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Genomic Structure of the Mouse δ Opioid Receptor Gene

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SUMMARY: Using mouse δ opioid receptor (DOR) cDNA sequence to probe genomic libraries in bacteriophage λ and P1 vectors, clones traversing the entire DOR coding sequence and 5' and 3' flanking regions were isolated. Genomic sequence encoding mature DOR message, including 5' and 3' untranslated sequence, is divided by two introns of 26 kb and 3 kb, resulting in the gene occupying 32 kb of chromosomal DNA. Multiple putative transcription initiation sites were located, by RNase protection assay, in TATA-less G+C rich sequence between 390 and 140 nucleotides upstream from the ATG translation start codon. A polyadenylation site was located 1.24 kb downstream from the TGA translation stop codon. Examination of 1.3 kb of 5' flanking sequence revealed potential binding sites for several known transcription factors including: Sp1, Ap-2, NF-κB, NF-IL6, and NGFI-B.

Pharmacological effects of exogenous opioid alkaloids and endogenous opioid peptides, e.g. antinociception, depression of respiratory and gastrointestinal functions, inhibition of immune responses, and psychological euphoria (1), are all known to initiate through G-protein coupled cell surface receptors (2). Although opioid receptors were identified in mammalian brain over two decades ago, molecular characterization of a functional opioid receptor remained an elusive goal until two years ago, when two independent laboratories reported the isolation of a cDNA encoding a δ opioid receptor (DOR) from mouse NG108-15 neuroblastoma X glioma cells (3, 4). Subsequent to these discoveries, molecules encoding the three major pharmacologically defined opioid receptor types, δ, κ, and μ, have been isolated from rodent and human cDNA libraries (5-15). Other novel cDNAs, sufficiently homologous to be included in the opioid subfamily of seven-transmembrane G-protein coupled receptors, but with as yet undefined ligands, have also been isolated (16, 17). Genomic structure of a mouse μ receptor gene has been reported (18), and two splice junctions in the human κ and μ receptors have been identified (5, 19). In addition, a splice junction in an orphan receptor gene has been identified by the discovery of a differentially spliced mRNA (16, 17).

To begin experiments designed to elucidate mechanisms regulating expression of opioid receptor genes we have obtained mouse opioid receptor genomic clones. We report here the exon-

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intron structure, possible transcription initiation sites, and 5' flanking sequence, of mouse DOR genomic DNA.

MATERIALS AND METHODS

Isolation and analysis of genomic clones. The λDOR11 clone was isolated from a library constructed in Lambda Dash II *Bam*HI arms (Stratagene, La Jolla, CA, cat. #247211), using 18-23 kb *Sau*3A fragments from a partial digest of DNA isolated from the liver of an adult C57 Black/6 female. The primary library was screened by *in situ* plaque hybridization, using ^{32}P-labeled mouse DOR-1 cDNA (3) containing the entire DOR coding sequence. The P1-1178 genomic clone was isolated from a c129 embryonic stem cell library by Genome Systems, Inc. Library Screening Service (St. Louis, MO). The library was screened for phage hybridizing to a 206 bp DNA fragment, selected from sequence in λDOR11 spanning the intron-exon junction at the upstream border of DOR exon 2 (Fig. 1).

