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Antisense mapping the MOR-1 opioid receptor: evidence for alternative splicing and a novel morphine-6β-glucuronide receptor

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Abstract Although MOR-1 encodes a mu opioid receptor, its relationship to the pharmacologically defined mu receptor subtypes has been unclear. Antisense mapping now suggests that these subtypes result from alternative splicing of MOR-1. Three oligodeoxynucleotide probes targeting exon 1 and another oligodeoxynucleotide directed against the coding region of exon 4 block supraspinal morphine analgesia, a mu_1 action, while five of six oligodeoxynucleotides directed against exons 2 and 3 are inactive. Inhibition of gastrointestinal transit and spinal morphine analgesia, two mu_2 actions, are blocked only by the probe against exon 4 and not by those directed against exon 1. In contrast, the analgesic actions of the extraordinarily potent mu drug morphine-6β-glucuronide are blocked by six different antisense oligodeoxynucleotides targeting exons 2 and 3, but not by those acting on exons 1 or 4. These results suggest that the mu_1 and mu_2 receptor subtypes originally defined in binding and pharmacological studies result from alternative splicing of MOR-1 while morphine-6β-glucuronide acts through a novel, previously unidentified receptor which is yet another MOR-1 splice variant.

Key words: Antisense; Morphine; Mu receptor; Mu_1 receptor; Mu_2 receptor; Morphine-6β-glucuronide; Analgesia; Gastrointestinal transit; Opioid receptor

1. Introduction

Morphine acts through mu receptors to elicit a variety of pharmacological actions, including analgesia, respiratory depression and the inhibition of gastrointestinal transit (for review, see [1]). Evidence from a variety of approaches has suggested that these actions are mediated through distinct mu (morphine) receptor subtypes initially proposed from traditional binding studies [2–5] and detailed computer modeling approaches [6–9]. Mu_1 actions, defined in large part by their sensitivity towards naloxonazine [10], include supraspinal morphine analgesia, while mu_2 receptors mediate spinal morphine analgesia, respiratory depression, the inhibition of gastrointestinal transit and most signs associated with morphine dependence [1]. However, the actions of another mu drug, morphine-6β-glucuronide [11,12], remain unclear. Binding experiments reveal that its affinity for mu receptors is slightly less than morphine while antagonist studies confirm that it acts through mu receptors [11,12]. Yet morphine-6β-glucuronide is at least 100-fold more potent than morphine in vivo, raising the possibility of a yet another mu receptor subtype.

Only one mu receptor, MOR-1, has been cloned [13–22]. When expressed, it shows the anticipated selectivity in both binding and functional assays for mu, as opposed to delta or kappa, ligands. Although a splice variant involving the terminal portion of the COOH terminus has been reported in both human and rat clones [23,24], these two variants do not explain the distinct mu subtypes inferred from the pharmacological studies [2–7,9].

Initial antisense approaches [15] establishing the pharmacological significance of the recently cloned mu (MOR-1) [25], $kappa_1$ (KOR-1) [26] and delta (DOR-1) receptors [27,28] were quickly confirmed [29–33]. Antisense oligodeoxynucleotides against DOR-1 downregulate mRNA and protein levels in both NG108–15 cells and intrathecally in mice [28], as predicted. Antisense probes are highly selective and specific. Downregulating one opioid receptor does not interfere with the activity of other subtypes. Indeed, changing the sequence of only four bases renders the oligodeoxynucleotide inactive. Equally important, the antisense probes are effective regardless of which region along the mRNA is targeted [27]. Morphine analgesia is blocked by an antisense oligodeoxynucleotide directed against the 5′-untranslated region of MOR-1 [25], while an antisense probe active against $kappa_1$ analgesia targeted the middle of the coding region of KOR-1 [26]. Five different antisense oligodeoxynucleotides directed against different regions of the DOR-1 mRNA lower [^{3}H]DPDPE binding in NG108-15 cells [27] and block intrathecal DPDPE analgesia equally well (G.R. Rossi and G.W. Pasternak, unpublished observations). The ability to effectively target any region of a cDNA with antisense oligodeoxynucleotides provides an opportunity to explore the functional relevance of partial cDNA sequences without cloning a full length cDNA, as illustrated by our recent cloning of KOR-3 [15,34,35], a fourth member of the opioid receptor family [15,36–43]. It also opens the possibility of using antisense to assess the presence of specific exons in the active protein, as we recently demonstrated with KOR-3 [34,35]. Thus, antisense mapping provides the opportunity to explore the possibility of functionally important splice variants which have not yet been identified from cloning studies. We now present evidence suggesting the presence of pharmacologically relevant splice variants of MOR-1 corresponding to mu_1 and mu_2 receptors as well as a novel receptor responsible for the actions of morphine-6β-glucuronide.

