

## Molecular Cloning, Expression, and Gene Localization of a Fourth Melanocortin Receptor\*

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The recent cloning of three melanocortin receptors suggests an unexpected diversity in this family of seven transmembrane G-protein linked receptors. Herein, we report the cloning, expression, and gene localization of a fourth human melanocortin receptor, the melanocortin-4 receptor. By Northern blot analysis and *in situ* hybridization, this receptor is expressed primarily in the brain, but its expression is notably absent in the adrenal cortex, melanocytes, and placenta. Agonist stimulation of COS-1 cells transiently transfected and L-cells permanently transfected with the coding region of the cloned melanocortin-4 receptor leads to increases in intracellular cyclic 3',5'-adenosine monophosphate. The profile of the responses of the melanocortin-4 receptor to different melanocortins distinguishes it from melanocortin receptors previously described. Using the technique of fluorescent *in situ* hybridization, the gene encoding the melanocortin-4 receptor was localized to chromosome 18 (q21.3).

Melanocortins, the products of pro-opiomelanocortin post-translational processing (1), are known to have a broad array of physiological actions. Aside from their well known effects on adrenal cortical function (adrenocorticotrophic hormone, ACTH)<sup>1</sup> and on melanocytes ( $\alpha$ -melanocyte stimulating hormone,  $\alpha$ -MSH), melanocortins have been shown to affect behavior, learning, and memory (2, 3), control of the cardiovascular system (4), analgesia (5), thermoregulation (6), and the release of other neurohumoral agents including prolactin, luteinizing hormone (7), and biogenic amines (8). Peripherally, melanocortins have been identified to have immunomodulatory (9) and neurotrophic properties (10) and to be involved in events surrounding parturition (11-13). Melanocortins mediate their effects through G-protein linked receptors. Using the technique of polymerase chain reaction with primers based on conserved areas of other members of seven transmembrane G-protein linked receptors, we recently iso-

lated several genes encoding an "orphan" subfamily of this receptor group which we and others identified to be specific for melanocortins. One was identified as specific for  $\alpha$ -MSH (melanocortin-1 (MC1) receptor) (14, 15) and the other for ACTH (melanocortin-2 receptor (MC2)) (14). We recently identified a third melanocortin receptor (MC3) that recognizes the core heptapeptide sequence of melanocortins. This receptor is expressed in the brain, placenta, and gut tissues (16), but not in adrenal cortex or melanocytes. In the work presented herein, we have identified and cloned a gene encoding a fourth human melanocortin receptor (MC4) that has a unique profile of pharmacological responses to melanocortin peptides.

### EXPERIMENTAL PROCEDURES

**Polymerase Chain Reaction**—Highly conserved sequences in the second intracytoplasmic loop and the seventh transmembrane domain of the other melanocortin receptors served as the basis for designing primers for the polymerase chain reaction (PCR) using mouse genomic DNA as the substrate to find other members of the melanocortin receptor gene family (5' PCR primer, TACGCA/GCTG/CCGCTACCACAGCATC, and 3' PCR primer, GAAG/AGCA/GTAT/GATGAA/GG/TGGGTCA/GAT). Oligonucleotides were synthesized by the Molecular Biology Core of the University of Michigan Gastrointestinal Peptide Research Center using an Applied Biosystems 380B DNA synthesizer. The conditions for the PCR were as follows: denaturation for 1.5 min at 94 °C, annealing for 2 min at 45 °C, and extension for 4 min at 72 °C. The reactions were carried out for 30 cycles, and then 20% of the products were added to fresh buffer and enzyme and subjected to an additional 30 cycles. The final reaction products were phenol-extracted and ethanol-precipitated. The DNA was cut with an appropriate restriction enzyme corresponding to the linker portion of the oligonucleotide and then electrophoresed on a 1% NuSieve, 1% Seaplaque gel (FMC, Rockland, ME). DNA bands were cut out of the gel and subcloned directly into the M13 sequencing vector. Dideoxynucleotide sequencing was then performed using Sequenase Version 2 (U. S. Biochemical Corp.).