RNase protection assay. Total RNA was prepared from NG108-15 cells using the Totally RNA kit (Ambion, Austin, TX). Poly(A)$^+$ RNA was prepared from C57 Black/6 mouse brains using the Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). To generate an antisense RNA probe for RNase protection analysis, the 1.3 kb *Sac*I/*Nco*I 5' flanking mouse DOR genomic DNA fragment (Fig. 2) from phage P1 1178 (Fig. 1), was blunted at the *Nco*I end, by filling in using Klenow enzyme and all four deoxyribonucleotides, and subcloned into *Sac*I/*Eco*RV digested pBluescript II SK (+) (Stratagene). 0.5 μg of the resulting plasmid, pRPD3N, was linearized by *Spe*I digestion and transcribed *in vitro* using T7 RNA polymerase and [α-^{32}P]CTP to make anti-sense riboprobe. This ^{32}P-labeled RNA was treated with RNase-free DNaseI, to digest the plasmid DNA template, and made 5M in guanidine isothiocyanate. An aliquot of anti-sense riboprobe (5 x 10^5 cpm) was hybridized to NG108-15 total RNA or mouse brain poly(A)$^+$ RNA, as well as to yeast RNA used as a negative control, in 5M guanidine isothiocynate solution as described previously (20). The mRNA·^{32}P-labeled antisense RNA hybrids were diluted 1:40 with RNase buffer and digested with various concentrations of an RNase A/RNase T1 mixture (RPAII kit, Ambion). After ethanol precipitation, fragments protected from RNase digestion were electrophoresed on a 6% polyacrylamide/7M urea denaturing gel along side dideoxy nucleotide sequenced DNA for size estimation (Fig. 2).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of 5' and 3' ends. Mouse brain poly(A)$^+$ RNA (1 μg) was used as template in RT-PCR assays using components of 5' and 3' RACE systems (GibcoBRL, Gaithersburg, MD, cat. nos. 18374-025 and 18373-019). In 5' assays, templates were reverse transcribed with Superscript II RNase H$^-$ reverse transcriptase at 42 °C, 50°C, or 55°C, using primers complementary to DOR-1 cDNA (3) (bases 33-55 or 375-395, numbered relative to the +1-ATG translation start codon). In one assay the RNA template was equilibrated in 5 mM methyl mercuric hydroxide for 5 min. at room temperature prior to being diluted to 2 mM in a 42 °C reverse transcription reaction with the 33-55 base primer. After removal of excess nucleotides and primer on a GlassMax spin column (GibcoBRL), the purified cDNAs were homopolymeric tailed with either dCTP or dATP using terminal deoxynucleotidyl transferase. Tailed cDNA was then amplified by PCR using the oligo-dC or oligo-dA specific primers provided in the RACE kits, and a nested gene specific primer complementary to DOR-1 cDNA bases 4-25 or 267-288. In the 3' assay, the RNA template was reverse transcribed using the oligo-dA specific primer at 42 °C. The resulting cDNA was PCR amplified twice using the amplification primer supplied in the kit and two DOR 3' untranslated region specific primers: first with a primer homologous to bases 1761-1777, and then with a nested primer at bases 2107-2125. Amplified PCR products were cloned in the pAMP1 plasmid vector using the Uracil DNA glycosylase/CloneAMP system (GibcoBRL), analyzed for insert size by restriction endonuclease digestion, and selected clones were sequenced to determine 5' or 3' ends. In some 5' assays, transformants were screened by colony hybridization with ^{32}P-end-labeled oligonucleotide probes specific to the 5' end of DOR-1 cDNA. Plasmids from positive colonies were further characterized by DNA sequencing to determine transcription start sites.

RESULTS

Exon-Intron Structure of the DOR Gene. An initial genomic clone, λDOR11 (Fig. 1), containing part of the DOR gene was obtained by screening 10^6 bacteriophage λ plaques from a primary mouse genomic library using a 1.8 kb fragment of mouse cDNA containing the

