimal staining with the *MOR-1* antiserum. The hypothalamus also revealed significant differences between the two antisera (Fig. 6, C and D). Although there was some *MOR-1* staining, the intensity of the *MOR-1C* immunoreactivity was far more intense in the arcuate nucleus and median eminence. Additional studies documented intense *MOR-1C* immunoreactivity in the trigeminal tract and the dorsal horn of the spinal cord, as well as in the periaqueductal gray (data not shown). Overall, the mouse distributions were very similar to those observed in the rat (Abbadie et al., in press).

Antisense Mapping Exons 6, 7, 8, and 9. Finally, we explored the functional significance of these new variants. Antisense mapping (Standifer et al., 1994; Pasternak and Standifer, 1995) has been extensively used to correlate opioid pharmacology with the *MOR-1* receptor (Rossi et al., 1994, 1995a,b; Kolesnikov et al., 1996). We examined the activity of antisense probes designed to target each of the four new exons against both morphine and M6G analgesia (Fig. 7), two μ drugs whose actions have been distinguished using antisense approaches (Rossi et al., 1994, 1995a,b). Control studies have documented that intrathecal administration of the antisense targeting exon 8 down-regulates *MOR-1C* immunohistochemistry in the dorsal horn by 40 to 50% (Abbadie et al., in press). All four antisense probes significantly lowered morphine analgesia (Fig. 7). A mismatched control based on

TABLE 1
[^{3}H]DAMGO binding in membranes from transfected cells
MOR-1, *MOR-1C*, *MOR-1D*, or *MOR-1E* was transfected into CHO cells, and stable transfectants were isolated. [^{3}H]DAMGO binding was then examined in stable lines expressing either *MOR-1* or *MOR-1C*. Saturation studies were performed and the binding parameters were established by nonlinear regression analysis. Results are the mean ± S.E. of at least three independent determinations.

Clone	K_D	B_{max}
	nM	*pmol/mg protein*
MOR-1	1.75 ± 0.44	0.16 ± 0.04
MOR-1C	0.57 ± 0.14	0.41 ± 0.07
MOR-1D	0.35 ± 0.07	0.54 ± 0.06
MOR-1E	0.74 ± 0.11	0.25 ± 0.04

the antisense targeting exon 7 was inactive, confirming the specificity of the response. The activity of antisense probes against all four exons implied that each was present in the mRNA encoding a receptor or receptors involved with morphine analgesia. The activity of the probe against exon 6 clearly implied the involvement of *MOR-1E* because it is the only variant containing this exon. At this point, it is not possible to determine whether *MOR-1C* or *MOR-1D* was involved because they both share exons 7, 8, and 9 with MOR-1E. Thus, the response may involve *MOR-1E* alone or a combination of *MOR-1C* and *MOR-1D*. In contrast to their significant blockade of morphine analgesia, none of the antisense probes significantly lowered M6G analgesia; thus, these exons were not a component of the postulated M6G receptor.

Discussion

Correlation of the actions of morphine and other μ opioids with cloned receptors has long been a goal in the opioid field. The cloning of the μ-opioid receptor quickly led to its association with many morphine actions using both antisense (Rossi et al., 1994; Pasternak and Standifer, 1995) and gene disruption techniques (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998; Schuller et al., 1999). Pharmacological studies have long suggested subtypes of μ receptors (Wolozin and Pasternak, 1981; Pasternak, 1993; Reisine and Pasternak, 1996), and recent studies have raised the possibility that some of these may reflect splice variants of the *MOR-1* gene (Pasternak and Standifer, 1995), the only identified gene encoding a μ receptor. Two *MOR-1* variants were identified shortly after the initial cloning of *MOR-1* (Bare et al., 1994; Zimprich et al., 1995). Our current results identified an additional three *MOR-1* splice variants that result from combinations of four new exons.

