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Cloning and Functional Characterization Through Antisense Mapping of a κ_3-Related Opioid Receptor

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SUMMARY

We have identified a putative opioid receptor from mouse brain (KOR-3), belonging to the G protein-coupled receptor family, that is distinct from the previously cloned μ, δ, and κ_1 receptors. Assignment of the clone to the opioid receptor family derives from both structural and functional studies. Its predicted amino acid sequence is highly homologous to that of the other opioid receptors, particularly in many of the transmembrane regions, where long stretches are identical to μ, δ, and κ_1 receptors. Both cyclazocine and nalorphine inhibit cAMP accumulation in COS-7 cells stably expressing the clone. Northern analysis shows that the mRNA is present in brain but not in a number of other organs. Southern analysis suggests a single gene encoding the receptor. A highly selective monoclonal antibody directed against the native κ_3 receptor recognizes, in Western analysis, the clone expressed in COS-7 cells. The *in vitro* translation product is also labeled by the antibody. Additional clones reveal the presence of several introns, including one in the second extracellular loop and another in the first transmembrane region. Antisense studies with an oligodeoxynucleotide directed against a region of the second extracellular loop reveal a selective blockade of κ_3 analgesia *in vivo* that is not observed with a mismatch oligodeoxynucleotide based upon the antisense sequence. The μ, δ, and κ_1 analgesia is unaffected by this antisense treatment. Antisense mapping of the clone downstream from the splice site in the first transmembrane region reveals that six different antisense oligodeoxynucleotides all block κ_3 analgesia. In contrast, only one of an additional six different antisense oligodeoxynucleotides directed at regions upstream from this splice site is effective. This strong demarcation between the two regions raises the possibility of splice variants of the receptor. An additional clone reveals an insert in the 3′ untranslated region. In conclusion, the antibody and antisense studies strongly associate KOR-3 with the κ_3-opioid receptor, although it is not clear whether it is the κ_3 receptor itself or a splice variant.

Pharmacological studies have identified three major classes of opioid receptors, μ, δ, and κ (1). Recently, the δ receptor was cloned (2, 3), followed shortly thereafter by the μ and κ_1 receptors (4–12). Expression of each of these receptors reveals binding characteristics consistent with those anticipated from traditional receptor binding studies. Functionally, all of the expressed opioid receptors inhibit cAMP formation with the agonist and antagonist selectivity predicted from studies in the brain and cell lines. The relevance of these receptors in opioid analgesia has been established using antisense techniques (13–16). Antisense oligodeoxynucleotides directed against the δ receptor clone DOR-1 selectively and specifically down-regulate binding in the NG108–15 cell line and δ analgesia *in vivo*, without interfering with μ or κ_1 actions (13–16). Similarly, an antisense oligodeoxynucleotide against the κ_1 receptor selectively blocks U50,488H analgesia, without affecting the actions of μ or δ drugs (15). Microinjection into the periaqueductal gray of rats of an antisense oligodeoxynucleotide directed against the 5′ untranslated region of the μ receptor prevents morphine analgesia (14). In all cases, mismatch antisense oligodeoxynucleotides in which four bases are switched are inactive, confirming the specificity of the action. Thus, these studies confirm three distinct families of opioid receptors.

Previously, based upon a series of binding and behavioral studies (17–21), we suggested the existence of a novel opioid

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ABBREVIATIONS: NalBzoH, naloxone benzoylhydrazone; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

receptor, termed κ_3, which corresponds to the nalorphine, or N, receptor first proposed by Martin (22) and which may correspond to a site subsequently described by another group (23). The binding profile of κ_3 sites using [^{3}H]NalBzoH is distinct, with a density (B_{max}) in brain approximately twice that of either μ or δ receptors. Pharmacologically, κ_3 receptors elicit analgesia supraspinally through mechanisms distinct from those of traditional receptors. κ_3 analgesia is insensitive to the μ antagonist β-funaltrexamine, as well as antagonists selective for κ_1 (norbinaltorphimine) and δ (naltrindole) receptors. Furthermore, it shows no cross-tolerance with either μ or κ_1 analgesia. κ_3 receptors are expressed in BE(2)-C neuroblastoma cells, where they inhibit adenylyl cylcase activity independently of μ or δ receptors (24). Together, these observations strongly support the existence of a distinct opioid receptor. We now report the cloning and expression of an opioid receptor within the κ_3 receptor family.

Materials and Methods

Chemicals and supplies. Male CD-1 mice (25–35 g; Charles River Breeding Laboratory, Bloomington, MA) were used in all studies. Animals were housed five/cage, maintained on a 12-hr light/dark cycle, and given food and water *ad libitum*. Morphine sulfate, DPDPE, DAMGO, and U50,488H were obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). NalBzoH was synthesized as described previously (25). Levallorphan was a gift from Hoffman-LaRoche (Nutley, NJ). Oligodeoxynucleotides were synthesized by The Midland Certified Reagent Co. (Midland, TX) or by the Core Facility at Memorial Sloan-Kettering Cancer Center. DNA modification and restriction enzymes were from GIBCO (Gaithersburg, MD), Stratagene (La Jolla, CA), or New England Biolabs (Beverly, MA). DNA sequencing kits were from United States Biochemicals (Cleveland, OH). Nylon membranes were purchased from Micron Separation Inc. (Boston, MA). GeneScreen Plus membranes and all radiochemicals were purchased from DuPont-NEN (Boston, MA) unless stated otherwise. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Measurement of cAMP accumulation. Experimental incubations were performed as described (24). Basal levels of cAMP accumulation in the presence of GTP were determined in membranes treated at pH 4.5 to minimize stimulatory activity (26), in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.5 mM), at 37° for 10 min, in the absence or presence of the stated concentration of opioid. The incubation was terminated by removing the membranes to a boiling water bath for 5 min. Samples were centrifuged, and the supernatant was assayed for cAMP levels (24).

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* (27).