2. Materials and methods

2.1. Materials

Male CD-1 mice (25–35 g; Charles River Breeding Laboratory, Bloomington, MA) were used in all studies. Animals were housed five per cage, maintained on a 12-h light/dark cycle and given ad libitum access to food and water. Morphine sulfate and morphine-6β-glucu-

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ronide were obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). Oligodeoxynucleotides were synthesized by The Midland Certified Reagent Co. (Midland, TX) or by the core facility at MSKCC. DNA modification and restriction enzymes were from Gibco (Gaithersberg, MD), Stratagene (LaJolla, CA) or New England Biolabs (Beverly, MA). DNA sequencing kits were from U.S. Biochemical Corp. (Cleveland, OH). Nylon membranes were purchased from Micron Separation Inc. (Boston, MA). GeneScreen Plus and all radiochemicals were purchased from DuPont-NEN (Boston, MA) unless stated otherwise. All other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. Halothane was purchased from Halocarbon Laboratory (Hackensack, NJ).

2.2. Cloning the mouse MOR-1

Cloned cDNA encoding the mouse homolog of MOR-1 was isolated from a 5′-RACE-ready cDNA (Clontech) by PCR using 5′ anchor sense primer and 3′ antisense primer (5′ CTC TAG ACT CCA GGA ATG ACT TTG AAT GCA GGA 3′) designed to the rat MOR-1 sequence (GenBank accession #L13069) at positions 1518–1537. The PCR fragment was subcloned into *Eco*RI/*Xba*I sites of Bluescript SK and sequenced. Comparison of the sequence (GenBank Accession # U26915) to the published mouse MOR-1 sequence [22] and an unpublished mouse MOR-1 sequence (Drs. D. Kaufman and C. Evans, unpublished observations) shows 99.9 and 99.2% identity, respectively. All the antisense oligodeoxynucleotides are complimentary to cDNA sequences identical among all three sequences, with the exception of Antisense K which is identical to that of Drs. Kaufman and Evans but differs from that of Loh and colleagues [22] by a single base difference at position 1566 (T/G).

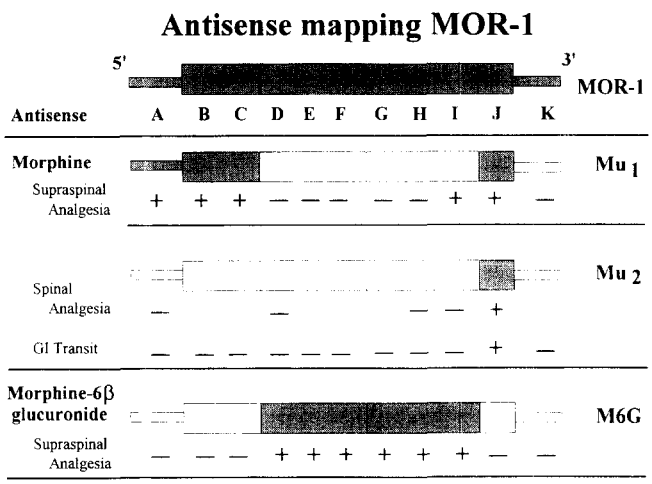

Fig. 1. Antisense mapping MOR-1. The four exons of MOR-1 are presented schematically. The various assays are given on the left, while the putative receptor classification is on the right. Activity of various oligodeoxynucleotides in each of the four assays are summarized with + representing a statistically significant blockade of activity and − representing no significant change. Exons corresponding to MOR-1 are shaded, while those probably reflecting alternatively spliced exons are not.