**Genomic Cloning**—A partial length PCR-derived clone corresponding to the novel receptor obtained as described above was randomized with <sup>32</sup>P and used as probe to screen a human EMBL3 phage library (Clontech, Palo Alto, CA). Under hybridization conditions (6 × SSC (0.9 M NaCl, 90 mM sodium citrate pH 7.0), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1 M Hepes, pH 7.0, salmon sperm DNA, 100 µg/ml, dextran sulfate, 10 mg/ml at 55 °C) and wash conditions (successive washes with 6 × SSC, 4 × SSC, and 2 × SSC at 50 °C), a single clone was isolated. Phage DNA preparations were made by the plate lysate method (17), and the inserts were restriction-mapped and examined by Southern blot analysis. Dideoxynucleotide sequencing of fragments containing the receptor coding regions subcloned into M13 was then performed.

**Receptor Expression**—The coding region of the novel receptor was subcloned into the eukaryotic expression vector CMVneo (18) using PCR according to methods previously described (19). The sequence was subsequently checked to ensure that no errors were induced by PCR. L-cells (derived from murine fibroblasts) were transfected using a calcium phosphate co-precipitation method (20). Permanently

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<sup>1</sup> The abbreviations used are: ACTH, adrenocorticotrophic hormone;  $\alpha$ -MSH,  $\alpha$ -melanocyte stimulating hormone; PCR, polymerase chain reaction; CMV, cytomegalovirus.

	1				50
MC3	MSIQKKYLEG	DFVFPVSSSS	FLRTLLEPQL	GSALLTAMNA	SCCLPSVQPT
MC4	.....	.....	.MVNSTHRGM	HTSLHLWNRS	SYRLHS....
MC2 (ACTH)	.....	.....	.....	.....	.....MKH
MC1 ( $\alpha$ -MSH)	.....	.....	.....	.....MAV	QGSQRRLGGS
	51			I	100
MC3	LPNGSEHLQA	PFFSNQSSSA	FCEQVFIKPE	IFLSLGIIVSL	LENILVILAV
MC4	..NASESLGK	GY....SDGG	CYEQLFVSPE	VFVTLGVISL	LENILVIVAI
MC2 (ACTH)	IINSYENINN	T....ARNNS	DCPRVVLPEE	IFFTISIVGV	LENLIVLLAV
MC1 ( $\alpha$ -MSH)	LNSTPTAIPQ	LGLAANQTGA	RCLEVSISDG	LFLSLGLVSL	VENALVVATI
	101		II		150
MC3	VRNENLHSPM	YFFLCSLAVA	DMLVSVSNAL	ETIMIAIVHS	DYLTTFEDQFI
MC4	AKNKNLHSPM	YFFICSLAVA	DMLVSVSNGS	ETIIITLLNS	T.DTDAQSFT
MC2 (ACTH)	FKNKNLQAPM	YFFICSLAIS	DMLGSLYKIL	ENILIILRNM	GYLKPRGSFE
MC1 ( $\alpha$ -MSH)	AKNRNLHSPM	YCFICCLALS	DLLVSGTNVL	ETAVILLEEA	GALVARAAVL
	151		III		200
MC3	QHMDNIFDSM	ICISLVASIC	NLLAIAVDRI	VTIFYALRYH	SIMTVRKALT
MC4	VNIDNVIDSV	ICSSLLASIC	SLLSIAVDRI	FTIFYALQYH	NIMTVKRVGI
MC2 (ACTH)	TTADDIIDSF	FVLSLLGSIF	SLSVIAADRY	ITIFHALRYH	SIVTMRRTVV
MC1 ( $\alpha$ -MSH)	QQLDNVIDVI	TCSSMLSSLC	FLGAIADVRI	ISIFYALRYH	SIVTLPRARQ
	201		IV		250
MC3	LIVAIWVCCG	VCGVVFIVYS	ESKMVIVCLI	TMFFAMMLLM	GTLYVHMFLF
MC4	IISCIWAACT	VSGILFIIYS	DSSAVIICLI	TMFFTMLALM	ASLYVHMFLM
MC2 (ACTH)	VLTVIWTFCT	GTGITMVIFS	HHVPTVITFT	SLFPLMLVFI	LCLYVHMFLF
MC1 ( $\alpha$ -MSH)	AVAAIWVASV	VFSTLFIAYY	DHVAVLLCLV	VFFLAMLVLM	AVLYVHMLAR
	251		3i		300
MC3	ARLHVKRIAA	LPPADGVAPQ	QHSCMKGAVT	ITILLGVFIF	CWAPFFLHLV
MC4	ARLHIKRIAV	LPGTGAI..R	QGANNMGAIT	LTILIGVFVV	CWAPFFLHLI
MC2 (ACTH)	ARSHTRKIST	LPR.....	..ANMKGAIT	LTILLGVFIF	CWAPFFLHLV
MC1 ( $\alpha$ -MSH)	ACQHAQGIAR	LHKRQ.RPVH	QGFGLKGAVT	LTILLGIFFL	CWGPFFLHLT
	301		VII		350
MC3	LIITCPTNPY	CICYTAHFNT	YLVLIMCNSV	IDPLIYAFRS	LELRNTFREI
MC4	FYISCPQNPY	CVCFMSSHFN	YLILIMCNSI	IDPLIYALRS	QELRKTFFKEI
MC2 (ACTH)	LMTFCPSNPY	CACYMSLFQV	NGMLIMCNAV	IDPFIYAFRS	PELRDAFKKM
MC1 ( $\alpha$ -MSH)	LIVLCPEHPT	CGCIFKNFNL	FLALIICNAI	IDPLIYAFHS	QELRRTLKEV
	351				
MC3	LGCNGMNLG				
MC4	ICCYPLGGLC	DLSSRY			
MC2 (ACTH)	IFCSRYW				
MC1 ( $\alpha$ -MSH)	LTCSW				

