MOLECULAR CHARACTERIZATION OF MELANOCORTIN RECEPTORS: ROLE OF THE MC5-R IN EXOCRINE GLAND FUNCTION

by

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LIST OF ABBREVIATIONS

AC	Adenylyl Cyclase
ACTH	Adrenocorticortropic Hormone
AKAP	A Kinase Anchoring Protein
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CaM K	Calcium-Calmodulin Dependent Kinase
cAMP	Cyclic 3',5'-Adenosine Monophosphate
CAT	Chloramphenicol Acetyltransferase
CBP	CREB Binding Protein
cDNA	Complementary Deoxylribonuclei Acid
cGMP	Cyclic 3',5'-Guanosine Monophosphate
CNS	Central Nervous System
CRE	Cyclic 3',5'-Adenosine Monophosphate Responsive Element
CREB	Cyclic 3',5'-Adenosine Monophosphate Responsive Element Binding Protein
CRF	Corticotropin-Releasing Factor
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxylribonuclei Acid
EC_{50}	Effective Concentration Producing a 50% Response
END	Endorphin
EP	Endorphin
ES	Embryonic Stem
FCS	Fetal Calf Serum
Gal	Galactosidase

GAP	GTPase-Activating Protein
GCV	Gancyclovir
GDP	Guanosine Diphosphate
GPCR	G Protein Coupled Receptor
GRF	Guanosine Diphosphate Releasing Factor
Gs	Stimulatory Heterotrimeric G protein α-Subunit
GTP	Guanosine Triphosphate
HSV	Herpes Simplex Virus
HTS	High Through Put Screening
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
IBMX	Isobutylmethylxanthine
IFN	Interferon
IL-1	Interleukin 1
IP_3	Inositol Triphosphate
iv	Intravenous
LPH	Lipotropic Hormone, Lipotropin
LPS	Lipopolysaccharide
MCn-R	The nth Melanocortin Receptor
mRNA	Messenger Ribonuclei Acid
MSH	Melanocyte-stimulating Hormone
NCS	Newborn Calf Serum
Neo	Neomycin Phospate
ONPG	*O*-Nitrophenyl-β-D-Galactopyranoside
PBS	Phosphate-Buffered Saline
PC	Prohormone Convertase

PCA	Perchloric Acid
PCR	Polymerase Chain Reaction
PGK	Phosphoglycerate Kinase
PIP_2	Phosphatidylinositol Biphosphate
PKA	Protein Kinase A
PKC	Protein kinase C
PLA	Phospholipase C
PLC	Phopholipase C
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulphonylfluoride
POMC	Pro-Opiomelanocortin
R	Receptor
RIA	Radioimmunoassay
RNA	Ribonuclei Acid
RPA	RNase Protection Assay
RT	Reverse Transcriptase
sc	Subcutaneous
SD	Standard Deviation
SDS	Sodium Dodecylsulphate
SEM	Standard Error of the Mean
TK	Thymidine kinase
TLC	Thin-Layer Chromatography
TNF	Tumor Necrosis Factor
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
TSH	Thyroid Stimulating Hormone
UV	Ultraviolet
VIP	Vasoactive Intestinal Peptide

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To Lixin and Jessica

ABSTRACT

In addition to the regulation of pigmentation in the melanocytes and steriodogenesis in adrenal cortex, the POMC derived melanocortins also affect many other physiological and behavioral processes. These other activities of melanocortins are not well defined, and are probably mediated by the three novel melanocortin receptors MC3-R, MC4-R, and MC5-R. It is also conceivable that these novel receptors may regulate other processes that have not yet been discovered. The goal of this thesis has been to determine the physiological role of MC5-R in the mouse. To fulfill the goal, multiple avenues of reseach were initiated aiming to specifically stimulate or inhibit the activity of the MC5-R by pharmacological and genetic means, namely agonist/antagonist administration and gain/loss-of-function mutations.

Mouse MC5-R has been cloned and its pharmacologic properties characterized. In the same cloning process, MC3-R and MC4-R were also cloned, and high levels of MC4-R expression was found during embryonic development by in situ hybridization studies. Sequence analysis of these receptors revealed extensive identity to their human counterparts, implying important functions for these receptors.

A high throughput system has been established for screening of agonists and/or antagonists of Gs-coupled receptors (Chen, et al., 1995). The system measures CREB activation-induced β-galactosidase expression as a readout of receptor activity. As a number of other signaling pathways, such as Gq-linked receptors, activate CREB upon stimulation, this system is a powerful tool for identification of agonist/antagonist for numerous receptors that regulate a wide spectrum of physiological systems. This system has been extensively used by this laboratory for the identification of specific melanocortin antagonists.

Modification of the above strategy has led to the development of a somatic cell genetic system for screening gain-of-function mutations of Gs- and Gq-coupled receptors. In addition, this very system is potentially capable of identifying other genes/alterations that activate CREB as well.

While MC5-R specific antagonists have not been obtained, a function for the MC5-R was identified using the gene-targeting approach. Generation of MC5-R deficient mice by gene targeting revealed its role in several exocrine glands. MC5-R is functionally expressed at high levels in sebaceous, preputial, lacrimal and Harderian glands. Loss of MC5-R function in sebaceous gland results in mice with markedly reduced sterol ester production. Consequently, MC5-R deficient mice displayed impaired water repulsion. As a result, the mutant mice develop hypothermia during exposure to water at 32 °C. Furthermore, loss of MC5-R function leads to defective thermoregulation in the cold, apparently due to impaired heat insulation. Lacrimal glands that lack MC5-R function fail to increase their rate of protein secretion in response to melanocortins. Harderian gland from MC5-R deficient mice produced nearly undetectable amounts of porphyrin compared to the amounts found in wild-type counterparts. Together, these data revealed a role for MC5-R in the coordinate regulation of the activity of multiple exocrine glands. Since the MC5-R is activated by relatively low levels of either α-MSH or ACTH, both of which are induced in response to dtress, MC5-R may be an integral part of the stress response. MC5-R activity in sebaceous, preputial, and Harderian glands, glands that are implicated in pheromone production, may provide stress-regulated olfactory cues for social interaction and for establishment of social hierarchy in rodents.

CHAPTER ONE

INTRODUCTION

1. FROM MELANOCORTINS TO MELANOCORTIN RECEPTORS: A BRIEF HISTORY

Melanocortins are a family of peptides derived from pro-opiomelanocortin (POMC) (Nakanishi et al., 1979; Roberts et al., 1979), a prohormone gene expressed mainly in the pituitary of vertebrates. The family consists of adrenocorticotropin (ACTH, or corticotropin), α-, β-, and γ-melanocyte stimulating hormones (MSH), named after the adrenal tropic activity and the skin darkening activity, respectively. Structurally, these peptides share an indispensable tetrapeptide core, HFRW, as shown in figure 1. In this section, I shall review briefly the historic development from identification of melanocortin peptides and their functions to the cloning of melanocortin receptors (For a more detailed review, see Eberle, 1988).

Figure 1. Sequence alignment of mammalian melanocortin peptides.

Pharmacophore is high lighted.

1.1. Discovery of Melanocortins

The production of melanotropic and adrenocorticotropic hormones by the pituitary was discovered early in this century. In 1912, Fuchs found that bovine pituitary extracts darken

frog skin (Fuchs, 1912). Using the frog skin system, Smith and Allen independently demonstrated that hypophysectomy results in skin decolorization (Allen, 1916; Smith, 1916). Atwell found that extracts from bovine intermediate lobe caused hyperpigmentation of the skin of hypophysectomized frogs (Atwell, 1921). A serious of partial hypophysectomy (Hogben, 1923; Hogben, 1923) and implantation (Allen, 1920) studies further defined the intermediate lobe as the source of pigmentary substances. The adrenal action of pituitary was not discovered until 1927. In this year, Smith successfully conducted hypophysectomy in rats and demonstrated atrophy of adrenal glands as a consequence of the operation. That the atrophy could be reversed by implantation of pituitaries prompted the purification of another trophic factor, adrenal corticotropin (ACTH), from the pituitary gland (Smith, 1927).

The molecular characterization of melanocortins began in the1950s. In 1955, the complete amino acid sequence of ACTH was determined to consists of 39 amino acids (Howard, 1955; Li, 1955). The following year, the amino acid sequence of α-MSH and β-MSH were resolved (Geschwind, 1956; Harris, 1956)). α-MSH consists of 13 amino acids, and β-MSH, 18. The discovery that the sequence of α-MSH is identical to the first 13 amino acids of ACTH suggested that α-MSH is derived from ACTH (Harris, 1957). In addition, all the three molecules share a MEHFRW sequence, suggesting a role of this core for the melanocyte stimulating activity common to all the three hormones. When Li and colleagues identified a new peptide, β-lipotropin (β-LPH), from pituitary in 1965 (Li et al., 1965), they discovered that β-LPH contains the entire β-MSH sequence, and postulated that β-MSH is a β-LPH derivative (Chretien, 1972). A large ACTH-like protein was found in a number of ACTH producing tumors by virtue of its ACTH immunoactivity

(Mains and Eipper, 1975; Orth et al., 1973; Yalow and Berson, 1971). The large ACTH was subsequently demonstrated to be the common precursor of ACTH and β-LPH (Eipper and Mains, 1978; Roberts and Herbert, 1977). In 1976, Li and co-workers discovered peptides with high opiate activty from camel pituitary (Li and Chung, 1976). This peptide was identical to the C-terminal 31 amino acids of β-LPH. It was named β-endophin, denoting endogenous morphine (Holaday et al., 1977). Chretien and colleagues named the precursor proopiomelanocortin, to describe the three principal biological activities encoded by peptides derived from the molecule (Chretien et al., 1979). This string of findings culminated in 1979 when the cDNA for the precursor was cloned and sequenced (Nakanishi et al., 1979; Roberts et al., 1979). The nucleotide sequence not only confirmed that ACTH/α-MSH and β-LPH/β-MSH originated from a common precursor, it also predicted the existence of another melanocortin-stimulating hormone, γ-MSH, based on the primary structure. The prediction was soon validated (Benjannet et al., 1980; McLean and Lowry, 1981). Moreover, it revealed that all the known peptides are flanked by two basic amino acid residues. The dibasic signal was later found to direct the cleavage of the many other prohormones.

1.2. Processing of POMC

POMC processing is initiated by cleavage at the dibasic sites by prohormone convertases (PC) and completed by carboxypeptidases. Figure 2 summarizes the processing of the POMC gene product. PCs are trypsin-like proteases that cut at the C-terminus of dibasic sites. Enzymes with PC activity have been isolated from intermediate lobe of bovine pituitary (Loh, 1985), and porcine anterior pituitary (Cromlish, 1986). Two genes that encode PC activity for POMC have been cloned (Seidah et al., 1991; Smeekens et al., 1991). Carboxypeptidase E removes the C-terminal basic residues resulted by prohormone

convertases. The gene for this enzyme has also been cloned and characterized (Fricker, 1986.)

The synthesis of α-MSH and ACTH is a result of differential processing of POMC gene product in the intermediate and anterior lobes of the pituitary. In the anterior lobe, ACTH and β-LPH predominate. In contrast, ACTH and β-LPH are further processed into α-MSH and β-endorphin, respectively, in the intermediate lobe (Eipper, 1980). This is due to the discrimination by the PCs of distinct dibasic residues. In the anterior lobe, Lys-Arg sites are favored for cleavage, while in the intermediate lobe, all dibasic sites are equally recognized (Smith and Funder, 1988). The molecular mechanism of the differential processing was elucidated by the cloning and characterization of two prohormone convertases, PC1 (also known as PC3) and PC2 (Seidah et al., 1991; Seidah et al., 1981; Smeekens et al., 1991). Both enzymes share a strong homology with a yeast prohormone convertase Kex-2 (Fuller et al., 1989). In situ hybridization demonstrated that PC1 is expressed in both anterior and intermediate lobes in the pituitary, while PC2 is highly

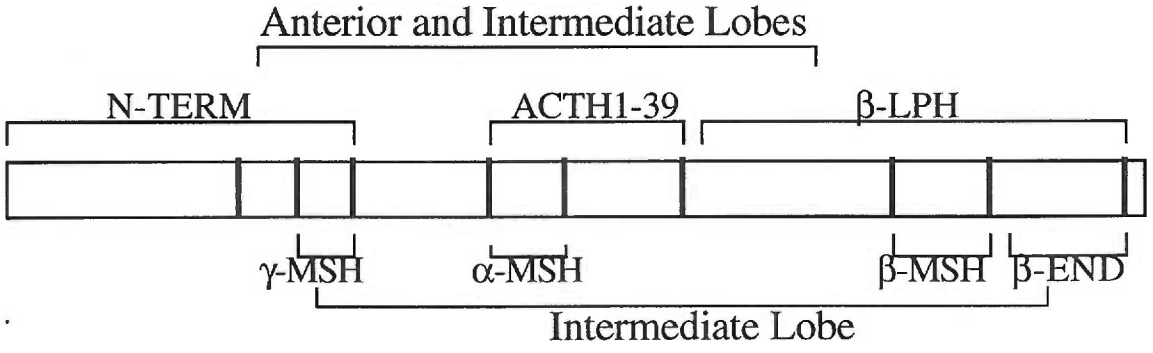


Figure 2. Processing of POMC in anterior and intermediate lobes of the pituitary. . | indicates dibasic residues.

abundant in the intermediate lobe. Very little PC2 mRNA was found in the corticotrophs in the anterior lobe (Day et al., 1992). Moreover, overexpression of PC1 or PC2 in processing-deficient BSC-40 cells resulted in processing of POMC to ACTH/β-LPH, or α-MSH/β-endorphin, respectively (Benjannet et al., 1991; Thomas et al., 1991). The molecular basis of substrate specificity of the two prohormone convertases is not clear.