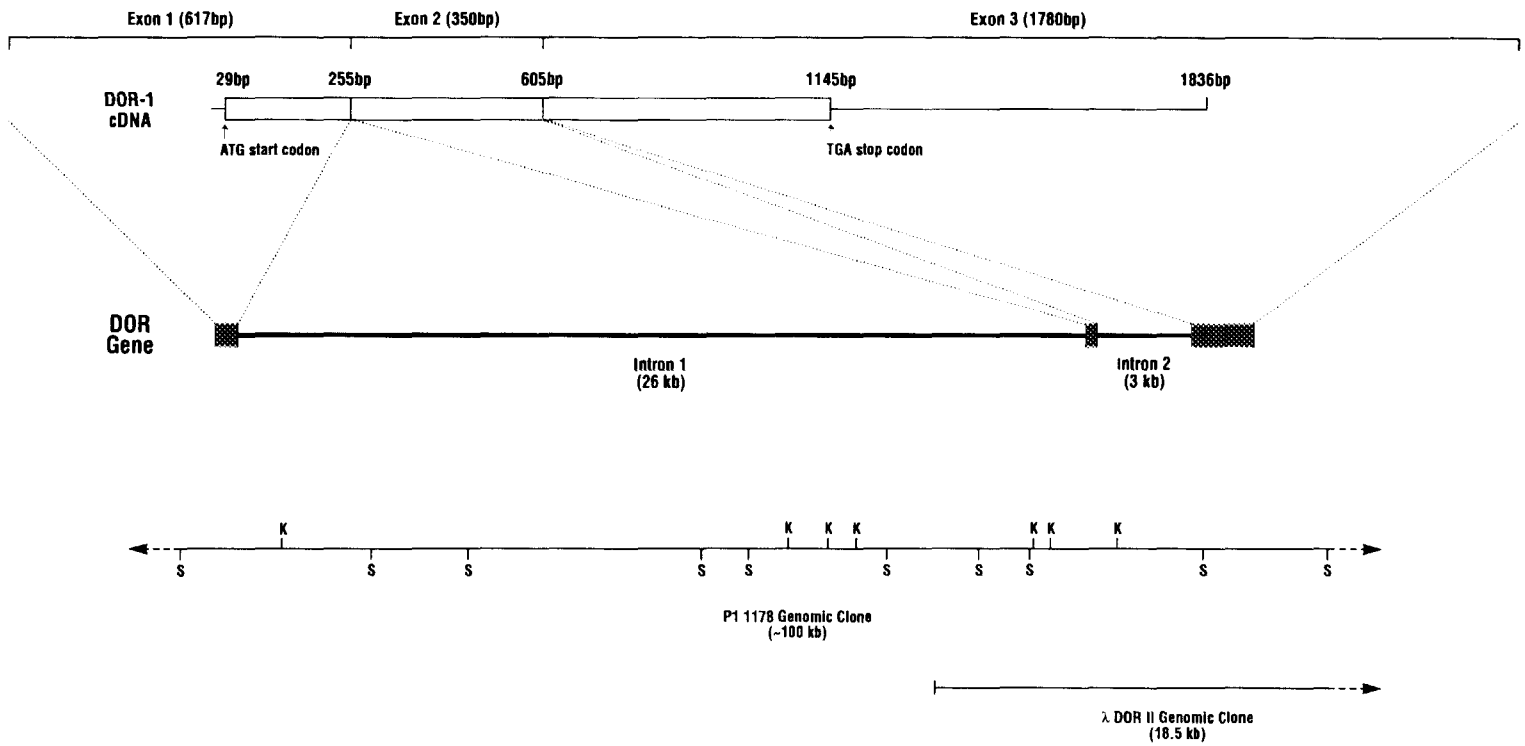

Figure 1. Structure of the mouse DOR gene. The center (bold) line represents mouse genomic DNA encompassing the DOR gene from 390 bp upstream of the ATG translation start codon in exon 1 to 1.24 kb downstream of the TGA translation stop codon in exon 3. The 1.8-kb DOR-1 cDNA molecule (3) is drawn above the DOR gene, relative to a fully spliced, undegraded DOR message (top line). Portions of genomic inserts from the two DOR phage clones isolated in this study (λDOR11 and P1 1178) are drawn to scale relative to the genomic DNA, with the DOR gene *Kpn*I (K) and *Sac*I (S) restriction map indicated on the P1 1178 insert.

entire DOR-1 coding sequence. Southern blot hybridization of DOR-1 cDNA to restriction endonuclease digests of λDOR11 DNA, and subsequent DNA sequencing, revealed exon-intron junctions corresponding to DOR-1 cDNA nucleotides 255/256 (amino acid #76) and 605/606 (amino acid #193) (Fig. 1).

To walk upstream from the λDOR11 genomic sequence, a 206 bp probe was generated from mouse genomic DNA by polymerase chain reaction using a primer pair consisting of one primer homologous to intron sequence upstream from DOR-1 nucleotide 255 and a second primer complementary to sequence from 275-294 in DOR-1 exon 2. This probe was used to screen a bacteriophage P1 mouse genomic library. A clone was obtained, P1-1178 (Fig. 1), containing approximately 100 kb of mouse genomic sequence. Restriction endonuclease mapping, and Southern blot hybridization of DOR-1 cDNA coding sequence to *Kpn*I and *Sac*I digested P1-1178 DNA, indicated P1-1178 homology to the mouse genomic sequence contained in the λDOR11 clone, and to upstream DOR-1 cDNA sequence not contained in the λDOR11 clone. Subsequent DNA sequencing identified the remaining 5' DOR-1 cDNA sequence contained in a single exon located 26 kb upstream of exon 2 (Fig. 1).