FIG. 1. Deduced amino acid sequences of the melanocortin receptors. The shading denotes amino acids shared by the melanocortin receptors. The putative transmembrane domains are denoted by overbars and Roman numerals. The GenBank accession number for the melanocortin-4 receptor is L08603.

transfected L-cells were selected by resistance to the neomycin analogue G418, and receptor mRNA expression was checked by Northern blot analysis. L-cell clones expressing the novel receptor at levels comparable to those expressing the melanocortin-1 and melanocortin-3 receptors as previously reported (16) were chosen for these studies. COS-1 cells which were transiently transfected with the CMVneo-MC4 gene construct by lipofection (21) using the Lipofectin Reagent (Life Technologies Inc.) were split onto 12-well plates after 24 h and assayed 36 h post-transfection.

**Northern Blotting**—Total RNA was extracted from cell lines and tissues using the acid guanidinium thiocyanate-phenol-chloroform method (22). Poly(A<sup>+</sup>) RNA was obtained using oligo(dT)-cellulose chromatography with the Poly(A) Quik mRNA Isolation Kit (Stratagene), and some poly(A<sup>+</sup>) was obtained directly from tissue using the Fast Track kit (Invitrogen, San Diego, CA). A commercially

available human multiple tissue Northern blot (Clontech) was also used. RNA was transferred to nitrocellulose and hybridized in 50% formamide, 5 × SSPE (0.75 M NaCl, 0.05 M NaH<sub>2</sub>PO<sub>4</sub>, 0.005 M Na<sub>2</sub>EDTA, pH 7.4), 10 × Denhardt's solution, 100 µg/ml salmon sperm DNA, and 2% sodium dodecyl sulfate for 18 h according to standard methods (23). A random primed <sup>32</sup>P-labeled human or rat probe of the novel receptor gene was used for hybridization of human and canine tissues, respectively. Human tissue was hybridized at 42 °C and canine tissue at a lower temperature (40 °C) to compensate for cross-species sequence mismatches. Blots were exposed to XAR-5 film for 24–72 h.

**In Situ Hybridization**—Adult male mice were killed by cervical dislocation, and their brains were removed and frozen in liquid isopentane (−30 °C) for 30 s. Frozen tissue was sectioned on a Slee cryostat (15 µm), thaw-melted onto polylysine-coated slides, and

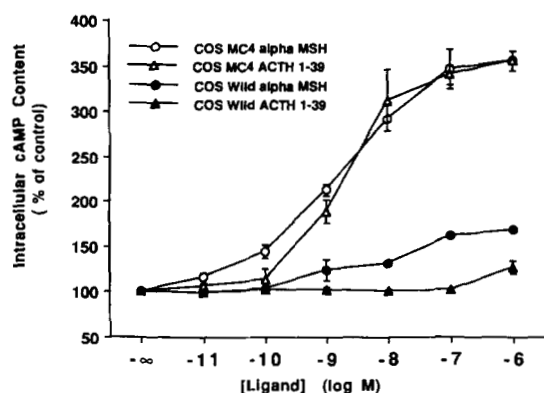


FIG. 2. Generation of cAMP in response to ACTH and  $\alpha$ -MSH in wild type COS-1 cells and COS-1 cells transiently transfected with the melanocortin-4 receptor gene. Wild type cells have a small endogenous response to the peptides. COS-1 cells transfected with the CMV vector without a receptor insert had a response comparable to the wild type cells. The data represent an average of duplicate samples from two different experiments.