A number of post-translational modifications occur to processed melanocortins (Smith and Funder, 1988). They include α-amidation, acetylation, and glycosylation. The biological significance is not clear for melanocortin peptides. However, C-terminal amidation and N-terminal acetylation have profound influence on biological activity of melanocortins. For example, acetylated α-MSH displays considerably more potency than the desacetylated form on melanocytes (Guttmann, 1961). C-terminal amidation of α-MSH is also essential for its biological activity. Amidation is thought to protect the peptide from digestion by carboxypeptidases.

1.3. Extra-pituitary Melanocortins

In addition to pituitary, a number of other tissues also express POMC peptides. POMC immunoreactive peptides have been found in the CNS, pancreas, gastrointestinal tract, placenta, thyroid, mast cells, skeletal muscle and skin (Chen et al., 1984; Chen et al., 1984; DeBold et al., 1988; DeBold et al., 1988; Saito et al., 1983; Saito and Odell, 1983). In the brain, POMC mRNA and immunoreactivity have been found in the arcuate nucleus of the hypothalamus (Jacobowitz and O'Donohue, 1978; Nilaver et al., 1979; Watson and Akil, 1979) and brainstem (Bronstein et al., 1992).

The presence of melanocortins in extra-pituitary tissues indicates these peptides may also function in paracrine and autocrine fashion. This is certainly true at least in brain as pituitary borne melanocortins hardly cross the blood-brain barrier (Wilson et al., 1984). In peripheral tissues, however, it is still controversial whether functional melanocortins are produced outside of the pituitary. Some POMC mRNA positive tissues such as skin, testis and tumors, express an aberrant transcript that is smaller than that in the pituitary (Chen et al., 1984; de Keyzer et al., 1989; DeBold et al., 1988; Lacaze-Masmonteil et al., 1987). Furthermore, even when melanocortin-immunoreactive substances are found, questions still remain as to the source and molecular identity of the antigen. The low levels of immunoreactivity may be due to receptor retention of blood-borne melanocortins. Even if the immunoreactivity is truly of local origin, the protein may be not functionally processed in other tissues. Furthermore, immunoreactivity does not necessarily prove the presence true POMC products. For instance, the α-MSH immunoreactivity in axons is due to neurofilament crossreactivity (Drager et al., 1983). Nevertheless, functional forms of melanocortins have been identified in several tissues such as placenta, thyroid, pancreas, gastrointestinal truct, male and female reproductive trucks, and immune system (Smith and Funder, 1988). Therefore, it should be kept in mind that there are possible autocrine and paracrine mechanisms for melanocortin actions, even in peripheral tissues. This may be important in primates, as the α-MSH producing intermediate lobe of the pituitary regresses after birth (Daniel, 1975).

Except in the brain, the processing of POMC gene product in extra-pituitary tissues has not been carefully studied. In the hypothalamus, POMC processing is similar to that in the intermediate lobe of pituitary (Emson et al., 1984; Smith and Funder, 1988). In peripheral tissues, the processing varies (Smith and Funder, 1988).

1.4. Possible Functions of Melanocortins

Expression of POMC in the CNS prompted searches for its functions in the brain. Administration of melanocortins in the rodent brain resulted in a number of behavioral changes. Among the changes are facilitation of active and passive avoidance behaviors (De Wied, 1964; Weiss, 1970), induction of grooming and stretching-yawning (Ferrari, 1958; Gispen et al., 1976), and alterations in social and sexual behavior (Bertolini et al., 1969; Thody and Wilson, 1983). When administered centrally, melanocortin have also been reported to be potent antipyretic (Glyn and Lipton, 1981; Lipton et al., 1981), anti-inflammatory (Lipton et al., 1994; Lipton et al., 1991; Macaluso et al., 1994), and analgesic (Smock and Fields, 1981; Wiegant et al., 1977) agents. Alpha-MSH and related peptides also inhibit food intake in rats (Vergoni et al., 1986) and mice (Fan et al., 1997). Recent data using an antagonist of the neural melanocortin receptors has demonstrated this effect to be specifically mediated by melanocortin receptor stimulation (Fan et al., 1997). Central administration of melanocortins also affect cardiovascular functions (Gruber and Callahan, 1989; Li et al., 1996).

Systemic injection of melanocortins also affects many physiological and behavioral systems in the rodent other than adrenocortical steroidogenesis and pigmentation. In the skin, α-MSH has also been implicated in regulation of sebum production (Ebling et al., 1970; Thody and Shuster, 1971). Intraperitoneal injection of α-MSH in mice elicited aggressive behavior from uninjected cohorts due to release of an olfactory cue (Nowell et al., 1980). Peripheral injection of α-MSH also inhibits inflammation (Hiltz and Lipton, 1990). However, the anti-inflammatory effect may be centrally mediated (Ceriani et al., 1994; Macaluso et al., 1994). High dose of i.p. ACTH releases alarm substances independent of adrenal function (Abel, 1994).

Other reported activities of melanocortin peptides include natriuretic and cardiovascular effects (Aldinger, 1973; Callahan et al., 1984; Klein et al., 1985; Llanos et al., 1983; Sedlakova et al., 1974). In these two activities, γ-MSH is at least as potent as α-MSH (Callahan et al., 1984; Klein et al., 1985; Lymangrover et al., 1985). Intracarotid injection of γ-MSH produces robust pressor and tachycardic effects, which are not elicited by α-MSH. For adrenal gland, α-MSH induces aldosterone secretion in hypophysectomized rats (Shenker, 1985), and enhances zona glomerulosa growth when administered chronically (Robba, 1986). Finally, a few activities of melanocortins have been found in cultured cells. These include the mitogenic activity on skeletal muscle progenitor cells (Cossu et al., 1989; De Angelis, et al., 1992), prosecretory activity in lacrimal gland (Jahn, 1982; Leiba et al., 1990), and inhibitory activity on macrophages (Star et al., 1995) and neutrophils (Catania et al., 1996). These melanocortin effects will be discussed further along with melanocortin receptors later in this chapter.

1.5. Cellular and Molecular Mechanism of Melanocortin Action

Melanocortin peptides exert their actions through their cognate receptors. Adrenal cortical cells have been the model system for characterizing the ACTH receptor, while frog skin and mouse melanoma cells are the models for studying the MSH receptor. Abe et al. first demonstrated experimentally that α-MSH acts via a unique receptor (Abe et al., 1969). Using a frog skin pigment dispersion assay, they showed that whereas α-MSH in the medium quickly scattered the pigment granules, intracellularly administered α-MSH had no effect. With the development of bioactive radio-labeled ligand, a number of laboratories demonstrated high affinity α-MSH binding sites on melanoma membranes (Siegrist et al.,

1988; Siegrist et al., 1989), and ACTH binding sites in adrenal cortical cells and in rabbit adipocytes (Buckley and Ramachandran, 1981; Catalano et al., 1986; Ramachandran et al., 1987). The distribution of binding sites in vivo for melanocortin peptides was also assessed by Tatro and colleagues (Tatro, 1990; Tatro and Reichlin, 1987), who demonstrated specific retention of iodinated NDP-α-MSH, a superpotent and superstable α-MSH analog (Hruby, 1980; Hruby et al., 1993), in a number of peripheral tissues and in the brain (Tatro, 1990; Tatro and Reichlin, 1987).

The predominant mechanism of transmembrane signaling by α-MSH and by ACTH in melanocytes and adrenal cortex, respectively, is activation of adenylyl cyclase and elevation of intracellular cAMP. As early as 1958, Hayne demonstrated that ACTH stimulates cAMP production in bovine adrenal slices (Haynes, 1958; Haynes, 1958). In 1965, Bitensky and Burstein found that application of cAMP on frog skin causes skin darkening (Bitensky and Burstein, 1965). Four years later, Abe et al. demonstrated that α-MSH stimulates cAMP production in frog skin, and postulated that cAMP is the mediator of α-MSH signaling (Abe et al., 1969). Soon after, the same connection was established in mammalian melanoma cells (Bitensky, 1970).

Molecular characterization of the receptors for melanocortin peptides started in 1987. Scimonelli and Eberle first reported specific photoaffinity labeling of a 45 Kd membrane protein by a photoreactive ^{125}I-[Nle^4,D-Phe^7,Trp-(Naps)^9]-α-MSH (Scimonelli and Eberle, 1987). Salomon and co-workers identified a β-MSH binding protein of the same size from M2R cell membrane with a similar approach (Gerst et al., 1988). Using degenerate primers designed based on all the G protein coupled receptors known in 1991, Mountjoy et

al. cloned two related sequences from cDNA derived from a human melanoma known to express high levels of α-MSH receptor (Mountjoy et al., 1992). The molecular and pharmacological characteristics of the two receptors fit that of the classical α-MSH receptor and ACTH receptor, respectively (Mountjoy et al., 1992). Chhajlani and Wikberg independently cloned the human MC1-R using a similar approach (Chhajlani and Wikberg, 1992). This was followed by the cloning of three additional receptors based on homology using low stringency hybridization or degenerate PCR primers (Barrett et al., 1994; Chen et al., 1995; Chhajlani et al., 1993; Desarnaud et al., 1994; Fathi et al., 1995; Gantz et al., 1993; Gantz et al., 1993; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994; Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993). The receptors were named MC1-R to MC5-R, based on their order of discovery, with the classical MSH-R and ACTH-R now being called MC1-R and MC2-R, respectively.

The availability of the cloned melanocortin receptors offers a unique opportunity to validate previous findings on melancortin functions, to assign the described effects attributed to melanocortins to individual receptors, to understand the mechanism of action at the molecular levels, and to explore new physiological functions of these peptides.

2. G PROTEIN COUPLED RECEPTORS

All five melanocortin receptors are G protein coupled receptors (GPCR). Before detailing what we know about the melanocortin receptors, a general introduction to GPCRs is necessary.

2.1. Characteristics of G Protein Coupled Receptors

GPCRs are a superfamily of membrane proteins that contain seven hydrophobic membrane spanning regions. All the members of the family are thought to have a membrane topology similar to that of bacterial rhodopsin. The N-terminus of GPCRs is extracellular, and the C-terminus intracellular. There may be differences between GPCRs and bacterial rhodopsin in the placement of the seven helices (Baldwin, 1993; Baldwin, 1994; Schertler and Hargrave, 1995; Schertler et al., 1993). Elling and Schwartz recently presented evidence from mutagenesis studies that suggests the seven helices are arranged in an anti-clockwise orientation, in contrast to clockwise arrangement of bacterial rhodopsin (Elling and Schwartz, 1996).

GPCRs convey a wide spectrum of extracellular chemical signals across the membrane. Among these signals are neurotransmitters, peptide hormones, odorants, pheromones, ions, and photons. The receptors are highly specific for their ligand, and the same ligand may have multiple receptors; there are more than 1000 estimated members of this superfamily (Gudermann, 1997).

2.2. Signal Transduction by GPCR

GPCRs transform the extracellular signal into a cascade of intracellular reactions. Not only do these receptors share structural topology, they also employ a limited set of signal

transduction pathways. The most proximal downstream component of the signalling machinery is a composite of trimeric G proteins, consisting of α-, β- and γ-subunits. There are twenty-three Gα subunits derived from seventeen genes, five Gβ subunits, and eleven Gγ subunits. These subunits display different patterns of expression (Bourne, 1995; Freissmuth et al., 1989).

Like other small G proteins, the activity of Gα is modulated allosterically by GTP/GDP (Bourne, 1995). GTP loaded Gα is the form which activates effectors while GDP occupied Gα is inert. The conversion of GTP to GDP is catalyzed slowly by the intrinsic GTPase activity of Gα. The inactivation is accelerated by GTPase activating proteins (GAP) (Freissmuth et al., 1989). Gα contains GAP activity within itself (Markby et al., 1993). However, at least two effectors, phospholipase C-β and the γ subunit of retinal cGMP- specific phosphodiesterase, also act as GAP for their activator (Ross, 1995). In addition, a family of proteins, termed RGS (regulator of G protein signaling), were recently discovered to display GAP activity specific to subfamilies of Gα to which they bind (Berman et al., 1996; Berman et al., 1996; Chen et al., 1996; De Vries et al., 1996; Dohlman et al., 1996; Druey et al., 1996; Hunt et al., 1996; Koelle and Horvitz, 1996; Neill et al., 1997; Watson et al., 1996; Yu et al., 1996). RGSs are essential for signal transduction by GPCRs (Dohlman et al., 1996; Koelle and Horvitz, 1996). As GDP binds G proteins with high affinity, the release of GDP is thermodynamically unfavored and requires free energy. For small G proteins, the energy is provided by GDP releasing factors (GRF) (Bourne, 1995). Ligand bound GPCRs appear to serve as GRFs for the heterotrimeric G proteins. Once GDP is liberated, GTP occupies the binding pocket, as the

concentration of GTP is approximately 10-fold higher than that of GDP in normal cellular environment.

Binding of ligand to GPCR initiates a cascade of signal transducing events. It is thought that receptor forms a ternary complex with GDP-charged $G\alpha\beta\gamma$ (Strader, 1987). Ligand binding to complex facilitates the exchange of GDP by GTP on $G\alpha$. The bound GTP destabilizes the complex, and $G\alpha$ dissociates from $G\beta\gamma$. The separated subunits can each activate effectors. Among the effectors are adenylyl cyclases, phospholipase C, and ion channels. In response, the effectors increase the intracellular concentration of second messengers by de novo synthesis or release from cytoplasmic compartments. The cascade of actions ultimately lead to changes of cellular physiology and gene expression in the nucleus.