PCR cloning. Two degenerate oligodeoxynucleotides were designed to encode the predicted protein sequence of the mouse δ receptor at positions 139–146 (sense orientation; primer 1, 5′-CTCACNATGWGFGTNGACQG-3′) and 314–321 (antisense orientation; primer 2, 5′-TCYTTFCGYATFTCFTGFCCYAA-3′, where Y is T or C, W is A or T, Q is A or C, F is C or I, and N is G, A, T, or C) and used in PCRs (28) to amplify opioid receptor cDNA fragments. The template was first-strand cDNA reverse-transcribed from mouse brain total RNA (Clontech, Palo Alto, CA) with random hexamers. The PCR products were subcloned into Bluescript plasmids and sequenced. The nucleotide and predicted peptide sequences of one cloned fragment, op20-mer, were highly homologous with those of the mouse δ receptor.

Library screening and DNA sequence analysis. The op20-mer fragment was ^{32}P-radiolabeled and used as a probe to screen a mouse brain λZAP cDNA library (Stratagene). Approximately 2.5 × 10^6 plaques were plated and nylon membrane replicates were probed at high stringency. One of the longest clones (13–2a4 or KOR-3a/SKII), with an approximately 2.6-kb insertion, was selected for further sequence analysis. The DNA sequences were determined using appropriate oligonucleotide primers in both directions. The DNA sequence analysis was performed using the personal computer package DNANALYZ (Gregory Wernke, University of Cincinnati College of Medicine) and the CLONE and ALIGN programs from Scientific and Educational Software.

Northern and Southern analyses. Using oligo(dT) chromatography (Pharmacia), poly(A)$^+$ RNA was isolated from total mouse RNA as described (29). Mouse genomic DNA was isolated from mouse liver tissue as described (30) and was digested with appropriate restriction enzymes. Northern and Southern blotting followed the protocol for GeneScreen Plus membranes (DuPont). A ^{32}P-labeled, 460-base, PCR fragment corresponding to the nucleotide sequence of 13–2a4 at positions 743-1208 was used in Northern blotting, whereas a 810-base fragment corresponding to the nucleotide sequence of 13–2a4 at positions 281-1208 was used in Southern blotting.

Expression of mouse KOR-3 receptor. A *Xho*I-*Bam*HI fragment carrying the full length KOR-3 receptor cDNA was cut from 13–2a4 and subcloned between the *Xho*I and *Bam*HI sites of a simian virus 40 early promoter-based expression vector, pSRα (31). A *Hind*III fragment carrying the entire coding region of KOR-3 (KOR-3/SKII) was cut from 13–2a4 and subcloned into the *Hind*III site of pRcCMV (Invitrogen, San Diego, CA). The resulting plasmids, 13–2a4/pSRα and KOR-3/pRcCMV, respectively, were used to transfect COS-7 cells by the calcium phosphate co-precipitation method, as described (32). For transient expression, cells were collected 72 hr after glycerol or dimethylsulfoxide shock treatment. Stable transformants were obtained 6 weeks after incubation of the transfected cells with G418 (GIBCO). Stable transformants were monitored by both reverse transcription-PCR and Northern analysis, to ensure continued expression of the insert.

***In vitro* translation.** KOR-3/pRcCMV and KOR-3/SKII were linearized downstream of the stop codon, with *Xba*I and *Bam*HI, respectively, and were transcribed *in vitro* with T7 polymerase. The transcripts were translated for 1 hr at 30° with a rabbit reticulocyte lysate (GIBCO), with or without canine pancreatic microsomal membranes (Promega), in the presence of 0.04 mCi of [^{35}S]methionine (1000 Ci/mmol; Amersham, Arlington Heights, IL) or 30 μM unlabeled methionine, and the translation products were separated by SDS-PAGE.

Western analysis. Cell membranes from BE(2)-C neuroblastoma cells and transfected COS-7 cells and the *in vitro* translation products were subjected to 5–15% gradient SDS-PAGE and transferred to nitrocellulose membranes (Sigma). The membranes were blocked for 1 hr in PBS containing 5% nonfat dried milk, washed with PBS, and incubated with mAb 8D8[1] overnight at 4°. After washing in PBS containing 0.05% Tween-20, the membranes were incubated with peroxidase-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA), diluted 1/5000 in wash buffer with 1% bovine serum albumin, for 1 hr at room temperature. The signals were generated using Renaissance chemiluminescence reagents (New England Nuclear, Boston, MA).

Antisense testing *in vivo*. Antisense oligodeoxynucleotides (5 μg) were administered either intracerebroventricularly or intrathecally on days 1, 3, and 5, and analgesia was tested on day 6, as described previously (13–15). Analgesia was determined using the radiant-heat tail-flick technique, with a maximal latency of 10 sec (to minimize tissue damage) (19–21). Analgesia was defined quantally as a latency, 30 min after opioid injection, that was at least double the base-line latency for each mouse (typically 2–3 sec). Intrathecal

[1] A. I. Brooks, K. M. Standifer, J. Mathis, and G. W. Pasternak. Generation and characterization of a monoclonal antibody selective for the κ_3-opioid receptor. Submitted for publication.

and intracerebroventricular injections were performed under halothane anesthesia (20), which rapidly wears off and does not interfere with testing. Analgesia data were analyzed using Fisher's exact test.

Results

Cloning of KOR-3. We initiated our studies by PCR cloning of opioid receptor fragments using two degenerate oligonucleotides based upon the mouse δ-opioid receptor DOR-1. One cloned fragment of 0.5 kb (op20-mer) has nucleotide and predicted peptide sequences highly homologous, but not identical, to those of the mouse δ receptor. We then designed an antisense oligodeoxynucleotide (antisense oligodeoxynucleotide I) to examine the relevance of the sequence (Table 1). Administered into the brain, the antisense oligodeoxynucleotide potently blocked NalBzoH analgesia, whereas the corresponding sense oligodeoxynucleotide was without effect (Fig. 1A). A mismatch antisense oligodeoxynucleotide with the same base composition, in which the sequence of four bases was switched, also was inactive, confirming the selectivity of this action. In contrast to its effects against NalBzoH analgesia, the antisense oligodeoxynucleotide was inactive against μ, δ, and κ_1 analgesia, using paradigms in which the correct antisense oligodeoxynucleotides block their respective analgesic drugs (Fig. 1B) (13–15).