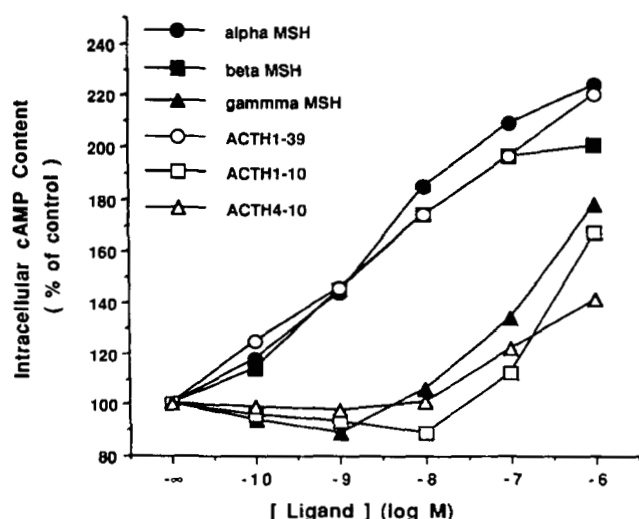


FIG. 3. Generation of cAMP in L-cells transfected with the melanocortin-4 receptor gene (MC4). Responses to  $\alpha$ -,  $\beta$ -, and  $\gamma$ -MSH, ACTH(1-39) ( $n = 5$  to 8 separate experiments), and the truncated peptides ACTH(1-10) and ACTH(4-10) ( $n = 3$  separate experiments) were measured. Each point represents the average of the total number of experiments obtained with each agonist. Standard errors were less than 12% for each point.

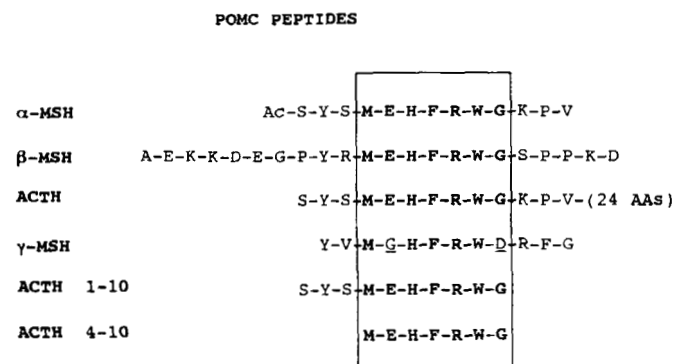


FIG. 4. Amino acid sequences of the melanocortin peptides. Standard single letter abbreviations are used to denote the amino acids. The core heptapeptide amino acids are highlighted. POMC, pro-opiomelanocortin.

stored at  $-80^{\circ}\text{C}$ . For further investigations, slides were transferred directly to 4% buffered formaldehyde for 60 min at room temperature. Following three rinses in  $2 \times \text{SSC}$ , the sections were treated with proteinase K (1  $\mu\text{g}/\text{ml}$  in 100 mM Tris, pH 8.0, 50 mM EDTA) for 10 min at  $37^{\circ}\text{C}$ , then rinsed in water and treated with a mixture of 0.1 M triethanolamine, pH 8.0, and acetic anhydride (400:1, v/v) with stirring for 10 min. The sections were again rinsed in water, dehydrated through graded alcohols, and air-dried. As a control, prior to treatment with proteinase K, some sections were incubated with RNase A (200  $\mu\text{g}/\text{ml}$  in 100 mM Tris, pH 8.0, and 0.5 M NaCl) for 30 min at  $37^{\circ}\text{C}$ . A sense strand control was also used to verify signal specificity. All sections were hybridized with a [ $^{35}\text{S}$ ]UTP- and [ $^{35}\text{S}$ ]CTP-labeled riboprobe generated from a 461-base fragment of the MC4 clone. cRNA probes were diluted in hybridization buffer (75% formamide, 10% dextran sulfate,  $3 \times \text{SSC}$ , 50 mM  $\text{Na}_2\text{PO}_4$ , pH 7.4,  $1 \times \text{Denhardt's}$ , 0.1 mg/ml yeast tRNA, 10 mM dithiothreitol) to a final concentration of  $2 \times 10^6$  dpm/30  $\mu\text{l}$ . After a  $55^{\circ}\text{C}$  overnight hybridization, sections were rinsed in  $2 \times \text{SSC}$  and treated with RNase A (200  $\mu\text{g}/\text{ml}$ ) for 60 min at  $37^{\circ}\text{C}$ . The slides were rinsed in  $1 \times \text{SSC}$ , washed in  $0.1 \times \text{SSC}$  at  $68^{\circ}\text{C}$  for 90 min, rinsed in water, dehydrated in graded alcohols, and air-dried. Sections were then exposed to Kodak XAR-5 film for 10 days and developed.