The identification of the new four exons indicated that the *MOR-1* gene contained at least nine exons spanning over 200 kb. There was extensive splicing, with six variants differing only at the intracellular carboxyl terminus. In MOR-1A, exon 4 was missing, leaving an extended exon 3 that encodes four additional amino acids (Bare et al., 1994). MOR-1A was first detected in a human cell line, but a similar murine variant also has been isolated (Y.-X.P., J.X., and G.W.P., unpublished observations). MOR-1B, isolated from the rat, contains an alternatively spliced exon 5 instead of the original exon 4. Exon 5, which is located between exons 3 and 4 (Fig. 2), now has been identified in the murine gene (Y.-X.P., J.X., and G.W.P., unpublished observations).

Unlike the other variants, the new ones consisted of two to four exons in place of the original exon 4. These exons were quite short, ranging from only 66 to 388 bases, and they were widely separated from the original exons comprising *MOR-1* and from each other. The amino acid sequences of all the new variants were different. Although exon 8 contained coding sequences in both *MOR-1C* and *MOR-1D*, the 89 bases from exon 7 produced a reading frame shift in exon 8 of *MOR-1C* compared with *MOR-1D*. Exon 8 was not translated in *MOR-1E* because the termination codon was in exon 6. The sequences encoded by *MOR-1D* and *MOR-1E* were both relatively short: 7 and 15 amino acids, respectively. *MOR-1C* had an extended sequence, 52 amino acids, which was far longer than *MOR-1* itself.

The differences between the new variants and *MOR-1* itself were restricted to the terminal portion of the intracellular tail of the receptor. The transmembrane regions of G protein-coupled receptors are critical in the binding selectivity of the receptors. Because all three new variants shared the exons encoding all seven transmembrane regions, it was not surprising that they all selectively bound μ opiods and had poor affinity for δ and κ drugs, yet the variants did have subtle binding profile differences, particularly for the endogenous opioids dynorphin A and β-endorphin. It is not yet possible to establish the reasons underlying these differences, which might reflect a generalized structural change in the receptor or possibly coupling to a different G protein. This question needs further examination. The presence of additional phosphorylation sites in *MOR-1C* and *MOR-1E* also raises questions about the regulation of their function. Fi-

TABLE 2

Selectivity of *MOR-1* and *MOR-1C* in receptor binding assays

[^{3}H]DAMGO binding was performed in stable transfectants containing the indicated cDNAs. ANOVA was performed to determine whether there were differences among the various clones for each competitor, followed by Tukey's post hoc analysis. Competition studies were performed using at least three concentrations of the indicated competitor. Results are the mean ± S.E. of at least three independent determinations.

Ligand	K_i Value				ANOVA	Tukey	
	MOR-1	*MOR-1C*	*MOR-1D*	*MOR-1E*		MOR	*p*
	nM						
Morphine	5.3 ± 2.0	2.4 ± 0.6	1.5 ± 0.2	2.3 ± 0.4	<.05	1 vs 1D:	<.05
M6G	5.2 ± 1.8	4.1 ± 1.2	4.8 ± 0.8	5.6 ± 0.7	N.S.		
DAMGO	1.8 ± 0.5	0.93 ± 0.19	0.71 ± 0.11	1.2 ± 0.5	N.S.		
DADLE	2.1 ± 0.3	3.2 ± 1.9	1.3 ± 0.4	2.5 ± 0.7	N.S.		
DSLET	12.5 ± 3.6	8.1 ± 1.2	3.6 ± 0.6	6.7 ± 1.4	<.05	1 vs 1D:	<.05
Naloxone	4.3 ± 0.9	2.8 ± 0.8	0.92 ± 0.08	2.8 ± 1.4	N.S.		
Endomorphin 1	2.1 ± 0.8	1.4 ± 0.4	1.8 ± 0.3	2.4 ± 0.1	N.S.		
Endomorphin 2	4.2 ± 1.8	1.6 ± 0.2	2.0 ± 0.3	4.4 ± 0.8	N.S.		
β-Endorphin	10.8 ± 2.9	5.8 ± 0.5	1.7 ± 0.4	5.0 ± 1.2	<.0003	1 vs 1D:	<.001
						1 vs 1E:	<.05
Dynorphin A	10.9 ± 0.5	5.6 ± 0.8	2.2 ± 0.8	8.9 ± 1.1	<.0001	1 vs 1C:	<.05
						1 vs 1D:	<.001
						1C vs 1E:	<.05
						1D vs 1E:	<.001
U50,488H	>1000	>1000	>1000	>1000			
DPDPE	>1000	>1000	>1000	>1000			

DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DSLET, [D-Ser^2, Leu^5]-enkephalin-Thr; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin.

nally, recent reports on the γ-aminobutyric acid$_B$ receptor found that the intracellular tail was important in forming receptor heterodimers necessary for a functional receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Opioid receptor dimerization also has been proposed (Cvejic and Devi, 1997). The potential role of these differences in the intracellular tail of the receptor in these interactions also needs to be explored.

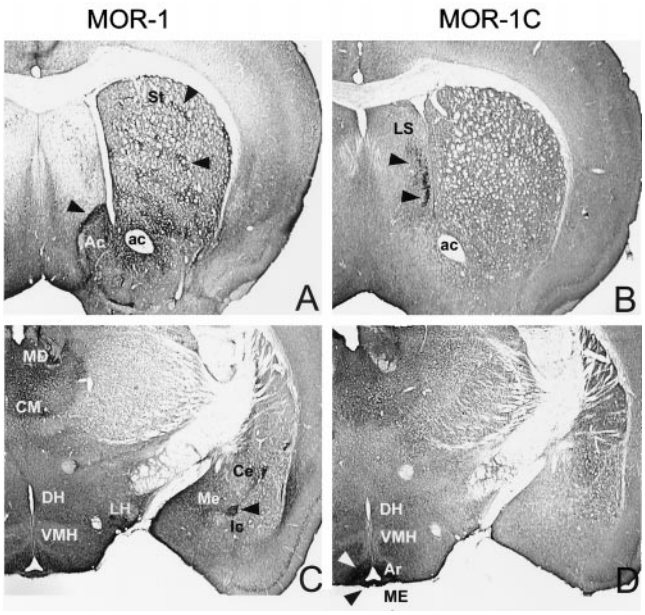


Fig. 6. Immunohistochemical localization of *MOR-1* and *MOR-1C* in mouse brain. Mouse brains were sectioned, and immunostaining for *MOR-1* and *MOR-1C* was determined as described previously (Abbadie et al., 1996, 1999a,b). Sections A and C and sections B and D were stained with *MOR-1* and *MOR-1C* antisera, respectively. Regions were (A and B) St, striatum; ac, anterior commissure; Ac, accumbens; and LS, lateral septum; (C) MD, mediodorsal thalamic nucleus; CM, centromedian thalamic nucleus; DH, dorsal hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; LH, lateral hypothalamic nucleus; Ce, central amygdaloid nucleus; Ic, intercalated amygdaloid nucleus; and Me, medial amygdaloid nucleus; and (D) Ar, arcuate nucleus; and ME, median eminence.