Having established the importance of the sequence of the PCR product in κ_3-opioid analgesia, we next obtained a full length clone. From the predicted protein sequence, the full length clone has high homology with rat μ-opioid (54%), mouse δ-opioid (52%), and mouse κ_1-opioid (53%) receptors. The sequence shows up to 95% identity with several other reported unidentified clones within the opioid receptor family (33–41). Kyte-Doolittle analysis predicts seven transmembrane regions, consistent with a traditional G protein-coupled receptor (Fig. 2). The homology between the predicted amino acid sequence of our receptor and the sequences of the previously cloned μ-, δ-, and κ_1-opioid receptors is greatest in the transmembrane regions. The amino and carboxyl termini and the region between the fourth and fifth transmembrane regions, which includes the second extracellular loop, display the most unique sequences.

In additional studies, we also isolated and sequenced two other clones (Fig. 3). One, 258, includes an intron in the

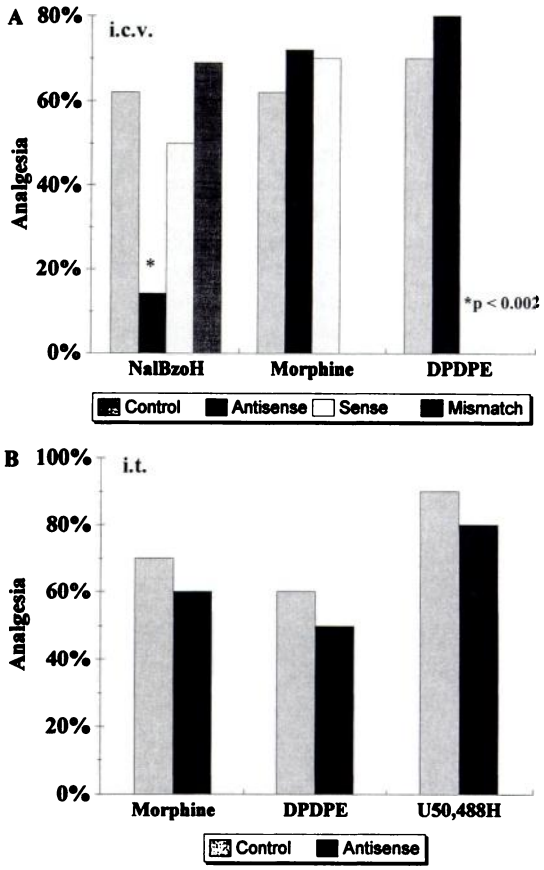

Fig. 1. Blockade of κ_3 analgesia by an antisense oligodeoxynucleotide. A, Mice were treated with saline alone, antisense oligodeoxynucleotide I, the corresponding sense oligodeoxynucleotide, or the mismatch (5 μg, intracerebroventricularly) on days 1, 3, and 5, and analgesia was assessed on day 6 with NalBzoH (15 μg, intracerebroventricularly), morphine (1 μg, intracerebroventricularly), or DPDPE (15 μg, intracerebroventricularly). Only the NalBzoH group showed a significant ($p < 0.002$) decrease in analgesia. Each group consisted of at least 30 mice. B, Mice received either saline alone or antisense oligodeoxynucleotide I (5 μg, intrathecally) on days 1, 3, and 5, followed by testing for morphine (1.6 μg, intrathecally), DPDPE (0.5 μg, intrathecally), or U50,488H (30 μg, intrathecally) analgesia. The antisense treatment did not significantly lower the analgesic response in any group. Each group consisted of at least 30 mice.

TABLE 1

Antisense oligonucleotides against the KOR-3 receptor

Oligonucleotide	Position	Sequence	T_m	GC content
				%
A	217–236	GCTGGGCCAAAACAGACCCT	59.6°	60
B	279–298	GCTGTCACCCTGCAAAGCCA	60.6°	60
C	301–321	GGGGCAGGAAAGAGGGACTCC	61.8°	67
D	381–401	GGAGCAGGTGATGGGGTACGG	62.1°	67
E	419–438	GTCCAAGGGGCAGGAAGGCA	62.9°	65
F	486–505	GACGAGGCAGTTCCCCAGGA	60.8°	65
G	610–630	CCCAGAAGGATGTCTGTGCCC	60.1°	62
H	890–909	GAGGGGCGGGGATCTCCACC	66.3°	75
I	1189–1208	GGGCTGTGCAGAAGCCGAGA	63.2°	65
I	Mismatch	GGGTCGTGCAGAGACCGAGA	39.2°	65
J	1319–1339	GCGCACACGATCAGAAACCTG	60.1°	57
K	1479–1497	AGCCATGGAGGCCACCTTG	59.3°	63
L	2192–2210	GTGCCCTGCCCTGCCCATA	62.4°	68
M[a]	52–71 (3′ insert)	CGCTCAGTTCAGGGTCAACC	56.7°	56

[a] This oligonucleotide corresponds to nucleotide sequence positions 3050–3069 of 6–5a1, which corresponds to bases 52–71 of the insert within the 3′ untranslated region.