To determine the downstream end of exon 3, mouse brain poly(A)$^+$ mRNA was subjected to 3' RACE using gene specific primers located within the 3' untranslated region of previously reported DOR cDNA sequence (4). All of sixteen RACE clones examined contained an approximately equal 3' untranslated restriction fragment length, and each of five clones selected for sequencing differed only in the length of their poly A tracts. Polyadenylation began within a group of four A residues located 1240-1243 bp downstream from the TGA stop codon. A canonical 5'-AATAAA polyadenylation signal is located 11 bp upstream from this site, and the four A residues are followed by a short G+T rich sequence usually found immediately downstream of polyadenylation sites (21).

Both introns have consensus GT/AG splice donor/acceptor boundaries. The amino acid coding portions of the exon sequence determined from P1-1178 and λDOR11 is 100% homologous to three (4, 15, 22) of the four mouse δ opioid receptor cDNA sequences currently in GenBank, however there are a number of differences among the four reported cDNAs in sequence flanking the coding sequence. We have carefully sequenced both DNA strands of the sequence we are reporting to the database.

Determination of the Transcription Initiation Site. RNase protection analysis of the 5' ends of mouse brain poly(A)$^+$ RNA and NG108-15 total RNA resulted in identical patterns of multiple protected fragments (Fig. 2). The protected fragments suggest the DOR gene is transcribed from initiation sites spanning 250 bp between 140 and 390 nucleotides upstream from the start of translation. Two of the protected fragments, representing putative start sites approximately 142 bp and 324 bp upstream from the ATG translation start codon, appeared 5- to 10-fold stronger than the others. Attempts to confirm the RNase protection results and more accurately locate the initiation sites by 5' RACE have failed repeatedly. This is most likely due to premature reverse transcriptase termination in the extremely G+C rich sequence at the 5' end of this gene. The 350 bp immediately upstream of the ATG translation start codon are 80% G+C. The 5' end of the longest 5' RACE product we have obtained is located 84 bp upstream of the start codon.

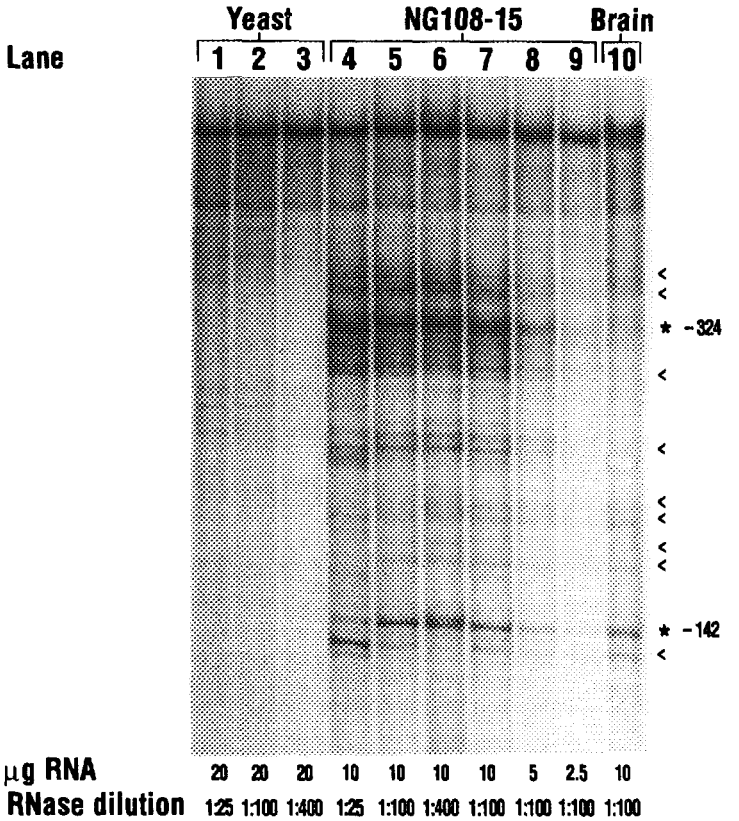

Figure 2. RNase protection analysis of transcription initiation sites. Autoradiograph of electrophoresed RNA protected from RNase digestion by a uniformly labeled antisense RNA probe generated from the *NcoI/SpeI* mouse DOR 5' flanking genomic DNA fragment indicated in figure 3. Mouse brain protected fragments are indicated to the right of the autoradiograph by bullets (<, minor) or asterisks (*, major). The two major bands are numbered relative to the ATG translation start codon.The RNA source is indicated above each lane: Yeast and NG108-15 are total RNA; Brain is poly(A)$^+$ mouse brain RNA.The amounts of RNA and dilution of stock RNase, added in equal volume to each reaction, are indicated below each lane. Fragment sizes were estimated relative to a dideoxynucleotide-sequenced DNA ladder (data not shown).