**cAMP Assays**—For our studies, we measured intracellular cyclic 3', 5'-adenosine monophosphate (cAMP) using a cAMP Assay Kit (TRK 432, Amersham). Cells transfected with the novel receptor gene were grown to confluence in 12-well ( $2.4 \times 1.7$  cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc.) containing 4.5 g/100 ml glucose, 10% fetal calf serum, 100 units/ml penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/ml geneticin. For assays, this medium was removed, and cells were washed twice with Earle's balanced salt solution containing 10 mM Hepes (pH 7.4), 1 mM glutamine, 26.5 mM sodium bicarbonate, and 100 mg/ml bovine serum albumin. An aliquot (0.5 ml) of Earle's balanced salt solution was placed into each well along with 5  $\mu\text{l}$  of  $2 \times 10^{-2}$  M isobutylmethylxanthine. Varying concentrations of agonist were added, and the cells were incubated for 30 min at  $37^{\circ}\text{C}$ . The following peptides obtained from Peninsula Laboratories, Inc. (Belmont, CA) were used as agonists for our studies: human ACTH, ACTH(1-10), ACTH(4-10),  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH,  $\beta$ -endorphin, Met-enkephalin, and porcine  $\beta$ -lipotropin. Ice-cold 30% trichloroacetic acid (500  $\mu\text{l}/\text{well}$ ) was added to stop the reaction and precipitate cellular protein. The cells were scraped and transferred to 16  $\times$  150 mm glass tubes, then placed on ice for 30 min. The precipitate was then centrifuged for 10 min at 1,900  $\times$  g, and the supernatant was ether-extracted, lyophilized, and resuspended in 50 mM Tris, 2 mM EDTA (pH 7.5). cAMP content was then measured by competitive binding assay according to the assay instructions.

**Chromosome Localization**—Chromosome localization was performed with assistance of the University of Michigan Genome Center using the fluorescent *in situ* hybridization (FISH) technique according to a modification of the protocols of Pinkel *et al.* (24), Lichter *et al.* (25), and Lemieux *et al.* (26). Metaphase chromosomes from a normal female were prepared from peripheral blood lymphocytes following overnight synchronization with 5-bromodeoxyuridine and thymidine release. Cells were harvested and slides were prepared using standard cytogenetic techniques. EMBL3 phage containing genomic inserts of between 10 and 20 kilobases of DNA surrounding and including the melanocortin receptor sequences were biotinylated using a Bionick kit (Life Technologies Inc.). Unincorporated nucleotides were removed using a Sephadex G-50 column. An aliquot (330 ng) of biotinylated DNA was precipitated with 3  $\mu\text{g}$  of Cot-1 DNA and 7  $\mu\text{g}$  of herring testes DNA and resuspended in 10  $\mu\text{l}$  of hybridization mixture (50% formamide/2  $\times$  SSC/10% dextran sulfate). The probe was denatured for 5 min at  $70^{\circ}\text{C}$  and preannealed for 15 min at  $37^{\circ}\text{C}$ . Slides were pretreated with RNase and proteinase K and fixed with 4% paraformaldehyde. They were then denatured in 70% formamide, 2  $\times$  SSC (pH 7.0) for 5 min, followed by dehydration in an ice-cold ethanol series. The preannealed probe mixture was applied to the denatured slide under a sealed 22-mm square coverslip, placed in a moist chamber, and incubated overnight at  $37^{\circ}\text{C}$ . Post-hybridization washes were at  $37^{\circ}\text{C}$  in 50% formamide, 2  $\times$  SSC (pH 7.0), followed by washes in  $0.1 \times \text{SSC}$  at  $42^{\circ}\text{C}$ , and a final wash of 4  $\times$  SSC at room temperature. Slides were preblocked with 4  $\times$  SSC, 3% bovine serum albumin for 60 min at  $37^{\circ}\text{C}$ . Signal detection was achieved by incubations of 30 min at  $37^{\circ}\text{C}$  with fluorescein goat anti-biotin and fluorescein-labeled anti-goat IgG (Vector, Burlingame, CA) in 4  $\times$  SSC/0.1% Tween/1% bovine serum albumin. Each incubation

FIG. 5. Tissue distribution of the MC4 receptor as evidenced by Northern blotting with RNA extracted from various tissues. The blot in panel A represents 5  $\mu$ g of canine poly(A<sup>+</sup>) RNA and in panel B, 35  $\mu$ g of human total RNA from the tissues listed.