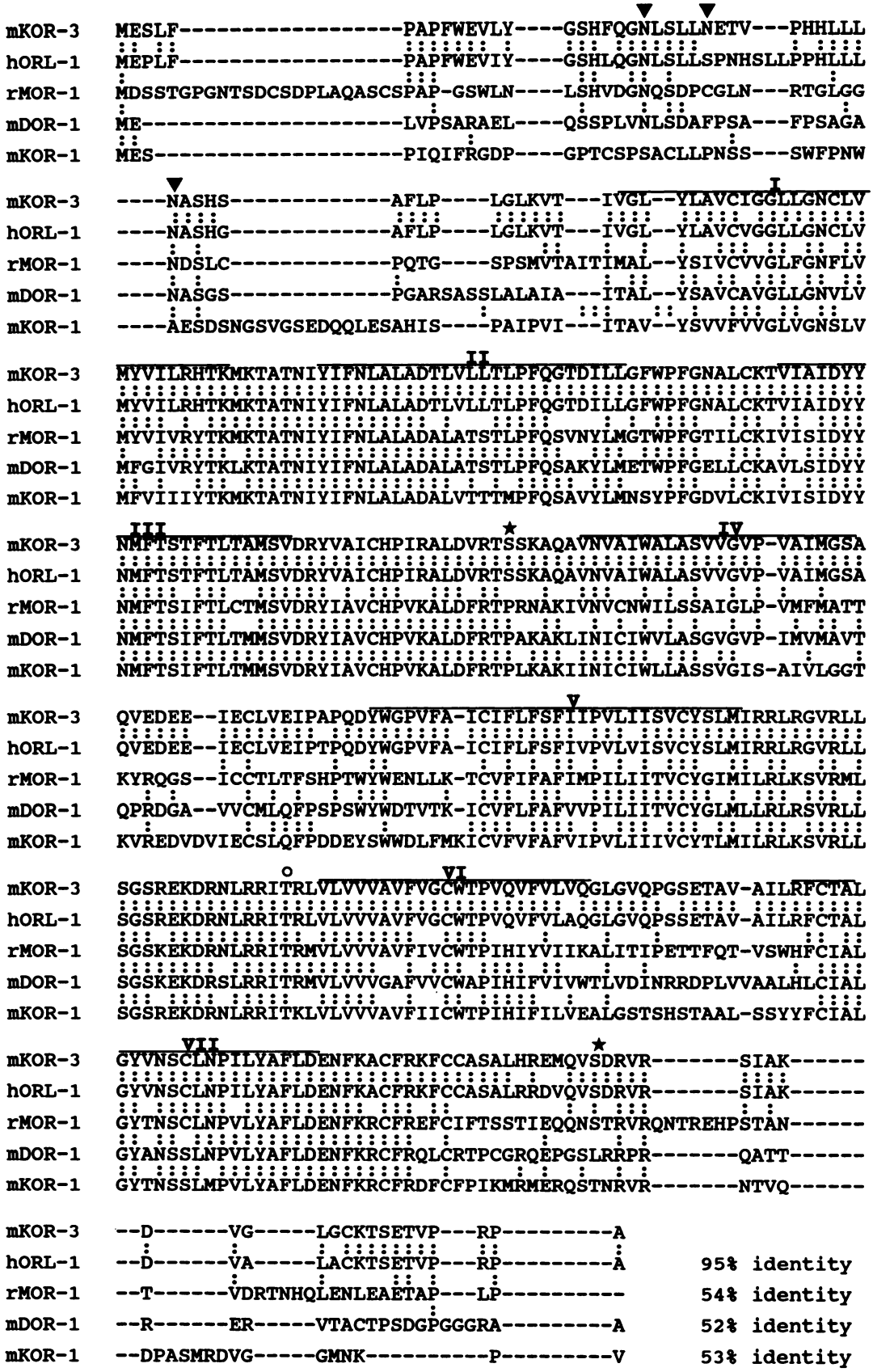

Fig. 2. Amino acid alignment of KOR-3 with other opioid receptors. *Dots*, identities in the sequences; differences are indicated by the indicated residues. *Overlined sequences*, putative transmembrane domains. The KOR-3 cDNA sequence has been submitted to GenBank under accession number U09421. ▼, Asparagine residues corresponding to potential *N*-linked glycosylation sites; potential phosphorylation sites for cAMP/cGMP-dependent kinase (○) and protein kinase C (★) are also noted.

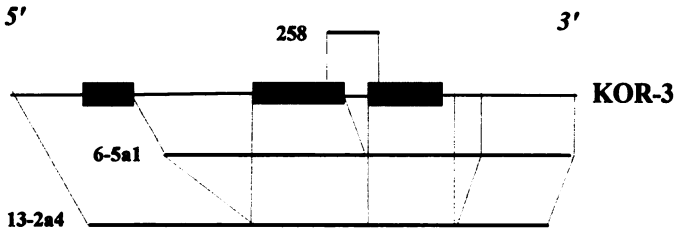

Fig. 3. Schematic diagram of different KOR-3 clones. The first clone sequenced, 13–2a4, is a full length clone; 258 is a small segment containing an intron of 81 bases between amino acids 193 and 194, and 6–5a1 corresponds to an additional clone that contains an intron of 2.1 kb between amino acids 75 and 76. We assume that this intron is truncated, because we have not obtained 5′ sequence corresponding to the coding region from 13–2a4. The 6–5a1 clone also contains a 117-base insert in the 3′ untranslated portion 35 bases downstream from the stop codon. With the exception of this insert, the 3′ untranslated region of 6–5a1 is identical to that of 13–2a4, as is the open reading frame.

second extracellular loop, similar to that reported for hORL-1 (33) and XOR-L (which has a 28-amino acid insertion at the same position) (34), whereas the other (6–5a1) has a 2115-bp truncated intron within the sequence of the first transmembrane domain and an insert of 117 bp in the 3′ untranslated region 35 bp downstream from the stop codon.

Northern and Southern analyses. Northern analysis (Fig. 4A) revealed the selective expression of this clone in the brain. The brain contains mRNA recognized by the probe with a size of approximately 3.4 kb. No evidence of mRNA corresponding to KOR-3a was observed in samples from spleen, muscle, or kidney.

Southern analysis (Fig. 4B) shows that genomic DNA restricted with *Apa*I, *Eco*RV, *Sst*I, *Eco*RI, and *Xba*I yielded a single band, strongly suggesting a single copy of the gene encoding KOR-3. Although there is a *Hind*III site in the 13–2a4 cDNA, it is not included within the probe. Thus, the two bands detected in the *Hind*III and *Bam*HI lanes probably reflect the presence of these sites within introns.

Expression of KOR-3. We have inserted the clone into several mammalian expression vectors and have used transient and stable expression systems to explore the pharmacology of the clone. Binding in transient tranfections was highly variable, making it difficult to assess. Traditional radioligands such as the antagonist [^{3}H]diprenorphine or agonists like [^{3}H]DAMGO did not demonstrate significant reproducible binding. Although we occasionally saw some [^{3}H]NalBzoH binding, it was not sufficiently reproducible for determination of accurate K_d and B_{max} values, along with detailed binding selectivities. Our inability to obtain consistent reproducible binding cannot be attributed to problems with the vector or transfection efficiency, because expression studies performed using the same vectors containing the MOR-1 clone (a gift from Dr. Lei Yu, University of Indiana) as a positive control showed typical μ binding, as already reported (4, 12). Expressing 13–2a4 in a variety of other cell lines and/or using additional vectors produced similar results. Truncating or removing the 5′ and/or 3′ untranslated portions of the clone also did not improve binding.