Nucleotide Sequence Analysis of the 5' Flanking Region. To identify sequence elements in the DOR promoter region that might be involved in regulation of transcription, we sequenced 1.3 kb of DNA upstream from the ATG translation start codon and compared this sequence to the Transcription Factors Database (23) at 100% homology. There are no CCAAT or TATA boxes in the promoter region, however there are numerous potential Sp1 (24) binding sites located throughout the region of transcription initiation identified by RNase protection (Fig. 3). Also located within this region, clustered about the two major transcription start sites observed in the RNase protection experiment, are a number of potential binding sites for the cAMP responsive transcription enhancer AP-2 (25). A site for NF-κB (26), a ubiquitous gene activator that is induced in response to various extracellular signals, is located at -163 immediately upstream of a strong transcription initiation site. A site for the nerve growth factor induced transcription activator NGFI-B (27) occurs at -817. Two sites for NF-IL6 (28), a factor that activates transcription of

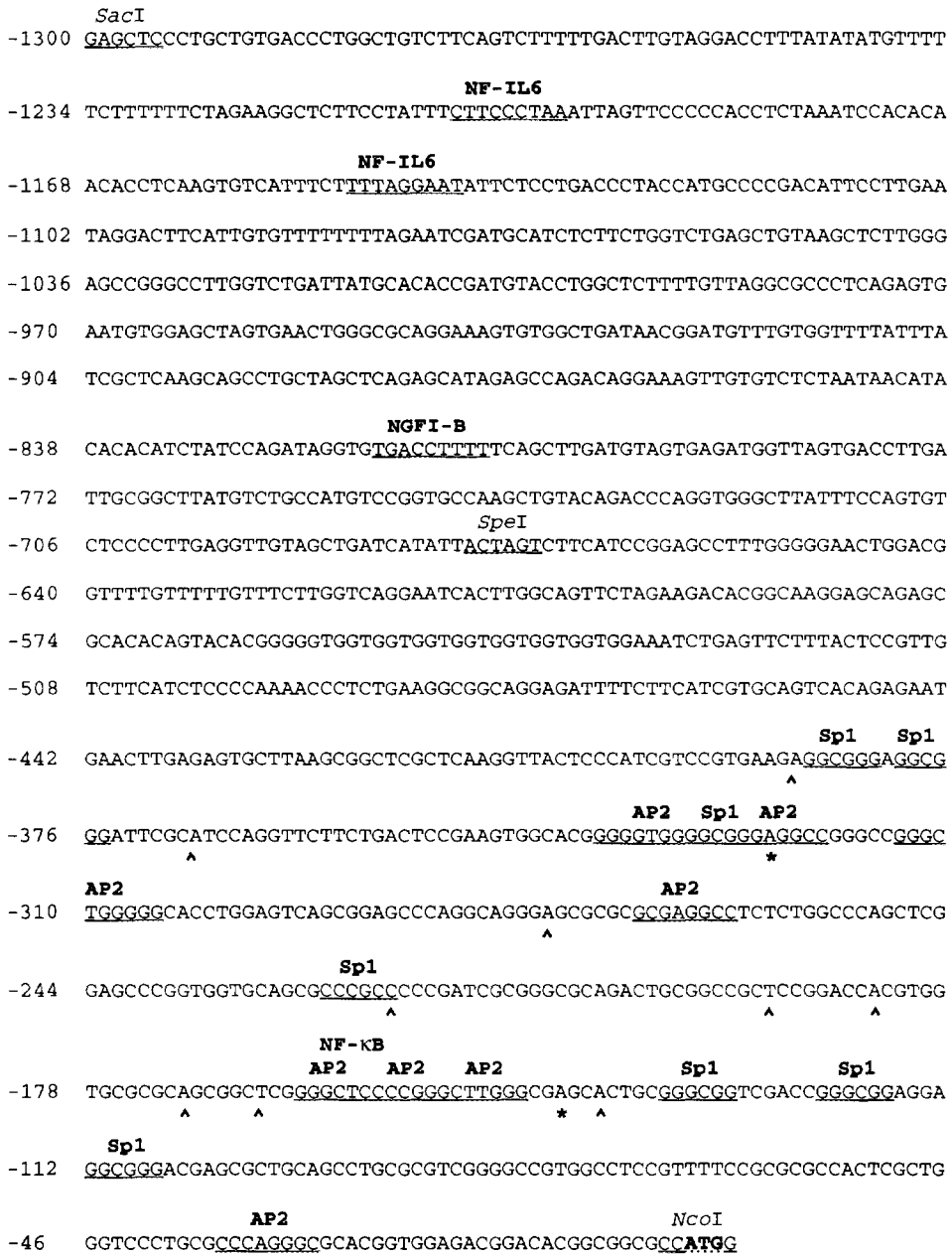

Figure 3. Nucleotide sequence of DOR gene 5' flanking region. Sequence is numbered relative to +1 representing the A nucleotide in the ATG translation start codon, indicated by bold type and dotted underline. The *Nco*I, *Spe*I, and *Sac*I restriction sites used to subclone and linearize the RNase protection probe template are indicated centered at +1, -675, and -1298, respectively. Positions of minor transcription initiation sites determined from estimates of RNase protected fragment sizes are indicated with bullets (^). The two strongest transcription start sites are indicated by asterisks (*) at positions -142 and -324. Sites of consensus sequence for possible cis-acting transcriptional regulatory elements are underlined and labeled above the sequence.

cytokine genes involved in immune responses and hematopoiesis, are located at -1149 and -1207 relative to the ATG translation start codon.

DISCUSSION

To describe the genomic structure of the mouse DOR gene we have: determined all exon-intron junctions, identified a 3' polyadenylation site, and examined 5' flanking sequence for transcription initiation sites and potential recognition sequences for known transcription factors.

The DOR gene spans 32 kb from transcription start sites located between 140 bp and 390 bp upstream of the ATG translation start codon to a polyadenylation site located 1.2 kb downstream of the TGA stop codon. The amino acid coding sequence is divided into three exons, with splice sites in Arg-76 and Asp-193. The first splice occurring at the N-terminal end of the first intracellular loop, and the second at the N-terminal end of the second extracellular