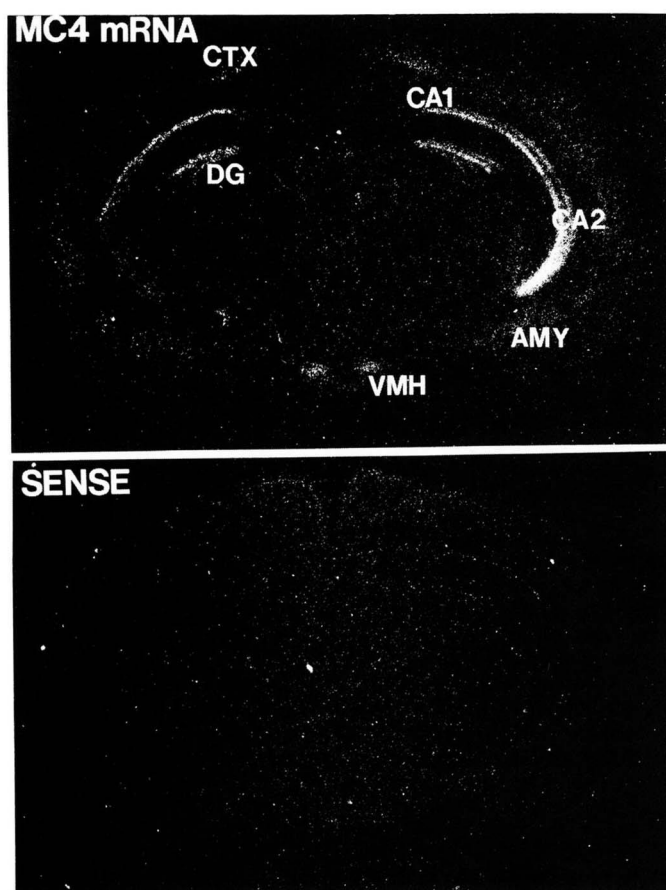
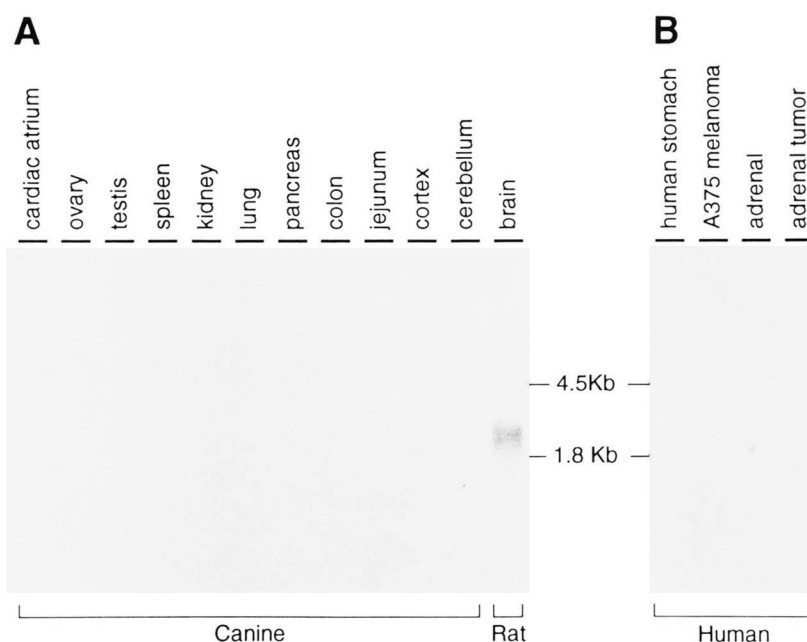


FIG. 6. Dark-field autoradiogram demonstrating *in situ* hybridization of an MC4 receptor riboprobe to mouse brain. MC4 mRNA can be visualized in CA1 and CA2 of the hippocampus, but is absent in CA3 and CA4. MC4 mRNA is also present in dentate gyrus (DG), cortex (CTX), ventral medial hypothalamus (VMH), and amygdala (AMY). Hybridization with a sense strand control cRNA (lower panel) produced no specific signal.