Both nalorphine and cyclazocine inhibited forskolin-stimulated adenylyl cyclase in transiently transfected COS-7 cells by >80%, with IC_{50} values of approximately 100 nM (data not shown). However, the variability in the sensitivity of different batches of transfected cells made the interpretation of these studies difficult. Modifying the vector and/or changing cell lines did not improve the reliability or reproducibility of these observations. We then examined the ability of opioids to inhibit basal cAMP accumulation in membranes from stably transfected cells (Fig. 5). To enhance the sensitivity of the response, we treated the cell membranes at low pH (26) to decrease stimulatory activity. Cyclazocine was inactive in COS-7 cells stably transfected with the vector lacking the insert (pSRα) or in COS-7 cells alone, regardless of the dose (Fig. 5A). In cells stably expressing the clone (13–2a4/pSRα),

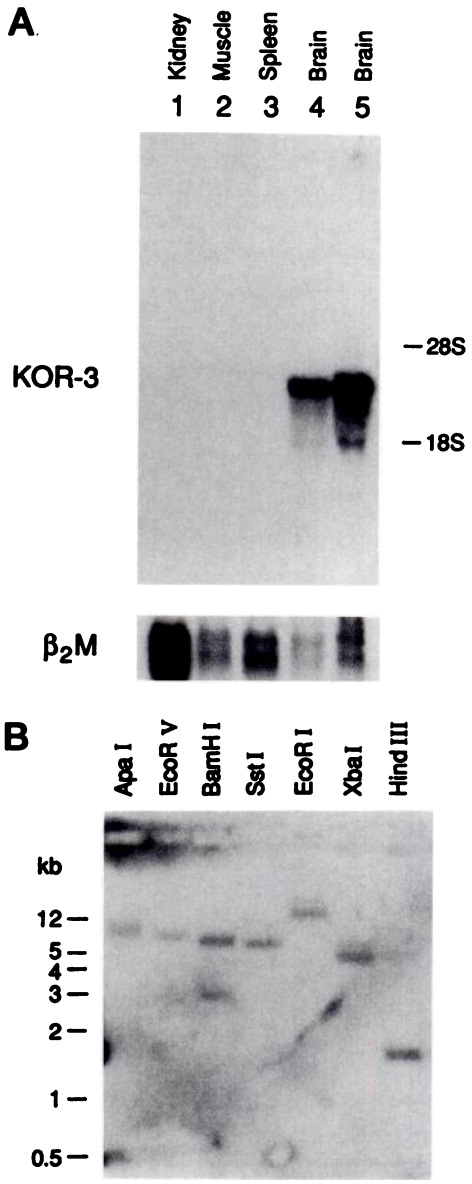

Fig. 4. Northern and Southern analyses of mouse KOR-3. A, Poly(A)$^+$ RNA was isolated from different mouse tissues as described in Materials and Methods [*lanes 1-4*, 15 μg of poly(A)$^+$ RNA; *lane 5*, 30 μg]. After hybridization with ^{32}P-labeled KOR-3a probe and exposure to film, the membrane was probed with a ^{32}P-labeled β_2-microglobulin (β_2M) PCR fragment to estimate the RNA loading. This is a representative experiment. It has been replicated four times, including several replications using different probes. B, Each lane was loaded with 15 μg of genomic DNA restricted with the indicated enzyme, as described in Materials and Methods. This is a representative experiment. It has been replicated three times.

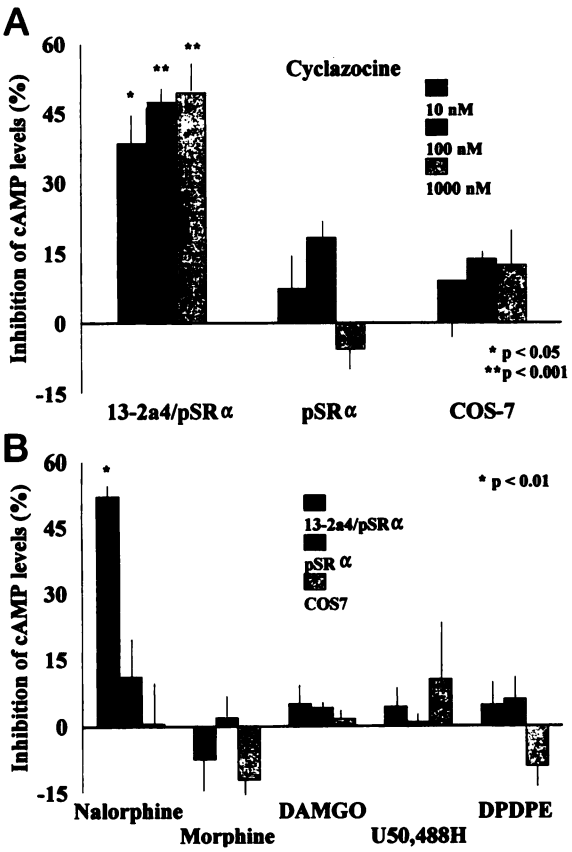

Fig. 5. Inhibition of basal cAMP accumulation by cyclazocine in stably transfected COS-7 membranes. A, Membranes were obtained from COS-7 cells, COS-7 cells stably transfected with the pSRα vector alone, or COS-7 cells stably transfected with 13–2a4 in the pSRα vector. cAMP levels were determined as described in Materials and Methods, after treatment of cells with the indicated concentration of cyclazocine. Results are the means ± standard errors of four independent experiments. Statistical signficance was determined using Students *t* test, by comparing 13–2a4/pSRα and pSRα values (i.e., the vector containing the insert and the vector alone). B, Membranes were obtained from COS-7 cells, COS-7 cells stably transfected with the pSRα vector alone, or COS-7 cells stably transfected with 13–2a4 in the pSRα vector. cAMP levels were determined as described in Materials and Methods, after treatment of cells with the indicated concentration of opioid. Results are the means ± standard errors of independent replications for COS-7 cells transfected with 13–2a4/pSRα (morphine, five experiments; DAMGO, three experiments; DPDPE, four experiments; U50,488H, four experiments), COS-7 cells transfected pSRα (morphine, four experiments; DAMGO, three experiments; DPDPE, three experiments; U50,488H, three experiments), and COS-7 cells (morphine, four experiments; DAMGO, two experiments; DPDPE, four experiments; U50,488H, three experiments). Statistical signficance was determined using Students *t* test, by comparing 13–2a4/pSRα and pSRα values (i.e., the vector containing the insert and the vector alone).