loop. The location of these splice sites are the same as those reported for the mouse μ opioid receptor (18) and the human κ and μ opioid receptors (5, 19). The Asp-193 splice junction also coincides with a differential splice site in a rat orphan opioid receptor gene (17). This suggests that an ancestral opioid receptor gene may have acquired these two introns prior to, or simultaneous with, it's divergence into multiple genes.

The identification of a polyadenylation site 1.2 kb downstream from the TGA translation stop codon dictates that a completely spliced and polyadenylated DOR mRNA molecule will not be much longer than 3 kb. However, Northern blot analysis of mouse brain poly(A)$^+$ RNA has indicated major DOR mRNAs at 8 kb and 12 kb with little or no signal in the 3 kb range (22). Perhaps additional 3' RACE experiments using primers located in exon 2 or downstream from the polyadenylation site identified in the present study will reveal additional exons or alternative polyadenylation sites.

The 80% G+C rich sequence encompassing transcription initiation sites in the DOR promoter contains an abundance of CpG dinucleotides, suggesting that this promoter may be subject to regulation by developmental and/or tissue specific methylation (29). In this context the existence of potential AP-2 binding sites may be significant, given the recent observations of CpG methylation inhibition of proenkephalin gene transcription through interference with AP-2 binding (30), and the increased expression of AP-2 in mouse embryonic neural crest cell lineages (31).The potential binding site for NGFI-B, a member of the steroid-thyroid hormone receptor superfamily known to be induced by nerve growth factor in neural crest cells (27), suggests this transcription factor may act in concert with AP-2 to promote neuron specific developmental expression of DOR. The involvement of AP-2 in cAMP-dependent signal transduction pathways affecting transcriptional activation (25), may provide a feedback mechanism by which activated delta opioid receptors, through inhibition of adenylyl cyclase, repress their own expression at the level of transcription. Other combinations of transcription factors with putative binding sites in the promoter may account for the recently reported expression of DOR in lymphocytes (32), e.g. NF-κB and NF-IL6 act synergistically in response to extracellular signals to induce cytokine gene expression in lymphocytes (33), and Sp1 is involved in regulating tissue specific gene expression in developing hematopoietic cells (34). The results of this study will facilitate the testing of these

hypotheses through construction of chimeric reporter genes to analyze expression from the DOR promoter in tissue culture and transgenic mice.

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