was followed by washes in 4  $\times$  SSC/0.1% Tween at 37  $^{\circ}$ C. Slides were counterstained with propidium iodide, rinsed in phosphate-buffered saline (2.68 mM potassium chloride, 1.76 mM anhydrous monobasic potassium phosphate, 137 mM sodium chloride, 10 mM anhydrous

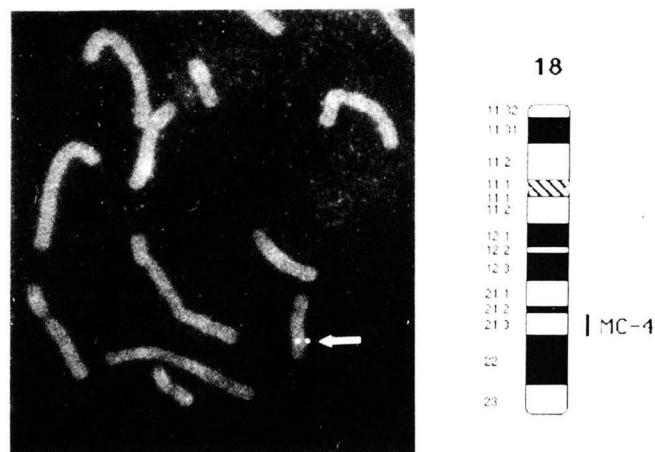


FIG. 7. Regional mapping of the melanocortin-4 gene to 18 (q21.3) by the fluorescent *in situ* hybridization technique. Hybridization signals were detected with fluorescein isothiocyanate on propidium iodide-stained R-banded chromosomes.

dibasic sodium phosphate) and coverslipped with PPD11 anti-fade solution (100 mg of *p*-phenylenediamine free base diluted in 100 ml of 9 parts glycerol to 1 part phosphate-buffered saline, adjusted to pH 11.0 with 1 M NaOH, and stored at  $-20^{\circ}$ C). Photographs were taken on Kodak ASA 400 Gold film using a Zeiss Axioskop epifluorescence microscope equipped with a Zeiss filter set allowing simultaneous visualization of fluorescein isothiocyanate and propidium iodide.

## RESULTS AND DISCUSSION

The single clone that we isolated from the human genomic library on the basis of homology to other melanocortin receptors was that of an intronless gene encoding a protein of 333 amino acids in length with apparent seven transmembrane topography. Its structural features characterized it as another member of the family of melanocortin receptors, hence we called it the melanocortin-4 receptor, to distinguish it from the melanocortin-1 ( $\alpha$ -MSH), melanocortin-2 (ACTH), and the previously reported melanocortin-3 (core melanocortin heptapeptide) receptors. The deduced amino acid sequences of the four members of the melanocortin receptor family are depicted in Fig. 1. The melanocortin-4 receptor is structurally

most similar to the melanocortin-3 receptor with which it shares 58% and 76% overall amino acid identity and similarity, respectively.

The pharmacological characteristics of the novel receptor confirm that it is, indeed, a member of the melanocortin receptor family. As shown in Fig. 2, COS-1 cells transiently transfected with the melanocortin-4 receptor gene demonstrated a marked increase in intracellular cAMP content in response to stimulation with both  $\alpha$ -MSH and ACTH. It is of note, however, that wild-type COS-1 cells appear to have a small endogenous response to melanocortins. COS-1 cells transfected with the CMV vector containing no insert had a response indistinguishable from that obtained with the wild-type cells.

For further examination of the pharmacological characteristics of the melanocortin-4 receptor, we permanently transfected its gene into L-cells, a murine fibroblast cell line, which demonstrate no endogenous response to melanocortins. As shown in Fig. 3, in the transfected cells, ACTH and  $\alpha$ -MSH elicited a dose-dependent increase in intracellular cAMP content with equal potency and efficacy. The equipotency of  $\alpha$ -MSH and ACTH in stimulating melanocortin-4 receptors is identical with the responses observed in L-cells transfected with the genes encoding the melanocortin-1 and melanocortin-3 receptors. The observed half-maximal effect of the two ligands ( $EC_{50} = 10^{-9}$  M) is consistent with previously published pharmacological studies of melanocortin receptors. Like the melanocortin-1 and melanocortin-3 receptors, the melanocortin-4 receptor did not respond to the other pro-opiomelanocortin-derived peptides Met-enkephalin or  $\beta$ -endorphin, although a small increase in cAMP was observed after stimulation with  $\beta$ -lipotropin.