cyclazocine inhibited cAMP accumulation in a dose-dependent manner. At only 10 nM, cyclazocine inhibited cAMP accumulation by almost 40%. Nalorphine at only 10 nM lowered cAMP levels by 50% in cells expressing the clone (13–2a4/pSRα), but not in cells transfected with the vector alone (pSRα) or in COS-7 cells (Fig. 5B). Traditional opiates acting at μ (morphine and DAMGO), κ_1 (U50,488H), and δ (DPDPE) receptors were inactive at 1 μM. This pharmacological profile clearly distinguishes this clone from the previously reported MOR-1, DOR-1, and KOR-1 clones.

Western analysis of KOR-3 expression. We next examined the expression of 13–2a4 using a mAb, 8D8, generated against κ_3 receptors natively expressed in the human neuroblastoma cell line BE(2)-C.[1] mAb 8D8 recognizes a diffuse band of approximately 70–80 kDa in Western analysis of BE(2)-C cell and brain membranes and inhibits κ_3 receptor binding in a variety of species, including mouse, rat, human, calf, and sheep. mAb 8D8 does not inhibit μ-, δ-, or κ_1-opioid receptor binding in brain tissue. Western analysis of COS-7 cells transfected with 13–2a4 revealed a diffuse band of 70–80 kDa, very similar to that seen in brain and BE(2)-C membranes (Fig. 6). Controls, including COS-7 cells alone (data not shown) and COS-7 cells transfected with the vector lacking the 13–2a4 insert, were negative.

To ensure that the protein recognized by mAb 8D8 reflected the clone itself and did not result from the activation of other genes by the clone, we examined *in vitro* expression (Fig. 7). *In vitro* expression of the clone with [^{35}S]methionine yielded a band on SDS-PAGE of approximately 36 kDa, slightly smaller than its predicted molecular mass. Addition of microsomal membranes resulted in a larger band (approximately 50 kDa), consistent with glycosylation. However, this larger band is still smaller than that seen in brain or BE(2)-C cells, probably reflecting the limited glycosylation possible in this system. Most importantly, the protein band seen with *in vitro* expression was recognized by the κ_3-specific antibody in Western analysis.

Functional characterization of KOR-3 by antisense mapping. Having demonstrated the selectivity of one antisense oligodeoxynucleotide *in vivo*, we proceeded to map the KOR-3 clones using a series of antisense oligodeoxynucleotides directed at various regions of the clones (Fig. 8). We initially chose six sites downstream from the splice site in the first transmembrane region. All six oligodeoxynucleotides, including two with sequences based upon the 3′ untranslated region of 13–2a4, dramatically lowered κ_3 analgesia in mice ($p < 0.0001$). As noted above, the 3′ untranslated region of 6–5a1 contains a 117-bp insertion. An antisense oligodeoxynucleotide based upon this insertion (Table 1, oligodeoxynucleotide M) did not lower κ_3 analgesia. We then designed antisense oligodeoxynucleotides against six regions of

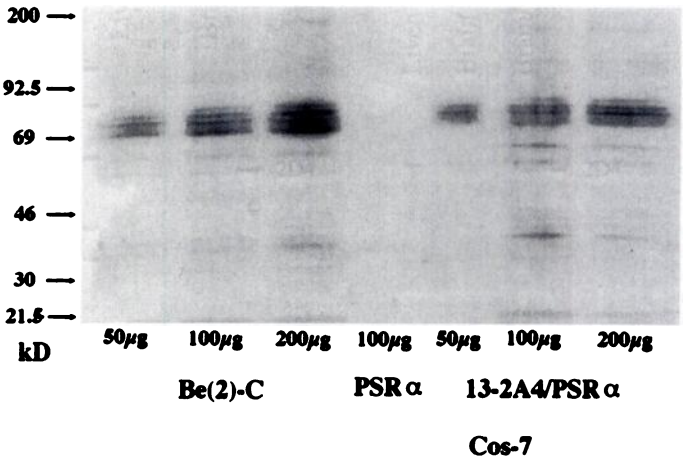

Fig. 6. Western analysis of KOR-3a expression. Membranes were obtained from BE(2)-C cells and COS-7 cells transfected with either the pSRα vector alone or vector with the 13–2a4 insert. The stated amount of membrane protein was loaded in each lane and separated by SDS-PAGE, followed by Western analysis using mAb 8D8, as described in Materials and Methods. This is a representative experiment, which has been replicated three times.

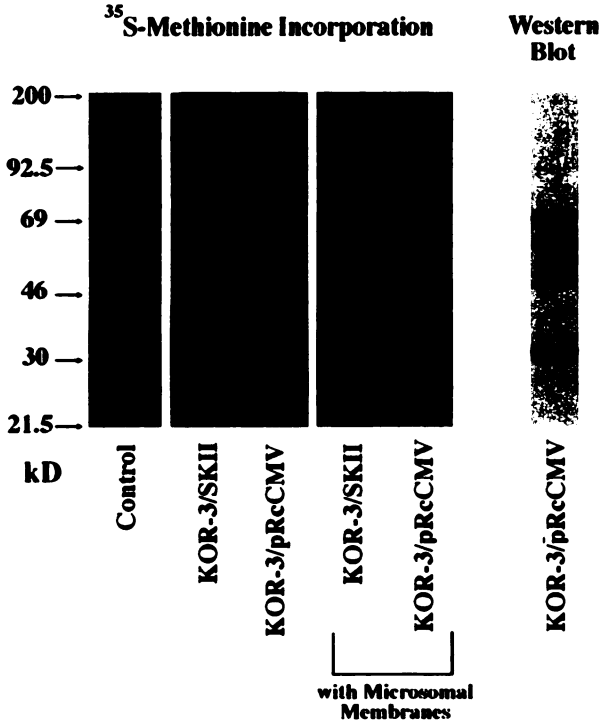

Fig. 7. *In vitro* translation of KOR-3a. *In vitro* translation was performed as described in Materials and Methods, with [^{35}S]methionine, using the stated expression vector. Similar samples were incubated with microsomal membranes to permit glycosylation. Additional *in vitro* translation was performed using unlabeled methionine. This product was run on SDS-PAGE, followed by Western analysis using mAb 8D8. This experiment has been replicated twice.