Table 1
Effects of antisense treatment on supraspinal morphine analgesia

		Analgesic response	*n*	*P*	
Control		74%	105		
Mismatch		60%	15		0.195
Antisense					
Exon 1	A	**26%**	70	**<0.0001**	
	B	**40%**	20	**0.008**	
	C	**25%**	20	**0.0001**	
Exon 2	D	75%	20		0.497
	E	74%	19		0.548
	F	72%	25		0.590
Exon 3	G	65%	20		0.368
	H	80%	45		0.197
	I	**35%**	20	**0.001**	
Exon 4	J	**20%**	20	**<0.0001**	
	K	80%	20		0.410

The antisense oligodeoxynucleotides were based upon our sequence of the mouse homolog of MOR-1. Their calculated T_m values and positions based upon our cDNA sequence (GenBank Accession # U26915) are as follows: A: CGC CCC AGC CTC TTC CTC T, 60.1°C, 195–213; B:GTT GAG CCA GGA GCC AGG T, 56.2°C, 357–375; C: GCC CAC TAC ACA CAC GAT AGA, 52.2°C, 508–528; D: TTG GTG GCA GTC TTC ATT TTG G, 58.4°C, 572–593; E: GCC TGT ATT TTG TGG TTG CCA T, 57.4°C, 895–916; F: GGA CCC CTG CCT GTA TTT TGT G, 59.1°C, 904–924; G: TGA GCA GGT TCT CCC AGT ACC A, 58.2°C, 959–979; H: CCA CCA GCA CCA TCC GGG, 61.5°C, 1118–1135; I: CAC TGT ATT AGC CGT GGA GGG, 55.8°C, 1402–1422; J: GGG CAA TGG AGC AGT TTC TG, 56.7°C, 1457–1476; K: CAG GAA ACC AGA GCC TCC CA, 58.5°C, 1558–1577. The mismatch oligodeoxynucleotide, based upon antisense A, had the following sequence with the switched bases underlined: CGC CCC GAC CTC TTC CCT T. Based upon the gene structure of mouse MOR-1 [22], the location of the antisense probes is as follows: Exon 1: A, B and C; Exon 2: D, E and F; Exon 3: G, H and I; Exon 4: J and K. Note that F spans the junction between exons 2 and 3. Since 17 bases are in exon 2 and only 5 are in exon 3, we assume that its activity is predominantly against exon 2. Groups of mice received the indicated antisense oligodeoxynucleotide on days 1, 3 and 5 as described in Section 2. Analgesia was assessed on day 6 with morphine (0.7 μg, i.c.v.). Significance in the analgesic assays was assessed using the Fisher exact test. Statistically significant results are bolded.

2.3. Antisense treatments

Groups of mice received the stated antisense (5 μg) or mismatch (5 μg) oligodeoxynucleotide or vehicle alone (0.9% saline) intracerebroventricularly (i.c.v.) or intrathecally (i.t.) under light halothane anesthesia on days 1, 3 and 5 as previously described (9–11). Analgesia was tested on day 6 in the tailflick assay 15 min after the injection of either morphine (0.7 μg, i.c.v. or 0.8 μg, i.t.) or morphine-6β-glucuronide (20 ng, i.c.v.).

Analgesia was determined quantally in the radient heat tailflick assay as a doubling or greater of baseline tailflick latencies, as previously described [4,11,12,25–27,34,35]. A maximal latency of 10 sec was used to minimize any tissue damage. Gastrointestinal (GI) transit [11] was determined by comparing the distance traveled by a charcoal meal in morphine-treated (0.7 μg, i.c.v.) mice compared to mice not receiving morphine. The control group received only vehicle without any oligodeoxynucleotide before receiving morphine.

3. Results

In the current study we have mapped MOR-1 using antisense oligodeoxynucleotides directed against 11 regions of the mouse MOR-1 mRNA which target all four exons (Table 1; Fig. 1). First, we cloned the mouse version of MOR-1 from a 5′-RACE ready cDNA. Using our sequence, we designed a series of antisense oligodeoxynucleotides targeting all four exons of MOR-1 and examined these probes against analgesia and gastrointestinal transit.

We examined the effects of antisense treatments on supraspinal morphine analgesia, a mu_1 action (Table 1). A mismatch oligodeoxynucleotide based upon the antisense A has no significant effect when compared to saline controls. In contrast, Antisense A, targeting the 5′-untranslated region of MOR-1, dramatically blocks morphine analgesia, confirming prior reports in the rat [25]. Antisenses B and C, which are both directed against the open reading frame of the first exon (Fig. 1).