$G\alpha$ subunits fall into four classes, Gs, Gq, Gi, and G_{12}, based on their sequence homology and biochemical properties (Helpler and Gilman, 1992; Simon et al., 1991). Activation of Gs results in stimulation of adenylyl cyclase activity. Cholera toxin achieves a similar effect by covalently modifying an Arg residue of $Gs\alpha$. The modification inhibits GTPase activity and thus locks $Gs\alpha$ in a stimulated conformation. In contrast, stimulation of Gi inhibits adenylyl cyclase. Pertussis toxin modifies the Cys residue in the C-terminus of some members of Gi family. This modification blocks the release of GDP and thus inhibits signal transduction. GTP loaded Gq increases phospholipase C activity, and thus stimulates the production of phosphoinositol (PIP_2) and diacylglycerol (DAG). So far, the effector for G_{12} is unknown.

In reality, however, the signalling is not as clear cut. The complexity comes from the participation of Gβγ in signalling and the cross-talk among the principal pathways. Not only can one receptor elicit more than one Gα class, but Gα and Gβγ can also have distinct effectors (Clapham and Neer, 1993). For example, human TSH receptor links to Gs and Gq (Allgerier et al., 1994). In vitro, it is possible for one receptor to activate G proteins of all four families reported (Laugwitz et al., 1996). Activation of Gi coupled receptors may potentiate adenylyl cyclase stimulation by Gs, as Gβγ released upon ligand binding activates protein kinase C (PKC) (Iiri et al., 1995) and PKC in turn phosphorylates adenylyl cyclase and renders it more sensitive to induction (Lustig et al., 1993). The dual specificity and cross-talk may not be very common in vivo, however, as both require very high density of receptor molecules in the membrane (Zhu et al., 1994).

2.3. Cyclic AMP Signaling

Cyclic AMP (cAMP) is the second messenger produced by adenylyl cyclases. Elevated cAMP levels in the cell binds allosterically to regulatory subunits (R) of protein kinase A (PKA). As a result, the catalytic subunit of PKA (C) is free and phosphorylates a number of substrates in its vicinity. One means of achieving the specificity is to target the R/C tetramers of PKA to different cellular compartments by a family of proteins (A kinase anchoring protein, AKAP) (Faux and Scott, 1996). Protein phosphorylation changes the activity of the substrates, thus leading to a number of biochemical alterations, such as the activation of enzymes like tyrosinase. Another result of cAMP elevation is the induction of gene expression. Many genes induced by cAMP contain a cAMP responsive element (CRE) in the regulatory regions. The CRE binding protein (CREB) is a substrate of PKA (Gonzalez et al., 1989; Montminy and Bilezikjian, 1987). Phosphorylation of CREB at Ser-133 by PKA confers the ability to interact and thus activate the basal transcription

machinery (Gonzalez and Montminy, 1989). This connection is bridged by phosphorylated CREB binding protein (CBP) (Chrivia et al., 1993). CBP is a platform that many other enhancer binding proteins employ to access the basal transcription machinery (Arany et al., 1996; Arias et al., 1994; Bhattacharya et al., 1996; Chakravarti et al., 1996; Eckner et al., 1996; Janknecht and Hunter, 1996; Kamei et al., 1996; Kwok et al., 1996; Kwok et al., 1994; Missero et al., 1995; Oliner et al., 1996; Parker et al., 1996; Perkins et al., 1997; Smith et al., 1996; Yao et al., 1996; Zhang et al., 1996). It may facilitate transcription by virtue of its histone acetyltransferase activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996).

CREB is also a signal integrator. In addition to PKA, other kinases phosphorylate Ser-133 on CREB. These kinases include Calcium/calmodulin kinases (Matthews et al., 1994; Sheng et al., 1991; Sun et al., 1994), and RSK2, a newly identified ras induced kinase (Xing et al., 1996). Calcium/calmodulin kinase is a down stream effector of Gq coupled receptors, and also mediates gene expression induced by Ca^{2+} influx upon neurotransmitter stimulation (Sheng et al., 1991; Thompson et al., 1995). RSK2 kinase is activated by multiple environmental cues such as UVC irradiation, neurotrophins, and mitogens (Ginty et al., 1994; Iordanov et al., 1997; Tan et al., 1996; Xing et al., 1996). Therefore, CREB activity provides a measurement for multiple signaling pathways. It is also conceivable that there may be other unknown factors that regulate CREB activity directly or indirectly.

2.4. Activating Mutations and Mechanism of GPCR Activation

The structures of several components in the GPCR signaling pathway have been determined by X-ray crystallography. Along the Gs pathway, solution structure of Gαs GTPase domain is known (Benjamin et al., 1995). The general Gαβγ structure is readily

deduced from that of the Gi/tαβγ (Coleman et al., 1994; Lambright et al., 1996; Mixon et al., 1995; Wall et al., 1995). The catalytic core structure of adenylyl cyclase has just been determined (Zhang et al., 1997). Structural information has been published for both catalytic and regulatory subunits of PKA (Su et al., 1995; Zheng et al., 1993). However, the structure of a GPCR is hard to determine by x-ray crystallography, due to the difficulty of crystallizing extremely hydrophobic proteins. With the refinement of cryomicroscopy of two-dimensional crystals (Schertler and Hargrave, 1995; Schertler et al., 1993), it may be possible to determine the atomic structure of GPCRs without crystallization, if one could express the receptor at high enough levels. Thus far, GPCR structure is far from clear. Therefore, the conformational changes that convert the inactive GPCR to the active structure (*R) are not well understood.

The model of induced conformational changes predicts intra-molecular movements upon ligand binding for GPCRs. In fact, recently Khorana and co-workers have detected a rigid body movement between helices C and F in rhodopsin (Altenbach et al., 1996; Farahbakhsh et al., 1995; Farrens et al., 1996). However, a detailed picture of the conformational switch still awaits resolution of atomic structure.

A number of mutations in GPCRs have provided some insights into the mechanism of receptor activation (Lefkowitz et al., 1993; Samama et al., 1993). In humans, several disease states are results of mutations in GPCRs that cause increased basal activity or ligand independent activation (Clapham, 1993; Lefkowitz, 1993). These mutations are called constitutively active mutations or activating mutations. Among diseases caused by these are retina degeneration resulting from mutations in rhodopsin (Robinson et al., 1992), familial male precocious puberty due to activating mutations in the luteinizing hormone receptor (Shenker et al., 1993), hyperfunctioning thyroid adenomas caused by activating mutations in the receptor for thyroid stimulating hormone (TSH) (Parma et al.,

1994), and familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism associated with mutations in the calcium sensing receptor (Pollak, 1993). Activating mutations are also found in the MC1-R that lead to hyperpigmentation in a number of species such as mouse (Robbins et al., 1993), fox (Vage, 1997), panther (Cone, unpublished data), sheep (Vage, unpublished data), cow (Klungland et al., 1995), and chicken (Takeuchi, 1996). Mutagenesis studies in a number of receptors have also revealed changes that result in hyperactivity (Heinflink et al., 1995; Kjelsberg et al., 1992; Kosugi et al., 1996; Liu et al., 1996; Pei et al., 1994; Ren et al., 1993; Samama et al., 1993). These mutations have led the proposal that GPCRs contain some sort of "intrinsic constraint" that keep them from interacting with G proteins, with ligand binding serving to release the constraint and thus allow receptor G protein coupling (Robinson et al., 1992). For example, in rhodopsin, a part of the intrinsic constraint is the salt bridge formed by Lys 295 and Glu 113 (Robinson et al., 1992). Mutations that interfere the ionic interaction lead to constitutive activation. Artificial constraints can also be synthesized in mutagenized receptors. Helices C and F of bovine opsin have been immobilized by cross-linking artificially introduced cysteines (Farrens et al., 1996), and by chelating similarly acquired histidines with Zn^{2+} (Sheikh et al., 1996). With the synthetic constraints, light triggered signal transduction in rhodopsin could not be achieved, indicating a relaxation is required for activation of G protein. The constraint presumably results from unique intramolecular interactions in each receptor. Activating mutations, therefore, do so by disrupting the constraint. Although the constraint may take different forms in different receptor, the activating mutations are likely to display a common configuration. This is because all receptors must share the same or at least a related interface for G protein interaction. The common structural feature is not readily deduced from available information, because there is only a limited number of such mutations known, and these mutations are scattered among a variety of GPCRs. It may be possible, however, to build a structural consensus if a larger number of activating mutations are found in a single receptor.

It should also be emphasized that the structure of GPCRs is dynamic. The receptor may constantly be at equilibrium between R and R* (Lefkowitz et al., 1993; Samama et al., 1993). At basal condition, R is predominant and activity is low. However, spontaneous receptor-G protein interaction can be detected in the absence of agonist (Chidiac, 1994; Costa, 1989; Samama et al., 1994). In addition, the spontaneous activation could be inhibited by some negative antagonists, or inverse agonists (Chidiac, 1994; Samama et al., 1994). In a sense, an agonist achieves activation merely by stabilizing the receptor in the R* conformation. The same may be true for point mutations that result in constitutive/hyperactive signaling.

2.5. GPCRs and Drug Discovery

As GPCRs convey a vast variety of environmental signals to a cell to regulate its physiological functions, they are a major target for drug intervention. Eighteen of the top 100 pharmaceutical drugs listed by the International Marketing Survey audit sheets in 1995 act on GPCRs. The proportion is estimated to increase in the future. Therefore, it is of both scientific and commercial interest to isolate specific agonists and antagonists for the GPCRs.

The development of combinatorial chemistry is revolutionizing the drug discovery process. Combinatorial libraries are collection of molecules synthesized using the combination of multiple monomeric building blocks at each reaction. Therefore, a library of n-mers built by m blocks would have a complexity of m^n. For example, a hexapeptide library made of 20 natural amino acids contains 20^6 species. In general, the probability of finding a "lead" for a given target is proportional to the number of species the target encounters. Therefore, combinatorial libraries greatly increased the chance of identifying a lead. Combinatorial libraries were originally used to synthesize a large number of peptides by repeated split-

and-recombine method using supporting resin beads (Houghten, 1994; Houghten, 1985; Houghten et al., 1992; Houghten et al., 1991). As the majority of peptide ligands are prone to degradation in vivo, it is important, however, to obtain libraries constructed of small, stable compounds. For this reason, the method has now been adopted to make a large number of organic compounds from small organic building blocks. Libraries made in this way may be extremely useful for providing lead compounds for development of new drugs. However, as there is only a tiny amount of each species, it is not a means of choice for optimization of "lead" compound and for drug development. A newer synthesis scheme, parallel unit synthesis, is more suitable. With this method, a library of 1 to 10 000 species is made in an addressable format, such as a 96-well microtiter plate. Such a library contains larger amounts of each compound. In addition, as the structural information at each address is known, and the format is compatible with that of most bioassays, structure activity data is readily deduced and optimized.

The availability of vast compound pools demands the development of high throughput screening (HTS) systems. As defined by Broach and Thorner, HTS is the process by which large numbers of compounds can be tested, in an automated fashion, for activity as inhibitors (antagonists) or activators (agonists) of a particular biological target, such as a cell surface receptor or a metabolic enzyme (Broach and Thorner, 1996). Different targets require different bioassays. For GPCRs, cell-based assays are the best as these receptors require membranes for their function. Although many biochemical and immunological methods have been developed for measuring activation of GPCRs, very few of them can easily be adopted for HTS. For an assay to be applicable in HST, it should satisfy two criteria: simple manipulation in one tube, and small number of cells. A few assays have been developed for HTS recently that can be applied to Gs-coupled receptors. Lerner and co-workers developed a HTS assay that takes the advantage of pigment dispersion in response to intracellular cAMP in frog melanocytes (Lerner, 1994; Quillan et al., 1995).

Several groups of scientists have engineered S.cerevisiae so that it requires activation of GPCR signaling for growth (Broach and Thorner, 1996; Manfredi et al., 1996; Price et al., 1995; Price et al., 1996). With this system, compounds that stimulate yeast growth are likely leads for the expressed receptor.

3. MELANOCORTIN RECEPTORS

3.1. Structural Features of Melanocortin Receptors

Melanocortin receptors form a unique subfamily of GPCRs. Like other GPCRs, melanocortin receptors contain seven putative transmembrane domains. They also possess residues that are common for all GPCRs. Figure 3 shows the placement of putative transmembrane domains for melanocortin receptors according to Baldwin (Baldwin, 1993; Baldwin, 1994). All the receptors link to Gs, and thus stimulate the synthesis of intracellular cAMP upon activation. They have many unique features, however. They are among the smallest known GPCRs. This is mainly due to their short second extracellular and third intracellular loops, and also short N- and C-termini. As figure 3 indicates, there are few distinquishable residues in the extracellular loop 2. MC2-R is the smallest amongst melanocortin receptors, and only has 19 residues in the intracellular loop 3.

There is remarkable homology among melanocortin receptors. In addition to the fingerprint residues, a number of residues are conserved in all the melanocortin receptors. These residues may be important for the receptors to interact with the melanocortin pharmacophore (ME/GHFRW). Moreover, many residues are preserved specifically in each melanocortin receptor subtype from different species. These residues may contribute to the specificity of the ligand/receptor interaction. Within a species, the amino acid identity is 39% to 60% among the different subtypes. The identity of the same receptor from different mammals is much higher. For instance, the identity among the cloned MC1-Rs is more than 70% (Figure 4), and that of MC5-Rs is 80% (Figure 5). The cross-species conservation of all the receptors implies that each may play unique and important physiological functions. This notion is further supported by the difference found in the pharmacological properties and pattern of expression of the five melanocortin receptors. These differences provide the molecular basis for their unique physiological functions, as described in the following text.