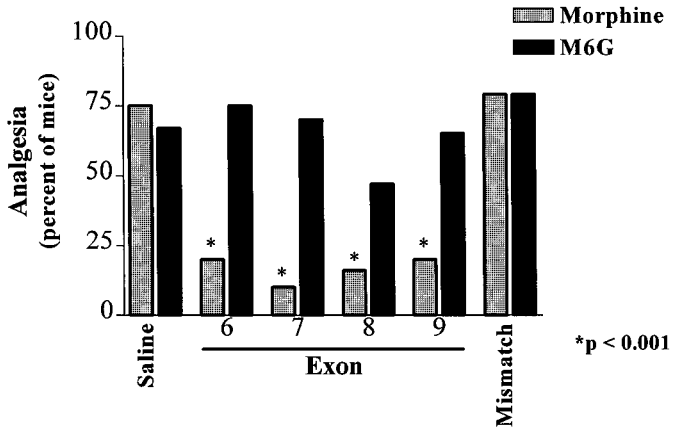


Fig. 7. Antisense mapping exons 6, 7, 8, and 9 of *MOR-1*. Groups of mice ($n \geq 20$) were administered either saline or the indicated oligodeoxynucleotide (10 μg i.c.v.) daily for 5 days and tested for analgesia on the sixth day with either morphine (700 ng i.c.v.) or M6G (15 ng i.c.v.) Analgesia was assessed quantally. Significant decreases for analgesia were determined by comparison with the control (saline) group using Fisher's exact test. Antisense oligodeoxynucleotide sequences: exon 6, 5′-GGCTCAAAGACAAGGGACAGGTCA-3′ (at position 1269–1293 of AF074974); exon 7, 5′-CCTGTAAAGATCTGGGCCACGC-3′ (at position 1361–1382 of AF074974); mismatch antisense of exon 7, 5′-CCGTTAAGAACTGTGGCACCGC-3′; exon 8, 5′-GGGCCATCATCAGGAAGAAGG-3′ (at position 1446–1466 of AF074974); and exon 9, 5′-GAA AGG CAT CTT CCC TCT CGC T-3′ (at position 180–201 of AF062752).

Anatomic studies in both mice and rats (Abbadie et al., in press) have demonstrated the presence of *MOR-1C* immunoreactivity in regions important in pain processing, including the dorsal horn of the spinal cord, the trigeminal nucleus, and the periaqueductal gray. The presence of *MOR-1C* receptors in this region was consistent with the antisense studies. Antisense probes targeting all four of the new exons lowered morphine analgesia. The activity of the exon 6 antisense probe clearly implicated *MOR-1E* in this action because this is the only variant containing this exon. The activity of the antisense probes targeting exons 7, 8, and 9 might be due to their actions against only *MOR-1E*, which contain these exons as well. Because there are no unique antisense probes for *MOR-1C* and *MOR-1D*, their involvement in morphine analgesia remains unclear. Pharmacological studies have suggested that morphine analgesia can involve more than one subtype of μ receptor (Paul and Pasternak, 1988; Pick et al., 1992). The blockade of morphine analgesia by antisense probes targeting the new variants, as well as probes based on exon 4, which is present in *MOR-1* (Rossi et al., 1994, 1995a,b), provide evidence at the molecular level for the involvement of at least two different μ receptor subtypes.

Our initial impetus into the search for additional splice variants was prompted, in large part, by the pharmacological differences between morphine and M6G (Rossi et al., 1995a,b, 1996, 1997). The antisense mapping results suggest that these new exons are not a component of the receptor responsible for M6G analgesia. However, the possibility remains for alternative splicing at other exons as well, leading to an even greater diversity of *MOR-1* variants. It will be interesting to see whether other variants will be uncovered.

References

Abbadie C, Brown JL, Mantyh PW and Basbaum AI (1996) Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. *Neuroscience* **70:**201–209.
Abbadie C, Pan Y-X and Pasternak GW (1999a) Distribution in rat brain of the mu opioid receptor splice variant *MOR-1C*: Evidence for region specific-specific processing (in press).
Abbadie C, Skinner K, Mitrovic I and Basbaum AI (1999b) Neurons in the dorsal column white matter of the spinal cord: Complex neuropil in an unexpected location. *Proc Natl Acad Sci USA* **96:**260–265.
Atweh SF and Kuhar MJ (1977a) Autoradiographic localization of opiate receptors in rat brain. I. Spinal cord and lower medulla. *Brain Res* **124:**53–67.
Atweh SF and Kuhar MJ (1977b) Autoradiographic localization of opiate receptors in rat brain. II. The brain stem. *Brain Res* **129:**1–12.
Atweh SF and Kuhar MJ (1977c) Autoradiographic localization of opiate receptors in rat brain. III. The telencephalon. *Brain Res* **134:**393–405.
Bare LA, Mansson E and Yang D (1994) Expression of two variants of the human μ