Table 2
Reversal of the inhibition of gastrointestinal transit by morphine by antisense

		Inhibition of GI transit	n	P	
Control		59.6 ± 1.3%	65		
Antisense					
Exon 1	A	54.8 ± 1.2%	50		NS
	B	46.3 ± 2.7%	10		NS
	C	46.8 ± 2.9%	10		NS
Exon 2	D	33.6 ± 3.0%	10		NS
	E	53.1 ± 3.1%	8		NS
	F	65.1 ± 1.8%	20		NS
Exon 3	G	46.0 ± 2.3%	9		NS
	H	46.1 ± 1.7%	27		NS
	I	48.2 ± 2.7%	10		NS
Exon 4	J	**4.5 ± 3.7%**	10	**<0.05**	
	K	66.5 ± 1.4%	20		NS

Groups of mice were treated intracerebroventricularly with the indicated antisense oligodeoxynucleotide on days 1, 3 and 5 and the effects of morphine (0.7 μg, i.c.v.) on gastrointestinal transit determined on day 6, as described in Section 2. The transit distance in mice not receiving morphine was 29.4 ± 1.01 cm (n = 56) and the transit distance in the morphine alone treated mice was 11.9 ± 0.84 cm. The sequences of the oligodeoxynucleotides and their positions are provided in Table 1. ANOVA revealed significant differences among groups for the inhibition of GI transit and Sheffe's posthoc test revealed that only Antisense J was different.

also effectively lower morphine analgesia. However, none of the antisense oligodeoxynucleotides based upon exon 2 are active. Of the three directed against exon 3, only Antisense I, located close to the junction with exon 4, lowers morphine analgesia. Antisense J, directed against the coding region of exon 4, potently blocks analgesia, but Antisense K from the 3′-untranslated region does not. However, the significance of the inactivity of Antisense K is not clear since it is inactive against all actions examined (see below).

We next examined two morphine actions mediated through mu_2 receptors. Supraspinally, morphine inhibits gastrointestinal transit through mu_2 receptors [1,44]. Only Antisense J, which targets exon 4, is effective against the inhibition of gastrointestinal transit (Table 2). Despite their potent blockade of supraspinal morphine analgesia, none of the probes directed against exon 1 block the inhibition of gastrointestinal transit. We see similar results with spinal morphine analgesia, another mu_2 action (Table 3). Again, Antisense J potently blocks spinal analgesia, while all the other antisense oligodeoxynucleotides tested are inactive. Thus, the antisense activity profile for both mu_2 actions is the same and distinct from supraspinal mu_1 analgesia.

Morphine-6β-glucuronide (M6G) is an exceedingly potent morphine metabolite which also acts through mu receptors [11,12]. However, questions have arisen regarding its actions. In vivo, morphine-6β-glucuronide is over 100-fold more potent an analgesic than morphine despite a lower affinity for mu receptors in binding assays [11,12] and in cyclase studies in neuroblastoma cell lines (K.M. Standifer, J. Ryan-Moro and G.W. Pasternak, unpublished observations). Thus, the far greater potency of morphine-6β-glucuronide in vivo compared to morphine does not correspond to either increased binding affinity or intrinsic activity, raising the possibility that it might be acting through a novel, previously unrecognized mu receptor subtype. Antisense mapping yields a very different picture for morphine-6β-glucuronide analgesia (Table 4). The probes targeting exons 1 and 4 which potently block morphine analgesia are without effect on morphine-6β-glucuronide analgesia. Conversely, antisense probes directed against exons 2 and 3 all effectively block morphine-6β-glucuronide actions.

4. Discussion

Antisense mapping can provide important insights in the correlation of the molecular biology and pharmacology of opioid receptors. Antisense mapping of DOR-1 in NG108-15 cells and intrathecally in mice reveals similar activities of all the antisense oligodeoxynucleotides regardless of which exon is targeted [27], consistent with the suggestion that virtually all regions of mRNA are accessible to antisense oligodeoxynucleotides [45]. Thus, the inactivity of antisense probes directed at a specific exon raises the possibility of an alternative exon, as illustrated by our prior studies with KOR-3 [34,35]. However, inactivity of an antisense oligodeoxynucleotide might result from a variety of technical factors, such as unanticipated secondary mRNA structures. This concern is eliminated when the antisense oligodeoxynucleotide is active in at least one assay. All the antisense probes used in the current study were active in at least one assay, with the exception of Antisense K which targets the 3′-untranslated region. Its inactivity in all assays makes its actions impossible to interpret. However, the activity of all the other probes in at least one assay confirm that almost the entire length of the MOR-1 mRNA is accessible to the antisense oligodeoxynucleotides and that technical factors cannot be responsible for the differences in activity among the various antisense probes.