Figure 3. Amino acid sequence alignment of all five human melanocortin receptors. Shaded residues are conserved in most of the human melanocortin receptors. Bars indicate putative transmembrane domains according to Baldwin.

```
hmc1r          MAV QGSQRRLLGS LNSTPTAIPQ LGLAANQTGA RCLEVSISDG LFLSLGLVSL VENALVVATI
hmc2r                        MKHIINSYE NINNTARNNS DCPRVVLPEE IFFTISIVGV LENLIVLLAV
hmc3r          MNA SCCLPSVQPT LPNGSEHLQA PFFSNQSSSA FCEQVFIKPE IFLSLGIVSL LENILVILAV
hmc4r    MVNSTHRGM HTSLHLWNRS SYRLHSNASE SLGKGYSDGG CYEQLFVSPE VFVTLGVISL LENILVIVAI
hmc5r          MNS SFHLHFLDLN LNATEGNLSG PNVKNKSSP. .CEDMGIAVE VFLTLGVISL LENILVIGAI

hmc1r   AKNRNLHSPM YCFICCLALS DLLVSGTNVL ETAVILLLEA GALVARAAVL QQLDNVIDVI TCSSMLSSLC
hmc2r   FKNKNLQAPM YFFICSLAIS DMLGSLYKIL ENILIILRNM GYLKPRGSFE TTADDIIDSL FVLSLLGSIF
hmc3r   VRNGNLHSPM YFFLCSLAVA DMLVSVSNAL ETIMIAIVHS DYLTFEDQFI QHMDNIFDSM ICISLVASIC
hmc4r   AKNKNLHSPM YFFICSLAVA DMLVSVSNGS ETIIITLLNS TD.TDAQSFT VNIDNVIDSV ICSSLLASIC
hmc5r   VKNKNLHSPM YFFVCSLAVA DMLVSMSSAW ETITIYLLNN KHLVIADAFV RHIDNVFDSM ICISVVASMC

hmc1r   FLGAIAVDRY ISIFYALRYH SIVTLPRAPR AVAAIWVASV VFSTLFIAYY DHVAVLLCLV VFFLAMLVLM
hmc2r   SLSVIAADRY ITIFHALRYH SIVTMRRTVV VLTVIWTFCT GTGITMVIFS HHVPTVITFT SLFPLMLVFI
hmc3r   NLLAIAVDRY VTIFYALRYH SIMTVRKALT LIVAIWVCCG VCGVVFIVYS ESKMVIVCLI TMFFAMMLLM
hmc4r   SLLSIAVDRY FTIFYALQYH NIMTVKRVGI SISCIWAACT VSGILFIIYS DSSAVIICLI TMFFTMLALM
hmc5r   SLLAIAVDRY VTIFYALRYH HIMTARRSGA IIAGIWAFCT GCGIVFILYS ESTYVILCLI SMFFAMLFLL

hmc1r   AVLYVHMLAR ACQHAQGIAR LHKRQR.PVH QGFGLKGAVT LTILLGIFFL CWGPFFLHLT LIVLCPEHPT
hmc2r   LCLYVHMFLL ARSHTRKIST LP........ .RANMKGAIT LTILLGVFIF CWAPFVLHVL LMTFCPSNPY
hmc3r   GTLYVHMFLF ARLHVKRIAA LPPADGVAPQ QHSCMKGAVT ITILLGVFIF CWAPFFLHLV LIITCPTNPY
hmc4r   ASLYVHMFLM ARLHIKRIAV LPGTGAI..R QGANMKGAIT LTILIGVFVV CWAPFFLHLI FYISCPQNPY
hmc5r   VSLYIHMFLL ARTHVKRIAA LPGAS..SAR QRTSMQGAVT VTMLLGVFTV CWAPFFLHLT LMLSCPQNLY

hmc1r   CGCIFKNFNL FLALIICNAI IDPLIYAFHS QELRRTLKEV LTCSW
hmc2r   CACYMSLFQV NGMLIMCNAV IDPFIYAFRS PELRDAFKKM IFCSRYW
hmc3r   CICYTAHFNT YLVLIMCNSV IDPLIYAFRS LELRNTFREI LCGCNGMNLG
hmc4r   CVCFMSHFNL YLILIMCNSI IDPLIYALRS QELRKTFKEI IC.CYPLGGL CDLSSRY
hmc5r   CSRFMSHFNM YLILIMCNSV MDPLIYAFRS QEMRKTFKEI IC.CRGFRIA CSFPRRD
```

Figure 4. Amino acid sequence alignment of all cloned MC1-R. Shaded residues are conserved in most of the MC1-R. Bar spanned regions are putative transmembrane domains according to Baldwin. * incomplete sequence.

```
  chickmc1r             PWNASE GNQSNATAGA GGAWCQGLDI PNELFLTLGL VSLVENLLVV AAILKNRNLH
       mmc1r MSTQEPQKSL LGSLNSN..A TSHLGLATNQ SEPWCLYVSI PDGLFLSLGL VSLVENVLVV IAITKNRNLH
     foxmc1r MSGQGPQRRL LGSPNATSPT TPHFKLAANQ TGPRCLEVSI PNGLFLSLGL VSVVENVLVV AAIAKNRNLH
   horsemc1r                   TLPA TPYLGLTTNQ TEPPCLEVSI PDGLFLSLGL VSLVENVLVV TAIAKNRNLH
       bmc1r MPALGSQRRL LGSLNCTPPA TLPFTLAPNR TGPQCLEVSS LDGLFLSLGL VSLVENVLVV AAIAKNRNLH
       hmc1r MAVQGSQRRL LGSLNSTPTA IPQLGLAANQ TGARCLEVSI SDGLFLSLGL VSLVENALVV ATIAKNRNLH

  chickmc1r SPTYYFICCL AVSDMLVSVS NLAKTLFMLL MEHGVLVIRA SIVRHMDNVI DMLICSSVVS SLSFLGVIAV
       mmc1r SPMYYFICCL ALSDLMVSVS IVLETTIILL LEVGILVARV ALVQQLDNLI DVLICGSMVS SLCFLGIIAI
     foxmc1r SPMYYFIGCL AVSDLLVSVT NVLETAVMLL VEAGALAAQA AVVQQLDDII DVLICGSMVS SLCFLGAIAV
   horsemc1r SPMYYFICCL AVSDLLVSMS NVLEMAILLL LEAGVLATQA SVLQQLDNII DVLICGSMVS SLCFLGSIAV
       bmc1r SPMYYFICCL AVSDLLVSVS NVLETAVMPL LEAGVLATQA AVVQQLDNVI DVLICGSMVS SLCFLGAIAV
       hmc1r SPMYCFICCL ALSDLLVSGT NVLETAVILL LEAGALVARA AVLQQLDNVI DVITCSSMLS SLCFLGAIAV

  chickmc1r DRYITIFYAL RYHSIMTLQR AVVTMASVWL ASTVSSTVLI TYYRNNAILL CLIGFFLFML VLMLVLYIHM
       mmc1r DRYISIFYAL RYHSIVTLPR ARRAVVGIWM VSIVSSTLFI TYYKHTAVLL CLVTFFLAML ALMAILYAHM
     foxmc1r DRYLSIFYAL RYHSIVTLPR AWRAISAIWV ASVLSSTLFI AYYNHTAVLL CLVSFFVAML ALMAVLYVHM
   horsemc1r DRYISIFYAL RYHSIMMLPR VWRAIVAIWV VSVLSSTLFI AYYNHTAVLL CLVTFFVAML VLMAVLYVHM
       bmc1r DRYISIFYAL RYHSVVTLPR AWRIIAAIWV ASILTSLLFI TYYNHKVILL CLVGLFIAML ALMAVLYVHM
       hmc1r DRYISIFYAL RYHSIVTLPR APRAVAAIWV ASVVFSTLFI AYYDHVAVLL CLVVFFLAML VLMAVLYVHM

  chickmc1r FALACHHVRS ISS.QQKQPT IYRTSSLKGA VTLTILLGVF FICWGPFFFH LILIVTCPTN PFCTCFFSYF
       mmc1r FTRACQHVQG IAQLHKRRRS IRQGFCLKGA ATLTILLGIF FLCWGPFFLH LLLIVLCPQH PTCSCIFKNF
     foxmc1r LARARQHARG IARLRKRQHS VHQGFGLKGA ATLTILLGIF FLCWGPFFLH LSLMVLCPQH PICGCVFQNF
   horsemc1r LARACQHARG IARLHKRQHP IHQGFGLKGA ATLTILLGVF FLCWGPFFLH LSLLILCPQH PTCGCVFKNF
       bmc1r LARACQHARG IARLQKRQRP IHQGFGLKGA ATLTILLGVF FLCWGPFFLH LSLIVLCPQH PTCGCIFKNF
       hmc1r LARACQHAQG IARLHKRQRP VHQGFGLKGA VTLTILLGIF FLCWGPFFLH LTLIVLCPEH PTCGCIFKNF

  chickmc1r NLFLILIICN SVVDPLIYAF RSQELRRTLR EVVLCSW
       mmc1r NLFLLLIVLS STVDPLIYAF RSQELRMTLK EVLLCSW
     foxmc1r NLFLTLIICN SIIDPFIYAF RSQELRKTLQ EVVLCSW
   horsemc1r KLFLTLILCS AIVDPLIYAF RSQELRKT
       bmc1r NLFLALIICN AIVDPLIYAF RSQELRKTLQ EVLQCSW
       hmc1r NLFLALIICN AIIDPLIYAF HSQELRRTLK EVLTCSW
```

3.2. MC1-R

MC1-R is the classical MSH receptor. It has been cloned from a number of mammals, including human (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992), mouse (Mountjoy et al., 1992), panther (Cone, unpublished), fox (Vage, 1997), cow (Vanetti et al., 1994), sheep [Vage, D.I. unpublished], and horse (Marklund,L.M. 1996, unpublished). The chicken MC1-R sequence was reported recently (Takeuchi et al., 1996). Pharmacologically, the order of potency of melanocortins at the typical mammalian MC1-R for cAMP production is α-MSH > $ACTH_{1-39}$ > β-MSH >> γ-MSH. The EC_{50} for α-MSH is about 1 nM. A synthetic ligand, [Nle^4, D-Phe^7]-α-MSH, is more potent than all the natural peptides (EC_{50} = 0.1 nM). Saturation binding with ^{125}I-NDP-α-MSH revealed a Kd of 0.1 nM for MC1-R. Similar results were obtained for α-MSH binding to the murine MC1-R expressed in HEK293 cells (Lu et al., manuscript in preparation). Human MC1-R is unique in its sensitivity to ACTH, responding equally well to α-MSH and ACTH (Mountjoy et al., 1994). This may be an evolutionary adaptation to the low α-MSH levels in the serum due to the lack of an intermediate lobe in the human pituitary (Daniel, 1975).

In mammals, the color of fur/skin is determined by the ratio of two pigments, eumelanin (brown/dark) and phaeomelanin (yellow/red), in the melanosome (Jackson, 1994). Both eumelanin and phaeomelanin are tyrosine derivatives. Phaeomelanin is the default product in melanocytes, whereas eumelanin synthesis requires elevated intracellular cAMP. α-MSH induces eumelanin synthesis, or dark pigmentation by stimulating cAMP production via the MC1-R on the membrane of melanocytes. Mutations in MC1-R cause pigmentation variants in a number of mammals (Cone et al., 1996). In the mouse, a frameshift mutation

results in yellow pigmentation, whereas mutations that lead to constitutive activation or higher basal activity cause dominant dark pigmentation (Robbins et al., 1993). Constitutively active MC1-R has also been found in dominant hyperpigmented animals in a number of species (Cone et al., 1996). In several cases, activation is due to a gain of positive charge around the upper portion of transmembrane helices two and three (Vage et al., 1997; Lu et al., manuscript in preparation). Interestingly, one of the constitutively active MC1-R alleles, E92K, was also found in hyperpigmented chicks (Takeuchi, 1996). The mechanism of constitutive activity is unclear so far, and is of tremendous interest as it may provide clues for understanding GPCR activation. In humans, several polymorphisms clustered in helix 2 are associated with red hair/fair skin (Koppula et al., 1997; Valverde et al., 1995). The functional significance of the variations is not clear. It is reported that a common polymorphism V92M is less sensitive to α-MSH than the "wild-type" allele (Xu et al., 1996), however, our laboratory does not find any pharmacological difference between the two (Koppula et al., 1997).

Pigmentation may be the primary physiological function of MC1-R. No other abnormality has been described in the recessive yellow mice, in which no functional MC1-R exists (Robbins et al., 1993). However, there could be some subtle defects that remain undetected in these mice. Star et. al. recently demonstrated that α-MSH inhibits LPS/IFN-γ induced nitric oxide production in cultured macrophage cells. These authors detected both POMC and MC1-R mRNA in these cells, and argued that MC1-R may mediate some of the anti-inflammatory action of α-MSH in an autocrine fashion (Star et al., 1995).