Despite these similarities, the melanocortin-4 receptor demonstrates subtle but important pharmacological differences from the other melanocortin receptors which could be elicited with the various ligands depicted on Fig. 4. We have previously reported that the melanocortin-3 receptor recognizes the ACTH(4-10) core of the melanocortins, thus it responds with equal potency and efficacy to  $\alpha$ -,  $\beta$ -, and  $\gamma$ -MSH and ACTH. In contrast,  $\gamma$ -MSH is unable to stimulate a full cAMP response via the melanocortin-1 receptor, and the potency of  $\beta$ -MSH at this receptor is 10-fold lower than that for  $\alpha$ -MSH and ACTH. Accordingly, the specificity of the melanocortin-1 receptor appears to depend on amino acid residues that extend in the carboxyl- and amino-terminal directions from the ACTH(4-10) core. This conclusion was supported by the observation that the truncated peptides ACTH(1-10) and ACTH(4-10) were fully efficacious agonists on melanocortin-3 receptors but not on melanocortin-1 receptors. In this respect, the melanocortin-4 receptor more closely resembles the melanocortin-1 receptor (Fig. 3). One key difference between melanocortin-4 and melanocortin-1 receptors, however, is that  $\beta$ -MSH is equipotent with ACTH and  $\alpha$ -MSH in acting on the former but not the latter. This observation implies that a portion of the  $\beta$ -MSH molecule at the carboxyl-terminal extension beyond the ACTH(4-10) core can determine the selectivity between melanocortin-1 and melanocortin-4 receptors. By structural analysis (Fig. 4), we hypothesized that Pro<sup>12</sup> of ACTH, which is shared by  $\alpha$ - and  $\beta$ -MSH and ACTH but not by  $\gamma$ -MSH, is critical for binding as a full agonist to the melanocortin-4 receptor. Because  $\gamma$ -MSH has a Phe substitution in the position corresponding to Pro<sup>12</sup> of ACTH, it is a less potent agonist than ACTH or  $\alpha$ -MSH. The tyrosine (Tyr<sup>2</sup>) residue of ACTH may also be important in defining activity at the melanocortin-4 receptor inasmuch as  $\gamma$ -MSH and ACTH(1-10) appear to have slightly

greater efficacy than ACTH(4-10) which is lacking this amino acid residue. These findings may have implications for the design of specific agonists or antagonists for the melanocortin-4 receptor.

Aside from these pharmacological characteristics, the members of the melanocortin receptor family can be differentiated on the basis of their tissue distribution. While the melanocortin-1 receptor is localized to melanocytes and the melanocortin-2 receptor to adrenal cortical cells, the melanocortin-3 and -4 receptors are found primarily in the brain (Fig. 5 and 6). In the brain, these latter receptors can be demonstrated by *in situ* hybridization in regions of the thalamus, hypothalamus, and hippocampus; however, there are distinct differences in the patterns of their expression. The extensive labeling of the melanocortin-4 receptor in the CA1 and CA2 regions of the hippocampus is of particular interest in view of the purported central nervous system functions of melanocortins in learning and memory. Notably, the melanocortin-4 receptor is not expressed in the placenta (data not shown), a tissue that expresses the melanocortin-3 receptor in large amounts.

The gene encoding the melanocortin-4 receptor was localized by fluorescent *in situ* hybridization to chromosome 18 (q21.3) (Fig. 7). Other genes localized to this site include those encoding the proto-oncogene *bcl-2* (27) and plasminogen activator inhibitor type II (28), neither of which has any relationship to G-protein-linked receptors. The gene encoding the melanocortin-2 receptor gene is found on the opposite arm of the same chromosome (18 (p11.2)).<sup>2</sup> By contrast, the melanocortin-3 receptor gene is found on a completely different chromosome (20 (q13.2-q13.3)). The chromosomal localization of the melanocortin-1 receptor gene has yet to be determined.

We have described through these studies a fourth member of the family of melanocortin receptors with unique structural and pharmacological characteristics, tissue distribution, and chromosomal localization. Further analysis of this receptor, particularly in light of its localization, may shed important insight into the function of melanocortins in cerebral neural function.

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