The selectivity profiles of the antisense probes are not consistent with a single receptor and imply alternative splicing of the MOR-1 gene (Fig. 1). Pharmacological studies indicate that mu_1 receptors produce supraspinal morphine analgesia while mu_2 receptors mediate spinal morphine analgesia and the inhibition of gastrointestinal transit [1]. The antisense studies reported here are consistent with two mu receptor subtypes. Both mu subtypes appear to contain exon 4 which blocks all three morphine actions examined, but the activity of Antisenses A, B and C against only supraspinal analgesia suggests that exon

Table 3
Effect of antisense treatment on spinal morphine analgesia

		Analgesic response	n	P	
Control		69%	50		
Mismatch		53%	15		0.229
Antisense					
Exon 1	A	70%	20		0.555
Exon 2	D	50%	20		0.129
Exon 3	H	75%	20		0.393
	I	56%	25		0.221
Exon 4	J	**36%**	25	**0.008**	

Groups of mice received the indicated antisense oligodeoxynucleotide intrathecally on days 1, 3 and 5 and were tested for morphine analgesia on day 6 (0.8 μg, i.t.), as described in Section 2. The sequences of the oligodeoxynucleotides and their positions are provided in Table 1. Significance in the analgesic assays was assessed using the Fisher exact test. Statistically significant results are bolded.

Table 4
Effect of antisense on morphine-6β-glucuronide analgesia

		Analgesic response	n	P	
Control		73%	60		
Mismatch		80%	10		0.497
Antisense					
Exon 1	A	55%	20		0.106
	B	72%	25		0.549
	C	55%	20		0.106
Exon 2	D	**5%**	20	**<0.0001**	
	E	**24%**	25	**<0.0001**	
	F	**34%**	35	**<0.001**	
Exon 3	G	**32%**	25	**<0.001**	
	H	**38%**	40	**<0.001**	
	I	**30%**	10	**0.012**	
Exon 4	J	90%	10		0.24
	K	52%	25		0.06

Groups of mice received the indicated antisense oligodeoxynucleotide i.c.v. on days 1, 3 and 5 and morphine-6β-glucuronide (20 ng, i.c.v.) analgesia assessed on day 6, as described in Section 2. The sequences of the oligodeoxynucleotides and their positions are provided in Table 1. Significance in the analgesic assays was assessed using the Fisher exact test. Statistically significant results are bolded.

1 is a component of the mu_1, but not the mu_2 receptor. Antisense I, which targets exon 3 near the junction with exon 4, downregulates supraspinal analgesia without blocking either of the two mu_2 actions, providing further support for differences between the two mu subtypes. It is not clear why Antisense I is active against supraspinal morphine analgesia while the other two antisense probes targetting exon 3 are not. The similar ability of all three to prevent morphine-6β-glucuronide analgesia makes technical problems related to the activity of the antisense unlikely. The results could be explained by an alternative exon 3 which shares some sequence with exon 3 in MOR-1. We cannot yet assess relationships of exons 2 in the putative mu_1 and mu_2 receptors.

The antisense profile against morphine-6β-glucuronide analgesia dramatically differs from that of morphine, implying that its actions result from yet another splice variant. The activity of the antisense oligodeoxynucleotides directed against exons 2 and 3 suggest that they are included within the morphine-6β-glucuronide receptor while exons 1 or 4 are not. The presence of a distinct morphine-6β-glucuronide receptor would help explain the apparent inconsistency between its binding and cyclase activity at traditional mu receptors and its extraordinary potency in vivo. The possibility that the mu receptor subtypes and the morphine-6β-glucuronide receptor represent splice variants leaves the important question of the role of MOR-1 in opioid pharmacology.

Despite the high abundance of its mRNA in the brain, its actions and pharmacological significance is unknown. In conclusion, these results suggest that the various mu receptor subtypes postulated from binding and pharmacological studies result from alternative splicing of MOR-1 and predict a novel receptor within the mu family for morphine-6β-glucuronide.

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