opioid receptor mRNA in SK-N-SH cells and human brain. *FEBS Lett* **354:**213–216.
Chen Y, Mestek A, Liu J, Hurley JA and Yu L (1993) Molecular cloning and functional expression of a μ-opioid receptor from rat brain. *Mol Pharmacol* **44:**8–12.
Cvejic S and Devi LA (1997) Dimerization of the delta opioid receptor: Implication for a role in receptor internalization. *J Biol Chem* **272:**26959–26964.
Giros B, Pohl M, Rochelle JM and Seldin MF (1995) Chromosomal localization of opioid peptide and receptor genes in the mouse. *Life Sci* **56:**PL369–PL375.
Goodman RR and Pasternak GW (1985) Visualization of mu_1 opiate receptors in rat brain using a computerized autoradiographic subtraction technique. *Proc Natl Acad Sci USA* **82:**6667–6671.
Guiramand J, Montmayeur JP, Ceraline J, Bhatia M and Borrelli E (1995) Alternative splicing of the dopamine D2 receptor directs specificity of coupling to G-proteins. *J Biol Chem* **270:**7354–7358.
Hsu SM, Raine L and Fanger H (1981) A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am J Clin Pathol* **75:**734–738.
Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA and Gerald C (1998) GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature (Lond)* **396:**674–679.
Kaufman DL, Keith DE Jr, Anton B, Tian J, Magendzo K, Newman D, Tran TH, Lee DS, Wen C, Xia Y-R, Lusis AJ and Evans CJ (1995) Characterization of the murine μ opioid receptor gene. *J Biol Chem* **270:**15877–15883.
Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A and Bettler B (1998) GABA(B)-receptor subtypes assemble into functional heteromeric complexes. *Nature (Lond)* **396:**683–687.
Kolesnikov YA, Jain S, Wilson R and Pasternak GW (1996) Peripheral morphine analgesia: Synergy with central sites and a target of morphine tolerance. *J Pharmacol Exp Ther* **279:**502–506.
Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A and Kornau HC (1999) Role of heteromer formation in GABAB receptor function. *Science (Wash DC)* **283:**74–77.
Liang Y, Mestek A, Yu L and Carr LG (1995) Cloning and characterization of the promoter region of the mouse μ opioid receptor gene. *Brain Res* **679:**82–88.
Llewellyn-Smith IJ and Minson JB (1992) Complete penetration of antibodies into vibratome sections after glutaraldehyde fixation and ethanol treatment: Light and electron microscopy for neuropeptides. *J Histochem Cytochem* **40:**1741–1749.
Loh HH, Liu HC, Cavalli A, Yang WL, Chen YF and Wei LN (1998) μ Opioid receptor knockout in mice: Effects on ligand-induced analgesia and morphine lethality. *Mol Brain Res* **54:**321–326.
Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.
Lucas JJ and Hen R (1995) New players in the 5-HT receptor field: Genes and knockouts. *Trends Pharmacol Sci* **16:**246–252.
Matthes HWD, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dollé P, Tzavara E, Hanoune J, Roques BP and Kieffer BL (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid-receptor gene. *Nature (Lond)* **383:**819–823.
Min BH, Augustin LB, Felsheim RF, Fuchs JA and Loh HH (1994) Genomic structure and analysis of promoter sequence of a mouse μ opioid receptor gene. *Proc Natl Acad Sci USA* **91:**9081–9085.
Namba T, Sugimoto Y, Negishi M, Irie A, Ushikubi F, Kakizuka A, Ito S, Ichikawa A and Narumiya S (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature (Lond)* **365:**166–170.
Pan Y-X, Cheng J, Xu J and Pasternak GW (1994) Cloning, expression and classification of a $kappa_3$-related opioid receptor using antisense oligodeoxynucleotides. *Regul Pept* **54:**217–218.
Pan Y-X, Xu J, Ryan-Moro J, Mathis J, Hom JSH, Mei JF and Pasternak GW (1996) Dissociation of affinity and efficacy in KOR-3 chimeras. *FEBS Lett* **395:**207–210.