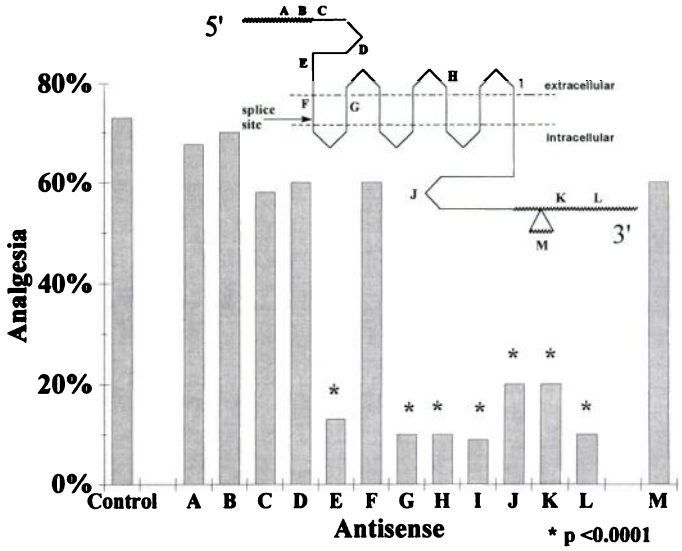

Fig. 8. Antisense mapping of KOR-3 blockade of κ_3 analgesia. Groups of mice were administered saline (control) or the indicated antisense oligodeoxynucleotide, as described in Materials and Methods. Analgesia was assessed 30 min after NalBzoH (50 mg/kg, subcutaneously). All experiments were performed blinded to the treatment condition, with groups of at least 10 mice/group. Controls were included with every antisense group and every replication. All treatments had at least two independent replications with at least 10 mice/experiment. The total number of animals tested with each treatment is as follows: control, n = 350; A, n = 40; B, n = 20; C, n = 30; D, n = 20; E, n = 30; F, n = 20; G, n = 20; H, n = 20; I, n = 70; J, n = 20; K, n = 20; L, n = 20; M, n = 20. *, Significantly different from controls ($p < 0.0001$), as determined by Fisher's exact test.

13–2a4 upstream from the splice site in the first transmembrane domain, including two based upon the 5′ untranslated region. Only one of these six oligodeoxynucleotides antagonized κ_3 analgesia. This difference between the two regions is quite dramatic and cannot be explained by differences in T_m values, base composition (Table 1), or predictable secondary structures.

Discussion

The first suggestion of multiple opioid receptors came from studies examining the interactions of morphine and nalorphine (42, 43). Martin (22) proposed distinct receptors for morphine (M receptors) and nalorphine (N receptors) and suggested that nalorphine was an antagonist at M receptors and an agonist at N receptors. The development of NalBzoH (25) led to the identification of a novel receptor, κ_3 (17–21), which corresponds pharmacologically to the nalorphine receptor of Martin (22).

The recent cloning of the δ receptor (2, 3) quickly led to the cloning of μ and κ_1 receptors (4–12), as well as cloning of another, highly homologous receptor from mouse, rat, and human brain (33–41). Our clone shows up to 95% identity, at the amino acid level, with the rat and human clones. Like the human clone ORL-1 (33) and the rat clone XOR (34), we have evidence for an insertion in the second extracellular loop of the mouse clone. However, this insertion may be an intron, because the sequence of the mouse clone suggests that it cannot be translated, due to the presence of a termination codon within the insertion. We also have identified a second intron in the first transmembrane region. In additional studies, we have isolated the gene for KOR-3 from a mouse genomic library and confirmed the presence of both introns.[2] *In situ* hybridization of KOR-3 in mouse brain (data not shown) gives a distribution similar to that reported in rats (39), providing further correlation between the mouse and rat clones. However, there are differences. Unlike with the rat clone, we did not observe KOR-3 mRNA in Northern analyses of several peripheral tissues (Fig. 4A). Furthermore, Northern analysis, by several different groups, of the rat clone in brain yields three bands. The lowest, 3.3–3.4 kb, is relatively constant among the reports, but the middle band ranges from 7.5 to 13 kb and the highest from 10 to 23 kb. The reasons for these differences are not clear. In mice, we observe a single band at 3.4 kb. The absence of the higher mRNA bands may reflect species differences.

The >50% homology of KOR-3 to μ (MOR-1), δ (DOR-1), and κ_1 (KOR-1) clones strongly emphasizes its association with opioid receptors. However, further classification has proven difficult, leading many to consider it an orphan member of the opioid receptor family. No group has been able to demonstrate reproducible binding, regardless of whether the clone is expressed transiently or stably. Only one other group has observed inhibition of adenylyl cyclase by an opioid (33). Our observation that antisense oligodeoxynucleotides against KOR-3 selectively blocked κ_3 analgesia strongly suggested an association between KOR-3 and κ_3 receptors (36, 37). Our current studies address the classification of KOR-3.