3.3. MC2-R

MC2-R is the classical ACTH receptor. The receptor has been cloned from several mammals (Clark and Cammas, 1996), including human (Mountjoy et al., 1992), mouse (Cammas et al., 1995; Kubo, 1995), cow (Raikhinstein, 1994), and hamster (Fleury, et al., unpublished data). This gene contains a single coding exon, however, recent data indicates that MC2-R may have a small untranslated exon 5' of the coding region (Naville et al., 1994). Whether other melanocortin receptor genes share this structural feature is unknown. MC2-R is expressed in adrenal cortex (Mountjoy et al., 1992) and adipocytes (Boston and Cone, 1996). In addition to its small size, MC2-R has unique phamarcological properties. In contrast to MC1-R, MC3-R, MC4-R, and MC5-R, the HFRW phamarcophore is not sufficient for binding to or activation of the MC2-R. Other determinants in middle portion of ACTH are also necessary for receptor binding and activation. Therefore, ACTH is the only agonist for MC2-R, and has an EC_{50} around 1 nM. Additionally, unlike other melanocortin receptors, functional expression of MC2-R in heterologous systems has met with some difficulties. Good pharmacological data for the human MC2-R have only been obtained from cells of adrenal origin. The peculiarity suggests that the human MC2-R may need an adrenal gland specific factor. However, the recently cloned mouse MC2-R appears to express well in heterologous cells (Cammas et al., 1995).

Variations in MC2-R are associated with some forms of ACTH resistance in humans. Tsigos and co-workers, and Clark et al. independently detected mutations in the MC2-R in patients with familial glucocorticoid deficiency (Clark et al., 1993 Weber et al., 1995; Tsigos et al., 1993). This syndrome is inherited in an autosomal recessive fashion. Patients are either homozygous or compound heterozygous for the mutant receptors. Some of the mutant receptors requires higher levels of ACTH for activation (Weber et al., 1993).

In addition, homozygous relatives of are more reactive to a CRH stimulation test, indicating that adrenal cells with two copies of MC2-R are more effective in glucocorticoid production (Tsigos et al., 1993; Weber et al., 1995).

Besides steroidogenesis, MC2-R may also mediate in ACTH-induced lipolysis in rabbit adipocytes (Ramachandran, 1976). The EC_{50} for this activity (0.2 nM) fits well with MC2-R pharmacology. Recently, Boston and Cone demonstrated functional expression of MC2-R in mouse adipocytes, further supporting the participation of the MC2-R (Boston and Cone, 1996).

3.4. MC3-R

MC3-R is a unique receptor that displays equal responsiveness to all the natural melanocortins with an EC_{50} of about 1 nM. It is, therefore, the only melanocortin receptor responsive to low levels of γ-MSH. The Ki values of human MC3-R expressed in COS7 cells for α-, β-, γ-MSH and ACTH are 4 nM, 13 nM, 7 nM, and 87 nM, respectively. It is expressed mainly in the brain and placenta. In the brain, MC3-R was found primarily in a number of hypothalamic and limbic system nuclei, with the highest expression in the ventromedial hypothalamic nucleus (Desarnaud et al., 1994; Gantz et al., 1993; Roselli-Rehfuss et al., 1993).

The expression in the hypothalamic and limbic system suggests that MC3-R may be involved in cardiovascular and neuroendocrine regulation, thermoregulation, as well as the regulation of motivational behaviors (Roselli-Rehfuss et al., 1993). Another candidate function of MC3-R is the regulation of natriuresis in the kidney. Intravenous injection of α- or γ-MSH induces natrieuresis in mouse and rat (Valentin et al., 1993). The natriuretic

effect of melanocortins could be blocked by co-injection of SHU9119, a MC3-R antagonist (Humphreys et al., manuscript in preparation).

Not all the activity of γ-MSH is mediated by MC3-R. For instance, intracarotid administration of γ-MSH induces a robust pressor and tachycardic effect. This effect can not be blocked by SHU9119 (Li et al., 1996). In addition, α-MSH is ineffective (De Wildt et al., 1993; De Wildt et al., 1994; Li et al., 1996). Together, they exclude the participation of any of the known melancortin receptors, and suggest the existence of additional receptors with high affinity for γ-MSH. The receptor that mediates the pressor function may be distantly related to the melanocortin receptors, as structure-function studies mapped the pharmacophore partially outside of the HFRW of γ-MSH (Van Bergen et al., 1995).

3.5. MC4-R

MC4-R is equally responsive to α-, β-MSH, and ACTH with an EC_{50} slightly less than 1 nM. γ-MSH is about 100-fold less potent than the other ligands. MC4-R is primarily expressed in the CNS (Gantz et al., 1993; Mountjoy et al., 1994). It is found in a large number of nuclei in every brain region. In the hypothalamus, MC4-R is found in structures that are implicated in neuroendocrine control, autonomic responses, feeding and reproductive regulation. The expression in the dorsal motor nucleus of the vagus in the brainstem suggests a role in controlling autonomic outflow to the heart (Mountjoy et al., 1994). These expression based predictions have been largely confirmed recently.

Two MC4-R antagonists have been discovered (Hruby et al., 1995; Lu et al., 1994). They are agouti signaling protein (ASP) and SHU9119, a synthetic cyclic lactam derivative of α-MSH. Agouti is another pigmentation regulator that promotes phaeomelanin synthesis. The mechanism of antagonism between α-MSH and agouti was not known. At least two hypotheses were proposed: antagonism of the same receptor or of a common downstream component (Conklin and Bourne, 1993; Jackson, 1993). With the cloning of genes for α-MSH receptors and agouti (Bultman, 1992; Miller, 1993), it was possible to distinguish these two possibilities by monitoring the effect of exogenously expressed agouti protein on the α-MSH stimulated cAMP production in cells containing melanocortin receptors. In doing so, Lu et al. demonstrated that agouti competes with α-MSH to bind MC1-R, and consequently inhibits cAMP production. Astonishingly, agouti exerts similar effect on MC4-R with an IC_{50} of 4 nM in the mouse. In an attempt to develop specific agonists/antagonists for the melanocortin receptors, Hruby et al. found an antagonist for MC3-R and MC4-R. This compound, SHU9119, is a cyclic lactam. Interestingly, SHU9119 is a full agonist for MC1-R and MC5-R (Hruby et al., 1995). In addition, as an antagonist, it is 10 fold more potent at the MC4-R (PA_2 = 9.3) than the MC3-R (PA_2 = 8.5).

MC4-R appears to be a component of the brain circuitry that regulates feeding behavior and metabolism. Two lines of evidence suggested a role for MC4-R in regulation of weight homeostasis: 1). it is expressed highly in hypothalamic structures involved in the control of feeding behavior; 2). ectopic expression of agouti results in obesity. Using different approaches, two groups have clearly proven the function of MC4-R in feeding control. Fan et al. showed that icv injection of MT II, a superpotent and super prolonged agonist for melanocortin receptors inhibits food intake in the mouse. This inhibition is overcome by

co-injection of SHU9119, suggesting MC4-R as the mediator. Moreover, injection of SHU9119 alone slightly stimulates feeding (Fan et al., 1997). Using a genetic approach, Huzar et al. deleted MC4-R gene in the mouse (Huszar et al., 1997). Loss of MC4-R function results in the development of the agouti obesity syndrome. Interestingly, heterozygous mice are significantly overweight compared to their wild-type littermates, particularly in old mice. Furthermore, both heterozygous and homozygous knockout obese mice also display increased linear growth, an association commonly seen in humans (Huszar et al., 1997). These findings suggest that perturbation in the MC4-R signaling pathway may be one factor in the etiology of obesity in humans.

MC4-R may mediate a number of other effects that α-MSH elicits in the CNS. MC3-R and MC4-R are the principle melanocortin receptors in the CNS. In fact, the summation of the expression sites for the two receptors superimpose well with ^{125}I-NDP-α-MSH binding sites (Tatro, 1990). It is therefore likely that effects that can be induced by α-MSH but not by γ-MSH, in the brain are due to activation of the MC4-R. One of these is the anti-pyretic and anti-inflammatory effect of α-MSH (Glyn and Lipton, 1981; Glyn-Ballinger et al., 1983; Lipton et al., 1981), possibly via MC4-R in the septum (Glyn-Ballinger et al., 1983). MC4-R may also be responsible for the melanocortin-induced grooming behavior. The structure-activity relationship (SAR) of various compounds in eliciting grooming correlates with the pharmacological profile of the heterologously expressed MC4-R (Adan et al., 1994). Unpublished data demonstrated that melanocortin-induced grooming is inhibited by SHU9119, further suggests the involvement of MC4-R [Adan, personal communication]. MC4-R may be also partially responsible for α-MSH-induced, SHU9119 blockable, hypotensive and bradycardic effects resulting from stereotaxic

injection in the medullary dorsal-vagal complex. In this case, γ-MSH is effective but less potent (Li et al., 1996). There is also some evidence that MC4-R may mediate the activity of melanocortins in antagonizing opiate dependence and tolerance behavior (Alvaro et al., 1996). Chronic administration of morphine in rats specifically suppressed the expression of MC4-R in striatum and periaqueductal gray, regions that are implicated in opiate behaviors.

3.6. Functions Unrelated to Cloned Melanocortin Receptors

There is, however, inconsistency between SAR of melanocortins and derivatives in several behavioral assays and in *in vitro* receptor binding/activation. Several "melanocortin" derivatives are superpotent in behavioral assays, such as Org2766 [H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH], yet are not agonists or antagonists of any known cloned melanocortin receptors (Adan et al., 1994; Gantz et al., 1993; Gantz et al., 1993; Roselli-Rehfuss et al., 1993). Although the stability of these unnatural compounds has been thought to be responsible for the superactivity, it may be necessary to reconsider their classification as melanocortin peptides. Inconsistency exists mostly in the passive and active avoidance assay. Peripheral administration of ACTH or α-MSH is capable of correcting the deficiency in passive and active avoidance in hypophysectomized rats. $ACTH_{4-10}$ is as potent as ACTH or α-MSH in facilitation of avoidance behavior. However, in in vitro receptor assay, it is several magnitudes less efficient than the natural peptides in activation of all the cloned receptors. There several derivatives are devoid of any activity at the cloned receptors, e.g. Org2776 (Adan et al., 1994; Gantz et al., 1993; Gantz et al., 1993; Roselli-Rehfuss et al., 1993), yet are much more potent than the native peptides (de Wied and de Kloet, 1988) in these behavioral assays. It is very unlikely that those compounds exert their function through the cloned melanocortin receptors. Although

pharmacological properties of receptors expression in cultured cells may not faithfully represent that in vivo, no major difference has been found thus far between in vivo and in vitro activity for other melanocortin compounds. Those compounds may elicit distinct signals by acting on a unrelated receptor (Hol et al., 1993). The receptor that is responsible for the avoidance behavior has not been defined and the site of action is not clear. The same is true for the melanocortin activity on neuromuscular systems in which the derivatives have been found to promote nerve regeneration, and neuromuscular junction development (Strand et al., 1993; Strand et al., 1994).

MC5-R is the main subject of this thesis, and its pharmacology and possible functions will be discussed separately in the next section.

4. MC5 RECEPTOR

4.1. Molecular Characteristics of MC5-R

MC5-R has been cloned from mouse (Barrett et al., 1994; Chen et al., 1995; Chhajlani et al., 1993; Fathi et al., 1995; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). Using degenerate primers based on known GPCRs, Chhajilani et al. cloned human MC5-R (Chhajlani et al., 1993). Similar degenerate primers let Barrett et al. clone the ovine MC5-R from pars tuberalis cDNA and Fathi et al. clone human and mouse MC5-R (Barrett et al., 1994; Fathi et al., 1995). Gantz et al. cloned murine MC5-R from genomic DNA using degenerate primers based on MC1-R to MC4-R (Gantz et al., 1994). Griffon et al. identified a fragment using a rat dopamine D3 probe at low stringency (Griffon et al., 1994). They used the fragment to pull out rat MC5-R from a striatum cDNA library. Labbe et al. obtained murine MC5-R from a genomic library using human MC3-R as a probe (Labbe et al., 1994).

There is remarkable identity between the MC5 receptors from different species, and figure 5 shows an amino acid alignment of the MC5-R from different species. The conservation suggests that MC5-R plays some essential conserved function in mammals. There are many speculations on MC5-R function based on its pharmacological properties and its site of expression.

MC5-R is responsive to all melanocortins other than γ-MSH. Although discrepancy exists among the data from different laboratories about the EC_{50} value of various melanocortins for stimulation of cAMP production via the MC5-R, all the reports agreed upon the order of potency for melanocortins: NDP-α-MSH > α-MSH⊇ACTH(1-24) > ACTH (1-39) = β-

MSH >> γ-MSH. It is also generally true that α-MSH is about 5 times less potent at the MC5-R than at MC1-R, MC3-R and MC4-R. The same is also true for ACTH at the MC5-R than at the MC2-R. The discrepancy in EC_{50} values may result from differences in cell lines used to express the receptor, and the species from which MC5-R is cloned. Fathi et al. found that α-MSH is about 10 times more potent at the murine receptor than the human receptor (Fathi et al., 1995). The high EC_{50} (> 10^7) for γ-MSH makes MC5-R a unlikely candidate for mediating effects ascribed to γ-MSH.

4.2. Possible MC5-R Functions

Predicting a single role for the MC5-R has also been made difficult by virtue of the wide distribution of expression of this receptor. Northern analysis consistently detected MC5-R mRNA in skeletal muscle, brain, lung, adrenal, and stomach. By in situ hybridization, MC5-R was found in rat adrenal cortex, in both zona glomerolusa and zona fasciculata (Griffon et al., 1994). In situ hybridization also detected another MC5-R abundant tissue, the submaxilary gland (Griffon et al., 1994). Using more sensitive methods, such as RNAse protection assay (RPA) and RT-PCR, however, a large number of tissues were MC5-R positive. These include cerebral cortex, cerebellum, brain stem, olfactory bulb, pituitary, adrenal cortex, skin, tongue, thymus, spleen, bone marrow, etc. Based on the expression pattern, a number of possible functions have been attributed to this receptor. As most expression sites of MC5-R are in the peripheral tissues, some of the non-centrally mediated effects of melanocortins, such as aldosterone secretion, muscle proliferation and nerve regeneration, etc., may be functions of the MC5-R.