Pasternak GW (1993) Pharmacological mechanisms of opioid analgesics. *Clin Neuropharmacol* **16:**1–18.
Pasternak GW and Standifer KM (1995) Mapping of opioid receptors using antisense oligodeoxynucleotides: Correlating their molecular biology and pharmacology. *Trends Pharmacol Sci* **16:**344–350.
Paul D and Pasternak GW (1988) Differential blockade by naloxonazine of two μ opiate actions: Analgesia and inhibition of gastrointestinal transit. *Eur J Pharmacol* **149:**403–404.
Pert CB, Kuhar MJ and Snyder SH (1976) Opiate receptor: Autoradiographic localization in rat brain. *Proc Natl Acad Sci USA* **73:**3729–3733.
Pick CG, Roques B, Gacel G and Pasternak GW (1992) Supraspinal μ_2 receptors mediate spinal/supraspinal morphine synergy. *Eur J Pharmacol* **220:**275–277.
Reisine T and Pasternak GW (1996) Opioid analgesics and antagonists, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Hardman JG and Limbird LE eds) pp 521–556, McGraw-Hill, New York.
Rossi GC, Brown GP, Leventhal L, Yang K and Pasternak GW (1996) Novel receptor mechanisms for heroin and morphine-6β-glucuronide analgesia. *Neurosci Lett* **216:**1–4.
Rossi GC, Leventhal L, Pan YX, Cole J, Su W, Bodnar RJ and Pasternak GW (1997) Antisense mapping of *MOR-1* in rats: Distinguishing between morphine and morphine-6β-glucuronide antinociception. *J Pharmacol Exp Ther* **281:**109–114.
Rossi GC, Pan Y-X, Brown GP and Pasternak GW (1995a) Antisense mapping the *MOR-1* opioid receptor: Evidence for alternative splicing and a novel morphine-6β-glucuronide receptor. *FEBS Lett* **369:**192–196.
Rossi GC, Pan Y-X, Cheng J and Pasternak GW (1994) Blockade of morphine analgesia by an antisense oligodeoxynucleotide against the mu receptor. *Life Sci* **54:**PL375–379.
Rossi GC, Standifer KM and Pasternak GW (1995b) Differential blockade of morphine and morphine-6β-glucuronide analgesia by antisense oligodeoxynucleotides directed against *MOR-1* and G-protein α subunits in rats. *Neurosci Lett* **198:**99–102.
Schuller AGP, King MA, Zhang J, Boland E, Chang A, Czick ME, Unterwald E, Pasternak GW and Pintar JE (1999) Retention of heroin and morphine-6B-glucuronide analgesia in *MOR-1* exon 1 knockout mice insensitive to morphine: Evidence for a novel heroin receptor. *Nat Neurosci* **2:**151–156.
Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL and Uhl GR (1997) Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci USA* **94:**1544–1549.
Standifer KM, Chien C-C, Wahlestedt C, Brown GP and Pasternak GW (1994) Selective loss of δ opioid analgesia and binding by antisense oligodeoxynucleotides to a δ opioid receptor. *Neuron* **12:**805–810.
van den Engh G, Sachs R and Trask BJ (1992) Estimating genomic distance from DNA sequence location in cell nuclei by a random walk model. *Science (Wash DC)* **257:**1410–1412.
Vanetti M, Kouba M, Wang X, Vogt G and Hollt V (1998) Cloning and expression of a novel mouse somatostatin receptor (SSTR2B). *FEBS Lett* **311:**290–294.
Wang JB, Imai Y, Eppler CM, Gregor P, Spivak CE and Uhl GR (1993) μ Opiate receptor: cDNA cloning and expression. *Proc Natl Acad Sci USA* **90:**10230–10234.
White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM and Marshall FH (1998) Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature (Lond)* **396:**679–682.
Wolozin BL and Pasternak GW (1981) Classification of multiple morphine and enkephalin binding sites in the central nervous system. *Proc Natl Acad Sci USA* **78:**6181–6185.
Zimprich A, Simon T and Hollt V (1995) Cloning and expression of an isoform of the rat μ opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1. *FEBS Lett* **359:**142–146.

Send reprint requests to: Dr. Gavril W. Pasternak, Department of Neurology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021. E-mail: pasterng@mskmail.mskcc.org