Two independent experimental approaches support the association of KOR-3 with κ_3-opioid receptors. The recognition

[2] Y.-X. Pan, J. Xu, and G. W. Pasternak. Manuscript in preparation.

of the expressed KOR-3 clone by mAb 8D8 is an important observation. mAb 8D8 was generated from a human neuroblastoma cell line [BE(2)-C] that expresses κ_3 receptors. It selectively blocks κ_3 binding in the BE(2)-C line and blocks the κ_3, but not μ, responses in adenylate cyclase assays. It inhibits κ_3 binding to brain membranes in several species, including mouse, and, when given intracerebroventricularly, mAb 8D8 antagonizes NalBzoH analgesia in mice without affecting morphine actions.[1] Its ability to recognize the KOR-3 clone in Western analysis after expression in COS-7 cells provides important evidence correlating KOR-3 with κ_3 receptors. These observations were strengthened even further by the *in vitro* translation studies. The ability of mAb 8D8 to recognize the product from the 13–2a4/pSRα cDNA ensured that the antibody was labeling the expressed receptor and not another protein induced by the transfection and/or expression of the clone. Furthermore, the expressed protein can be glycosylated, explaining the greater size of the protein expressed in COS-7 cells, compared with the predicted molecular mass. However, the degree of glycosylation obtained by adding canine pancreatic microsomal membranes is not as extensive as that seen in the COS-7 cells. These data support the association of KOR-3 with κ_3 receptors.

Functional expression of the clone has proven difficult. Like the other groups, we have encountered numerous difficulties in establishing a reliable binding assay. Initially, we were concerned about the level of expression after transfection, but the Western analysis with mAb 8D8 confirms that the protein is being expressed. Functional assays such as the inhibition of cAMP accumulation are often more sensitive than binding assays, leading us to explore this area in greater depth. Early studies with transiently expressed KOR-3 revealed that opioids such as cyclazocine and nalorphine could inhibit forskolin-stimulated cyclase activity in cells by >80%, with IC_{50} values of approximately 100 nM (data not shown). However, studies with transiently expressed KOR-3 were variable, making it difficult to define the pharmacology of the effect. We then went to COS-7 cells stably expressing KOR-3 and used a method previously noted to enhance the sensitivity of opioid inhibition of cAMP accumulation (26). Again, we observed a potent, dose-dependent inhibition of cAMP accumulation by cyclazocine. COS-7 cells alone or COS-7 cells transfected with the vector alone (pSRα) were not sensitive. Nalorphine, another κ_3 agent, at only 10 nM potently inhibited cAMP accumulation by 50%. NalBzoH also had activity in this assay (data not shown). The inactivity of morphine and DAMGO, two μ agonists, as well as the κ_1 drug U50,488H and the δ-selective compound DPDPE, further defines the pharmacological selectivity of this response and distinguishes KOR-3 from the previously reported clones MOR-1, DOR-1, and KOR-1, which all respond to their selective ligands in cyclase assays. Preliminary studies also indicate that naloxone has agonist activity in these assays (data not shown).

The strongest evidence correlating KOR-3 with κ_3 receptors comes from the antisense studies. Southern analysis indicates a single gene. This is consistent with the localization of the homologue Oprl to a single location on chromosome 2 (40). Oligodeoxynucleotide antisense probes >15 bases long should be specific for a single mRNA. With all of the available evidence indicating a single gene, the ability of seven different oligodeoxynucleotide antisense probes directed at various regions of the 13–2a4 cDNA to block κ_3 analgesia overwhelmingly argues that KOR-3 is derived from the same gene as is the κ_3 receptor. However, this does not prove that KOR-3 encodes the κ_3 receptor. Indeed, some of the data suggest that KOR-3 is a splice variant of the gene that encodes κ_3 receptors.

The ability of the antisense oligodeoxynucleotide probes to block analgesia is dependent upon their relationship to the first intron located in the first transmembrane region. Downstream from this splice site, all antisense oligodeoxynucleotides based upon 13–2a4, including those in the 3′ untranslated region, down-regulate κ_3 analgesia. Only one of the six different antisense oligodeoxynucleotides upstream from this site blocks κ_3 analgesia. It is unlikely that this difference can be explained by technical issues associated with the antisense technique. Studies with DOR-1 indicated that antisense oligodeoxynucleotides directed along the entire cDNA effectively down-regulated δ binding to the same degree in NG108–15 cells. Antisense oligodeoxynucleotides directed against the 5′ untranslated portion of the μ receptor (14) also potently blocked morphine analgesia *in vivo*. Indeed, it has been suggested that the most effective antisense probes are directed toward the amino terminus of the receptor (44). Thus, the location on the clone toward which the antisense oligodeoxynucleotide is directed is unlikely to explain the differences in action of the antisense oligodeoxynucleotides upstream and downstream from the splice site. Likewise, the structure of the various antisense oligodeoxynucleotides is unlikely to explain these differences. They all have similar base compositions and no identifiable secondary structures that might impair their ability to complex with the mRNA. Although secondary structure of the 5′ mRNA cannot be ruled out as a potential explanation for the differences, we have been unable to detect any predictable secondary structures upon analysis of the mRNA sequence. Furthermore, oligodeoxynucleotide antisense probes directed toward the 5′ region of the MOR-1 and DOR-1 clones successfully down-regulated μ and δ receptors, respectively. Thus, our results raise the possibility that 13–2a4 is an alternative splice variant from the gene encoding the κ_3 receptor. This would explain the sharp demarcation in antisense activity upstream and downstream from the splice site in the first transmembrane domain. We also might expect alternative first exons to share some sequence homology, consistent with the down-regulation of κ_3 analgesia by antisense oligodeoxynucleotide E, which lies upstream from the splice site.

The antisense studies looking at the 3′ untranslated regions are also interesting. Antisense oligodeoxynucleotides K and L designed from 13–2a4 are both effective. However, antisense oligodeoxynucleotide M, directed against a portion of the insert in the 3′ untranslated region from 6–5a1 that is not present in 13–2a4 (Fig. 3), is inactive, despite being closer to the open reading frame. This suggests that only mature mRNA is down-regulated by antisense oligodeoxynucleotides.

In conclusion, both the mAb and antisense studies strongly associate the KOR-3 clone with κ_3 receptors. The antisense work strongly implies that KOR-3 is derived from the same gene as κ_3 receptors, raising the possiblity of alternative splicing. If KOR-3 is a splice variant of the κ_3 receptor, it might have a very different binding profile. However, other

explanations remain, including the need for post-translational modifications of the protein or associated proteins not present in transfected cells.

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