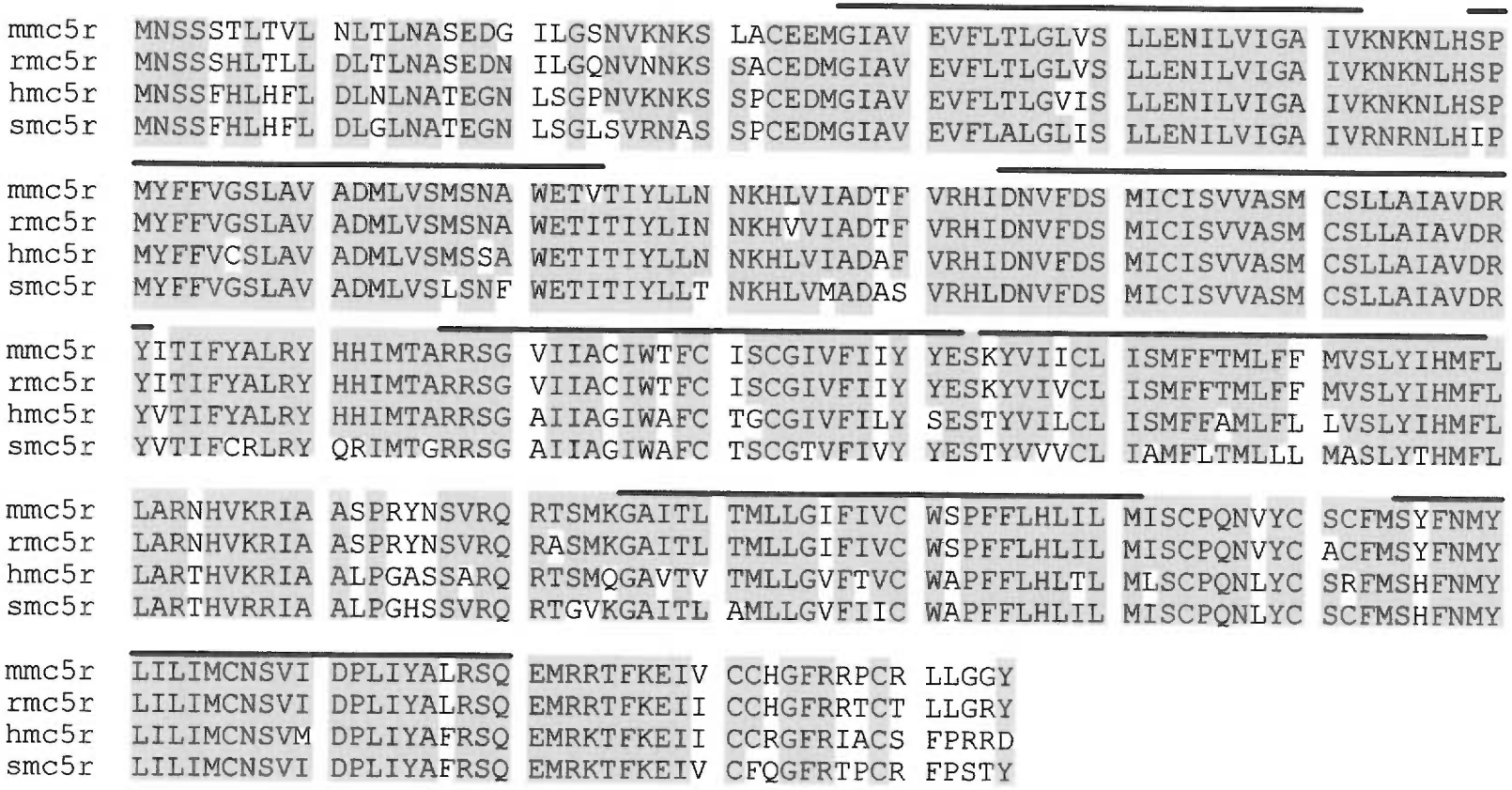


Figure 5. Amino acid sequence alignment of all cloned MC5-Rs.

Shaded residues are conserved in most of MC5-Rs. Bars span putative transmembrane domains according to Baldwin.

Systemic administration of melanocortins elicit a number of physiological and behavioral changes independent of adrenal gland function. As very little melanocortin peptides cross the blood-brain barrier (Wilson et al., 1984), these effects are conceivably due to activation of melanocortin receptors outside of blood-brain barrier. Although there are some domains of CNS that are outside of the barrier, many of these effects are presumably non-centrally mediated. These effects are therefore probably results of MC5-R activation, and are the subjection of discussion for the following sections. Whenever pertinent, the contribution from CNS and adrenal will also be mentioned.

4.2.1. Anti-inflammation

A component of the proposed peripherally mediated anti-inflammatory effects of melanocortins may involve MC5-R, as its mRNA is found in spleen and bone marrow (Labbe et al., 1994). Outside the anti-inflammatory activity of ACTH secondary to glucocorticoid production (Batemen et al., 1989), α-MSH also potently blocks inflammation. The anti-inflammatory action of α-MSH is thought to be a result of inhibiting IL-1β and TNF activity. α-MSH inhibits several IL-1β elicited effects. For example, it blocked IL-1β elicited HPA axis activation (Daynes, 1987), hypothermia, and elevation of serum amyloid P and circulating neutrophils (Robertson, 1986). α-MSH also antagonized IL-1β induced acute inflammation and hypersensitivity (Hiltz et al., 1992; Hiltz and Lipton, 1990). Intraperitoneal injection of α-MSH reduced κ-carrageenan induced paw edema and archidonic acid induced ear swelling in a dose responsive fashion in the mouse (Lipton et al., 1994). High dose of the endotoxin LPS results in lethality due primarily to massive TNF production. α-MSH and its derivative HP228 reduced LPS-

induced TNF synthesis and lethality in mice (Girten, 1994; Girten, 1995), and LPS-induced nitric oxide synthesis stimulation (Abou-Mohamed et al., 1995). There is some inconsistency in the literature as to whether the melanogenic pharmacophore is necessary for the anti-inflammatory actions. Several reports from Lipton and colleagues indicated that some anti-inflammatory activity resides in the three residues at the C-terminus (α-MSH 11-13) (Hiltz et al., 1992; Hiltz et al., 1991). There is a possibility that α-MSH may act upon a receptor outside the melanocortin receptor family to block inflammation. Another uncertainty is the site of action. Several lines of evidence suggest that the anti-inflammatory activity of α-MSH is largely centrally mediated (Macaluso et al., 1994). Central administration of α-MSH effectively inhibited IL-1 induced ear inflammation and κ-carrigeenan elicited hind paw edema. This action is mediated by descending β-adrenergic neurons. Both spinal transection and a β2-adrenergic antagonist markedly reduced central α-MSH activity (Macaluso et al., 1994). Further, the anti-inflammtory action of peripherally administered α-MSH is also largely dependent on intact spinal cord, suggesting it may also be centrally mediated (Macaluso et al., 1994).

4.2.2. Aldosterone Secretion

The expression of MC5-R in adrenal cortex, particularly in zona glomerulosa of adrenal gland, led Griffon et al. to postulate a role for the receptor stress-induced aldosterone secretion (Griffon et al., 1994). Several studies demonstrated a role of α-MSH in adrenal cortex . However, the steroidogenic activity of α-MSH in isolated rat adrenal cells was 100- 1000 times less potent than that of ACTH (Baumann, 1986; Vinson et al., 1983;

Vinson et al., 1981). α-MSH was also effective in vivo, albeit at very high dose. A single injection of 100 mg/kg α-MSH markedly increased serum aldosterone levels. Further increase of aldosterone was achieved by daily administration for 6 days (Robba, 1986). α-MSH also increased aldosterone production in hypophysectomized rats (Shenker, 1985) and the order of potency of melanocortins in induction of this activity roughly parallels their activity at the cloned MC5-R. However, the physiological significance of this α-MSH action is questionable as the dosage used for this studies are much higher than serum α-MSH levels and non-physiological.

4.2.3. Neuromuscular System

Another plausible function of MC5-R is mediation of the mitogenic activities of melanocortins on skeletal muscle cells. Both α-MSH and ACTH are mitogenic to satellite cells from skeletal muscle (Cossu et al., 1989; De Angelis, et al. 1992). As satellite cells are implicated in muscle regeneration, melanocortin-stimulated proliferation may therefore be a compensatory mechanism for muscle damage. Indirect evidence support this notion. Both the number of binding site and the quantity of α-MSH/ACTH immunoactivity in skeletal muscle were increased in mice with muscle pathology (Hughes and Smith, 1994; Hughes et al., 1992; Hughes et al., 1992; Smith and Hughes, 1995; Smith and Hughes, 1994). The presence of MC5-R in skeletal muscles also make it a candidate through which α-MSH and its derivatives accelerate neuromuscular maturation during embryonic development (Strand et al., 1993; Strand et al., 1994; Strand et al., 1993), and in peripheral nerve regeneration after crush (Bijlsma, 1983; Strand, 1980).

4.3.4. Sebotropic Activity

Being a peripheral receptor responsive to both ACTH and α-MSH, MC5-R mediated functions may under pituitary control. One function of pituitary melanocortin peptides is their sebotropic activity. In the rats, hypophysectomy diminished sebum production (Ebling et al., 1970; Thody and Shuster, 1970). The reduction was fully recovered by concomitant α-MSH and androgen administration (Ebling et al., 1975; Thody et al., 1976), while either one was only partially effective. Thus, Thody suggested calling α-MSH a sebotropic hormone (Thody and Shuster, 1973). α-MSH may increase sebum production by promoting lipogenesis in the sebaceous gland (Cooper et al., 1974; Thody et al., 1976). The mechanism of the synergism between α-MSH and adrogens is not clear. The preputial gland, a specialized sebaceous gland in rodents, is also α-MSH responsive, and α-MSH and androgens are synergistic in stimulating lipogenesis (Cooper et al., 1976; Thody et al., 1981). In the preputial gland, α-MSH may act by altering adrogen metabolism (Hay et al., 1982).

4.2.5. Pheromone release

The preputial gland is a source of pheromone for a variety of social interactions in rodents (Bronson and and Caroom, 1971; Chipman and and Alberecht, 1974; Orsulak, 1972). As preputial gland is an α-MSH target, it is conceivable that α-MSH may alter pheromone release. In fact, injection of α-MSH in rats has been shown to elicit several behavioral changes in the conspecific animals. Among these changes are altered sexual attraction of male rats toward female recipients (Thody et al., 1981; Thody and Wilson, 1983; Thody et al., 1981), and heightened aggression of conspecifics and increased submission of

precipitant male mice (Nowell et al., 1980; Paterson, 1980; Plotnikoff, 1976). The aggression was induced by an olfactory cue, possibly from urine (Nowell et al., 1980; Nowell et al., 1980). By the same token, α-MSH also decreases active social interaction in rats (File, 1978).

Stress also produces pheromones that serve as an alarm chemosignal in rodents. For example, non-stressed rats were able to recognize and avoid odors from stressed rats (Mackay-Sim, 1980; Valenta, 1968). The odors are presumably originated from skin (Mackay-Sim and Laing, 1981). Stress odors from rats also induced analgesia in non-stressed conspecies (Famselow, 1985). In another paradigm, rats exude an "alarm substance" into the water that alerted subsequent rats after a forced swim (Abel and Bilitzke, 1990). The secretion depends on pituitary but not on adrenal gland (Abel, 1994; Abel and Bilitzke, 1992). High dose ACTH in hypophysectomized rats produced the same substance (Abel, 1994). It is uncertain which receptor may mediate the secretion of the materials, as the site of release is not known. Similarly in the mouse, stress provided an olfactory cue that causes aversion in non-stressed conspecifics (Carr, 1970; Rottman, 1972). Experience of conspecific stress odors alters both cellular and humoral immune responses (Cocke, 1993). Furthermore, severe stress induces aggression of cohorts (Mugford, 1971), a behavior that is recapitulated by α-MSH injection (Nowell et al., 1980). As MC5-R is expressed in the periphery, and responds to both ACTH and α-MSH, it is a strong candidate for the release of the stress related pheromones.

4.2.6. Avoidance Behaviors

Another function of pituitary melanocortins are fear and avoidance response. Besides adrenal trophic activity and adipocyte lipolytic activity attributed to MC2-R activation,

pituitary ACTH also appears to be involved in a number of other adrenal independent physiological and behavioral process. The passive and active avoidance behaviors are impaired by hypophysectomy in the rats (De Wied, 1964; Mirsky, 1953; Weiss, 1970). Systemic administration of ACTH completely rectified the impairment. This is MC2-R independent, because: 1). adrenalectomy enhanced avoidance behavior presumably by elevating serum ACTH levels (Weiss, 1970), 2). nonsteroidogenic melanocortins were equivalently potent (De Wied and De Kloet, 1988). The site of action for these behaviors are not certain. The CNS has been take for granted as the target, although administration has always been peripheral, i.e. subcutaneously, intraperitoneally, or intravenously. The amount of α-MSH used to enhance avoidance behaviors is 10 to 20 μg, very low compare to the requirement of many other assays. Peripheral tissues are therefore equally possible sites for the action.

4.2.7. Lipolysis

MC5-R may be partially responsible for melanocortin-induced lipolysis in adipocytes (Ramachandran, 1976). The pharmacological profiles of this melanocortin effect varies among species. In rat adipocytes, α-MSH is much less potent than ACTH. In contrast, α-MSH is several times more potent than ACTH in rabbit fat cells (Ramachandran, 1976). The demonstration of MC5-R, albeit at low levels, in mouse fat tissues supports the notion that the difference between species may be a result of different llevels of expression of MC5-R in adipocytes (Boston and Cone, 1996). This hypothetical function of MC5-R was another potential explanation for obesity in A^y mice. Although ineffective in inhibition of MC5-R signaling in vitro (Lu et al., 1994), agouti may block MC5-R activation in vivo. Recent data, however, suggest that the MC4-R is the primary target of agouti in the induction of obesity (Fan et al., 1997; Huszar et al., 1997).

4.2.8. Tear Production

Using rat lacrimal gland acini as a model of exocytosis, Jahn et al. found α-MSH and ACTH potently stimulate protein secretion, with an EC_{50} of about 10 nM. That 3-isobutyl-1-methylxanthine (IBMX), a potent inhibitor of cAMP diesterase, potentiated the activity of melanocortins suggests the involvement of cAMP. Indeed, cAMP was increased 4 to 5 fold by ACTH and α-MSH (Jahn et al., 1982). Salomon and co-workers further characterized the receptor in rat lacrimal gland by radioactive NDP-α-MSH binding. The receptor has an equal affinity of 10 nM for both α-MSH and $ACTH_{1-24}$ (Leiba et al., 1990). In the same study, they also found a similar receptor in the Harderian gland (Leiba et al., 1990).

5. SCOPE AND AIMS OF THE THESIS

The previous introduction has elucidated the importance of melancortins in a number of physiological and behavioral processes in addition to the trophic activities on melanocytes and adrenal cortex. Many of these effects are likely through activation of the cloned melanocortin receptors. At the initiation of this project, none of the other melanocortin effects has been defined experimentally. Several issues were raised at that time: 1).Are the effects truly results of activation of known melanocortin receptors? If so, which receptor is responsible for a given effect. 2). Is there any functional overlap among the known receptors. 3). Do melanocortins partake in physiological processes other than what have been described?

The goal of this research has been to determine the physiological function(s) of MC5-R, and identify which, if any, of the reported biological activities are dependent on MC5-R signaling.

6. DESIGN OF EXPERIMENTS AND RATIONALES

To explore possible MC5-R functions in more details, more extensive characterization of its expression is necessary. Localization of MC5-R mRNA at the cellular level within receptor positive tissues may provide hints regarding its mechanism of action.

To surely define the physiological role of a gene, a common approach is to specifically manipulate the activity of the gene product in vivo and study the consequent pathophysiology. Two approaches have been commonly used to manipulate G protein coupled receptor activities, administration of specific agonists/antagonists, and gene deletion. Both approaches have advantages and disadvantages. The genetic approach completely abolishes the function of the gene of interest, but may result in embryonic lethality, which makes determination of functions in adulthood impossible. It may also activate compensatory mechanisms and mask the normal function of the gene. In contrast, the pharmacological approach is developmental stage independent. It is also not restricted by strains and omits time- and labor-consuming breeding. Targeting of the agent to an organ/tissue of interest is possible. However, not every tissue is equally accessible to exogenous compound. In addition, complete and receptor-specific inhibition by an antagonist is difficult to achieve, and no melanocortin antagonists were available at the onset of this study. Thus, both approaches were undertaken at the initial stage. The mouse is then the ideal experimental system as it is amenable to both types of manipulation. Chapter two describes the cloning of the MC3-R, MC4-R, and MC5-R from a mouse genomic library, as well as restriction enzyme mapping and sequencing of the murine MC5-R. Chapter three elucidates the development of a high through put screening system for Gs and Gq-coupled receptors, for the purpose of identifying specific melanocortin antagonists. Chapter four focuses on characterization of MC5-R expression and physiological abnormalities in mice with an MC5-R deletion. And finally, chapter five

illustrates an extension of the HTS that is potentially applicable in capture of CREB activating genes/alterations.

CHAPTER TWO

CLONING OF MURINE MC3-R, MC4-R AND MC5-R AND CHARACTERIZATION OF THEIR EXPRESSION DURING DEVELOPMENT

SUMMARY

The genes encoding mouse MC3-R, MC4-R, and MC5-R were cloned from a genomic library. Partial sequence of MC3-R and MC4-R revealed high homology to their human counterparts. The expression of these receptors during mouse development was examined by in situ hybridization. MC4-R mRNA was detected as early as embryonic day 13.5 (E13.5). At E16.5, MC4-R was found in many locations in the CNS, with the highest expression in the cortical plate, brain stem, thalamus, hypothalamus, spinal cord, and dorsal root ganglia. The expression of MC3-R and MC5-R was not detectable during the same period. The MC5-R containing clone was further characterized by restriction mapping and by sequencing of the coding region. The pharmacological properties of MC5-R expressed in HEK293 cells was also studied. The order of potency for melanocortins for activation of the MC5-R is: NDP-α-MSH > α-MSH > ACTH = β-MSH >> γ-MSH.

INTRODUCTION

Melanocortins are a family of peptide hormones that induce pigment dispersion/eumalanization in vertebrate melanocytes and corticosterone production in adrenal cortex (Eberle, 1988). They include α-, β-, γ-melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH, or corticotropin). These peptides are derived from a common precursor, pro-opiomelanocortin (POMC) (Nakanishi et al., 1979; Roberts et al., 1979) , by the concerted action of prohormone convertases and carboxypeptidases in the pituitary gland (Smith and Funder, 1988). Other tissues also produce melanocortins, albeit at much lower quanity. Among them are hypothalamus and NTS in the CNS, placenta, reproductive tracts, and a number of POMC expressing tumors in the periphery (DeBold et al., 1988; DeBold et al., 1988; Smith and Funder, 1988)..

In addition to stimulation of adrenal cortex and melanocytes, melanocortins are implicated in avoidance behavior, grooming, theromoregulation, feeding, aggression, sebum production, natriuresis, hemodynamics, analgesia, and lacrimal gland function (Eberle, 1988). These functions is likely mediated by receptors located outside of adrenal cortex and melanocyte. Recently, several receptors for melanocortin peptides have been cloned (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993; Gantz et al., 1993; Mountjoy et al., 1994; Mountjoy et al., 1992; Roselli-Rehfuss et al., 1993). They are all G protein coupled receptors that activate adenylyl cyclase. In addition to the receptors that are expressed in adrenal cortex and in melanocytes, three novel melanocortin receptors have been discovered. These receptor are named MC1-R to MC5-R, according to their order of discovery. MC3-R and MC4-R are primarily expressed in CNS and the summation of their expression sites superimposes well with the binding sites of melanocortin in the brain (Gantz et al., 1993; Gantz et al., 1993; Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993). Both receptors are sensitive to α-MSH. MC3-R is also

equally responsive to γ-MSH. Together, MC3-R and MC4-R may be responsible for all the CNS effects of melanocortins (Gantz et al., 1993; Gantz et al., 1993; Mountjoy et al., 1994; Mountjoy et al., 1992; Roselli-Rehfuss et al., 1993).

A number of melanocortin functions in peripheral tissues are independent of adrenal cortex and melanocytes. Among them are activation of sebaceous gland function (Ebling et al., 1970; Thody and Shuster, 1970), sexual and social behaviors (Bertolini and Gessa, 1981; Bertolini et al., 1986; Nowell et al., 1980; Thody et al., 1981), fear and avoidance behavior(De Wied, 1964; Mirsky, 1953; Weiss, 1970), anti-inflammation (Hiltz et al., 1992; Lipton et al., 1994), and lipolysis (Ramachandran, 1976). MC1-R and MC2-R display highly tissue specific expression (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992). MC1-R is expressed at high level in some melanoma (Mountjoy et al., 1992), but very low levels in CNS and macrophages (Chhajlani, 1996; Star et al., 1995). MC2-R presents predominantly in adrenal cortex, and at low levels in muscle and fat (Boston and Cone, 1996).

Consequently, peripheral functions outside of pigmentation and adrenocortical steroidogenesis are very likely results of activation of other melanocortin receptors. MC5-R is expressed in skeletal muscle and many other peripheral tissues (Barrett et al., 1994; Chhajlani et al., 1993; Fathi et al., 1995; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). It is therefore a candidate for mediating some of the effects caused by systemic administration of melanocortins. However, the functional significance of the detected MC5-R mRNA is not certain, as it is present at very low levels and the bearing of ligand binding sites in these tissues has not been demonstrated.

The structure-function relationships for some physiological and behavior assays differ from that for the cloned receptors expressed in culture. For example, ACTH and ACTH4-10

were found to be equally potent in facilitation of active and passive avoidance (de Wied and de Kloet, 1988). However, $ACTH_{4-10}$ is at least 3 mangnitudes less effective than ACTH in activation of all the cloned receptor (Adan et al., 1994; Gantz et al., 1993; Gantz et al., 1993; Roselli-Rehfuss et al., 1993). Furthermore, Org2766, a derivative of $ACTH_{4-10}$, is much more potent in the same assay than its parental compound (de Wied and de Kloet, 1988). However, Org2677 showed no activity at the cloned melanocortin receptors (Adan et al., 1994; Gantz et al., 1993; Gantz et al., 1993; Roselli-Rehfuss et al., 1993). In other cases, the activity determinants for physiological responses are mapped out side of the melanogenic pharmacophore, HFRW. α-MSH is a potent anti-inflammatory agent. However, Lipton and co-workers have mapped one component of the anti-inflammatory activity to the C-terminal tripeptide (Hiltz and Lipton, 1989). γ-MSH has very strong hypertensive and tachycardiac responses when given intravenously. The pressor and tachycardiac activity of γ-MSH has also been mapped to its C-terminus, only including the FRW of the tetrα-peptide core (Van Bergen et al., 1995). There are at least two possible explanations for the observed discrepancies. Either the receptors expressed in heterologous cells do not represent their native characteristics, or these other peptides work on unidentified receptors to achieve their physiological output. A ultimate approach to resolve the issue is to delete individual receptors from the genome and examine the consequences in the above mentioned aspects. In doing so, it is also possible to explore novel physiological functions for these receptors.

Since its introduction, gene "knock-out" technology has become mature and hundreds of genes have been disrupted (Soriano, 1995). We decide to use the method to determine the functions of individual novel melanocortin receptors in the mouse. In this chapter, I shall describe the results of cloning of murine genomic DNAs encompassing MC3-R, MC4-R

and MC5-R coding sequences along with characterization of their expression during mouse development and pharmacological properties of MC5-R.

MATERIALS AND METHODS.

Materials A mouse (strain 129 Sv/J) genomic library in λ FIX II was obtained from Stratagene, CA. Restriction endonucleases were products of Gibco BRL, (MD), Boehringer Mannheim, (IN), or NEB, MA. Riboprobe Gemini System, Prime-α-Gene Labeling system and pGEM-T were from Promega, (WI). Sequenase deoxynucleotide sequence kit is made by USB, (MD). pcDNA I Neo was from Invitrogene, (CA). Lipofectin reagents is a product of BRL, (MD).

Library Screen. One million pfu of phages were screened with equal amount of ^{32}P-labeled DNA fragments encompassing TM3-TM6 of rat MC3-R and rat MC4-R (Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993) at inter-mediate stringency (1 M NaCl. 10 x Denharts' solution, 50 mM Tris.HCl, pH7.4, 0.1% pyrophosphate, 0.2% SDS, 10% Dextran sulfate, 100 mg/ml salmon sperm DNA, 40% formamide at 42°C). The final wash for the filters was in 0.5 x SSC, 0.1% SDS at 60 °C for 15 min. Positive clones were selected by two more rounds of hybridization with the same probes. The stringency for the secondary and tertiary screen was raised by changing formamide to 45% in the hybridization solution, and 0.1 x SSC, 65°C for the final washes.

Isolation of λ DNA. Aliquots of about 10^5 pfu of phages from eluant of a single λ clone were plated onto 150 mm dishes. After overnight incubation at 37 °C, phage particles were eluted from plates with 10 ml of SM solution at room temperature for 2 to 6 hours. Eluates of the same clone were pooled for further processing. After removal of

debris by centrifugation at 10, 000 rpm for 15 minutes, the phage containing supernatant was carefully loaded over two layers of glycerol in a SW27 tube [layer 1, 8 ml 10% glycerol in 50 mM Tris.HCl, pH7.4, 10 mM $MgSO_4$ (TM); layer 2, 6 ml 40% glycerol in TM]. Phage particles were pelleted by 25,000 rpm, 2 hour centrifugation at 4 °C. The particles were re-suspended into 0.5 ml of TM. After addition of 12.5 ml 20% SDS and 20 ml 0.5 M EDTA, protein in the particles was removed by proteinase K digestion (5 mg/ml) at 55 °C for one hour, followed by several rounds of phenol/chloroform extraction. After addition of NaOAc to 0.3M, phage DNA was precipitated by addition of 2 volumes of ethanol and incubation at -20 °C for 1 hour. The DNA is pelleted, washed with 80% ethanol, air dried, disolved in TE (10 mM Tris.HCl, pH7.5, 0.1 mM EDTA). Restriction mapping and subcloning were conducted following standard protocols (Sambrook, 1989).

PCR amplification. Two degenerate primers, MCR1DNO and MCR2DNO, were used to amplify the melanocortin receptor sequences spanning TM2 to TM7 using PCR (Mullis et al., 1986; Saiki et al., 1986). The primers were based on all the melanocortin receptors known at the time. These included human MC1-R, MC3-R, MC4-R, MC2-R, MC5-R, mouse MC1-R, rat MC3-R, and bovine MC2-R. The sequences are: MCR1DNO:

5'-C/T/CGT/C/A/CGT/GT/C/ACG/C/C/CG/A/T/G/T/A/CT/T/AGT/CT/T/T

MCR2DNO:

5'-T/C/CT/AGT/G/AG/C/T/CG/CT/G/AG/A/AG/AGT/G/C/AG/T/A/AGT/A/T

PCR was carried out in 100 ml standard Taq polymerase buffer (Promega) suplemented with 200 mM dNTP, 3 mM of MgCl2, 5 μM of each primer, 2 units Taq polymerase (Promega). DNA was amplified in a MJ mini cycler for 35 cycles. One cycle consisted of 94 °C, 1 min, 50 °C, 1 min, 72 °C 1 min. PCR products were purified with Qiagen columns, cloned into pGEM-T vector and sequenced using Sequenase 2.0 kit. Sequences were analysed by GCG program (Devereux et al., 1984).

In situ hybridization. Sense and antisense RNAs were synthesized in vitro in the presence of ^{35}S-UTP [NEN, 3000 Ci/mmol] using Promega Riboprobe Gemini System as instructed. The probes were purified through a Pharmacia Nick translation column. Perfusion, tissue preparation and cryosectioning, prehybridization, hybridization and autoradiography were performed according to Simerly et al [[Simerly, 1990 #1633]. The anatomical structures of positive hybridization sites were identified according to Hoffman (anatomy,)

Transfection and Adenylyl cyclase assay An 1.6 kb Apa I/ Hind III fragment (see Figure) that contains the coding sequence of MC5-R was inserted in pcDNA I Neo under the control of CMV promoter. This resulted plasmid was used to transfect HEK 293 cells using lipofection reagents following manufacturer's instructions. Two days after transfection, cells were selected in 400 mg/l of G418 for 3 weeks. Selected cell populations were used to study melanocortin induced adenylyl cyclase activity. Adenylyl cyclase assays were carried out essentially following the method of Salomon (Salomon, 1991; Salomon et al., 1974). Briefly, cells at confluence in 24-well plates ($2.5x10^5$ cells/well) were labeled with ^{3}H-Adenine for 1 hour (5 μCi/well). Cells were then stimulated with hormones in incubation medium for 30 minutes. Cell lysates were prepared by treatment with 2.5% PCA at 4 ^{o}C for 30 minutes. Supernatants of a KOH precipitation were subjected to Dowex 50 and alumina chromatography to purify ^{3}H-cAMP. Results are described as % conversion of ^{3}H-adenine to ^{3}H cAMP, and are proportional to adenylyl cyclase activity in the absence of transport or degradation of cAMP.

Data Analysis. All data points are the means of triplicate determinations if not defined otherwise, and bars indicate standard deviation. Data was plotted using Prism Version

1.02 from GraphPad (San Diego, CA). Curves were fitted and EC_{50} values were determined by non-linear regression.

RESULTS

Cloning of Murine MC3-R, MC4-R and MC5-R.

The cloning process is outlined in figure 1. A primary screen identified about 100 positive plaques. Twenty of them with strong signal were carried on for further screening at higher stringency. Among those 8 yielded strong hybridization after tertiary screen. All the clones contained an insert of 14 to 18 kb. Restriction endonuclease digestion analysis of the λ DNAs identified five overlapping groups. Further analysis of PCR products from representive clones of each group by restriction digestion revealed only four groups. Three of the four groups were cloned and partially sequenced. Sequence analysis indicated that they are mouse MC3-R, MC4-R and MC5-R (Figure 2). The nucleotide sequence homology between mouse and human receptors is about 90% for both MC3-R and MC4-R in the sequenced region. At the amino acids level, the sequence identity is more than 95%.

Characterization of Murine MC5-R

The mouse MC5-R gene was further characterized. Only one of the λ clones contained MC5-R. The insert of this clone was mapped with several restriction endonucleases, as shown in Figure 3a. The 9.0 kb Sac I fragment was subcloned and mapped further with more enzymes (figure 3b). Hybridization with the PCR product revealed that the coding region resides in the 1.6 kb Apa I/Hind III fragment. This fragment was then cloned under

the control of a CMV promoter in pcDNA I Neo for expression. The nucleotide sequence of the entire reading frame was also progressively determined using primers based on known sequence. It was identical to the published murine sequence (Fathi et al., 1995; Gantz et al., 1994; Labbe et al., 1994). The translated amino-acid sequences showed features of a typical G protein coupled receptor, with seven putative transmembrane domains and signature residues (Figure 4). In addition, there is remarkable amino acid sequence homology (>80%) to human MC5-R. Several putative glycosylation sites and protein kinase C phosphorylation sites are present in the extracellular and intracellular domains of MC5-R, respectively. The conserved cysteine residue for myristylation is also present. These modifications may be of functional importance.

Expression of MC3-R and MC4-R during embryonic development.

Antisense riboprobes revealed expression of MC4-R in cortical plate as early as E13.5. At E16.5, prominent hybridization with MC4-R mRNA was seen in the cortical plate, brainstem, thalamus, hypothalamus, spinal cord, and dorsal root ganglia (Figure 5.) Intensive hybridization signal was also detected in the site of teeth primordia. Unfortunately, the hematoxylin/Eosin counter stain used for these sections does not allow detailed neuronal structural identification. In the spinal cord, the precise distribution is also not clear as only sagital sections were prepared. This work was not followed up because very little MC5-R, the focus of this thesis, was seen during embryonic development and the first postnatal day of life in the mouse.

Functional Expression and Characterization of MC5-R.

The MC5-R expression cassette in pcDNA I Neo was transfected into HEK293 cells to establish stable transfectants. A G418 selected cell population was assayed for their responsiveness to melanocortins in an adenylyl cyclase stimulation. Figure 6 demonstrates

that murine MC5-R is sensitive to all natural melanocortins except γ-MSH. The EC_{50} for α-MSH, β-MSH and ACTH are 7 nM, 29 nM and 34 nM, respectively. The synthetic, "superpotent" NDP-α-MSH displayed a lower EC_{50}, and also a somewhat lower Vmax. The degree of maximal stimulation by NDP-α-MSH varied from one experiment to another, but was consistently smaller than that of the other peptides.

DISCUSSION

Mouse MC3-R, MC4-R and MC5-R have been cloned from a genomic library. Striking homology was found within the sequenced region between each subtype of human and mouse melanocortin receptor. There is more than 95% identity between mouse and human MC3-R, MC4-R and MC5-R in the sequenced region (Figure 2). The identity among different subtypes within a species is much lower. In the mouse, the identity among the sequenced region of MC3-R, MC4-R and MC5-R is about 70% at nucleotide level, and only about 62% at amino acids level. This is also true for MC1-R and MC2-R The amino acid sequence identity between human and mouse MC1-R is 76%, MC2-R, 89%. The divergency among the subtypes provides molecular basis for pharmacological differences. Together, these data suggest that each of the melanocortin receptor may play unique yet important functions.

The library was made from mouse strain 129SV/J, from which most ES cells were derived. It has been noticed that isogenic DNA yields higher homologous recombination (te Riele et

al., 1992). Therefore, these genomic sequences will be useful in making constructs for gene deletion in the mouse.

The expression pattern of MC4-R in embryonic development in mouse more or less resembles that in adult rats (Mountjoy et al., 1994). All the major MC4-R expressing regions in rat brain synthesis MC4-R early in development. The timing of MC4-R expression corelates well with POMC expression. Detectable POMC gene mRNA is seen as early as E10.5 in mouse brain []. Therefore, it is likely that MC4-R may play a subtle role during ontogeny, since no major developmental defects were seen in the MC4-R deficient mice.

MC5-R displayed nM range of EC_{50} for all melanocortins except γ-MSH. This is similar to human MC1-R and human MC4-R (Gantz et al., 1993; Mountjoy et al., 1994; Mountjoy et al., 1992). It differs from human MC2-R, which is selective for ACTH (Mountjoy et al., 1992), and MC3-R , which is equally responsive to all melanocortins (Gantz et al., 1993; Roselli-Rehfuss et al., 1993). However, the EC_{50} for activation of adenylyl cyclase by MC5-R is somewhat higher than that of MC2-R, the adrenocortical ACTH receptor. Assuming data represent the pharmacological characteristics of MC5-R in vivo, this property may be functionally significant. It may make MC5-R a sensor of elevated levels of circulating ACTH, a status associated with stress and illness.

SUMMARY

In order to disrupt the three novel melanocortin receptor genes, mouse MC3-R, MC4-R and MC5-R sequence have been isolated from a 129SV/j genomic library. Preliminary characterization of these genes revealed the following:

1. The mouse receptors are highly homologous to their human counterparts, implying important function.

2. MC4-R expression begins early in development, suggesting a possible role for embryogenesis.

3. MC5-R is less sensitive to melanocortin than the other melanocortin receptors, indicating a possible role during stress, or suggesting possible paracrine/autocrine action.

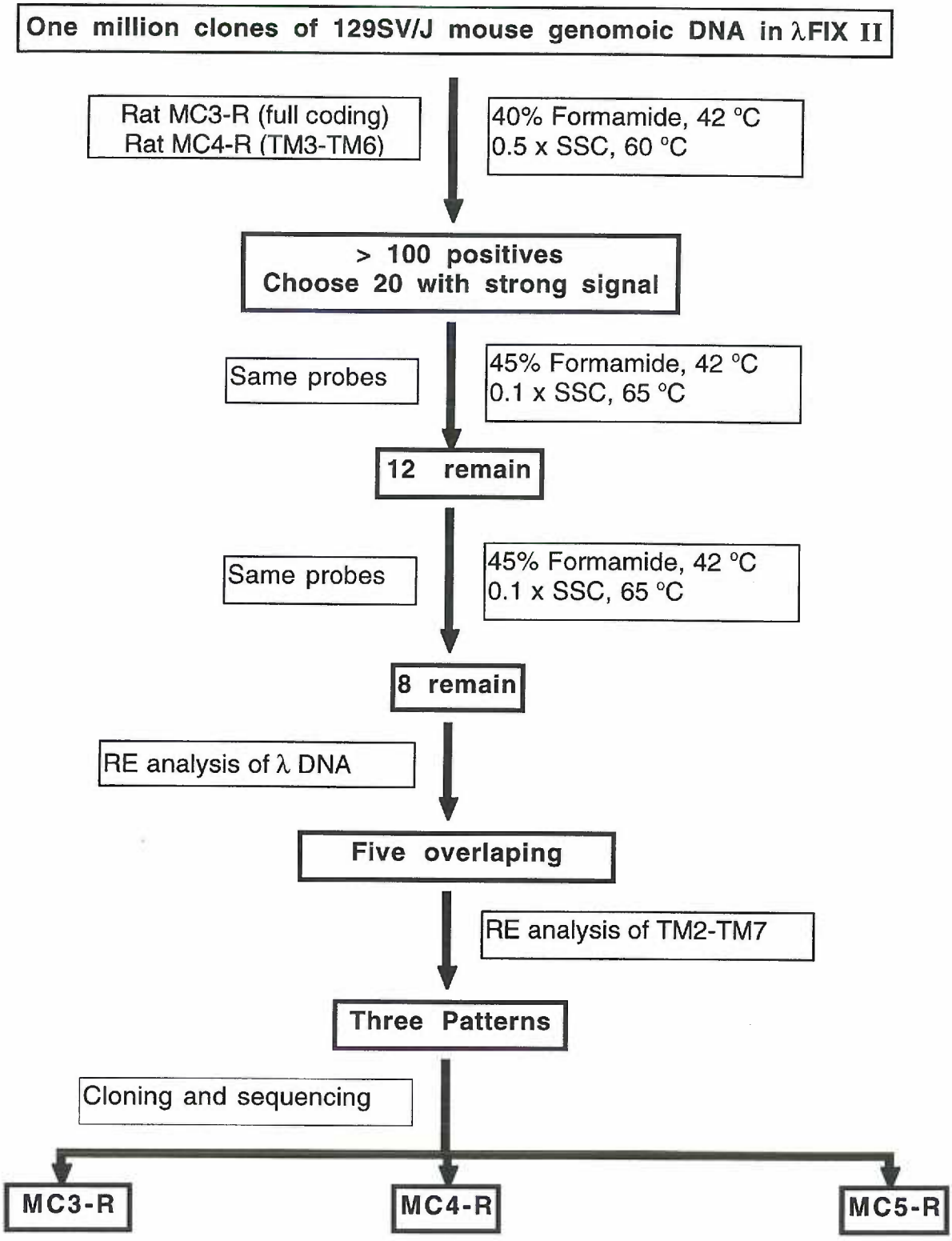


Figure 1. Outline of the screening process

A. Nucleotide sequence alignment of mouse MC4-R (start from extracellular loop 1) with that of human MC4-R

```
 1 CATCTGTAGCCTGGCTGTGGCAGATATGCTGGTGAGCGTTTCGAATGGGTCGGAAACCATCGTCATTACCCTGTTAAACA  80
   |||||| ||| |||||||||| |||||||||||||||||||| ||||| || |||||||| |||| ||||| |||||||
639CATCTGCAGCTTGGCTGTGGCTGATATGCTGGTGAGCGTTTCAAATGGATCAGAAACCATTATCATCACCCTATTAAACA 718

81 GTACGGATACGGATGCCCAGAGCTTCACCGTGAACATTGATAATGTCATTGACTCTGTGATCTGTAGCTCCTTGCTCGCA 160
   |||| |||||||||||| ||||| ||||| ||||| |||||||||||||||||||| ||||||||||||||||||| |||
719GTACAGATACGGATGCACAGAGTTTCACAGTGAATATTGATAATGTCATTGACTCGGTGATCTGTAGCTCCTTGCTTGCA 798

161TCCATTTGCAGCCTGCTTTCCATTGCGGTGGACAGGTATTTCACTATCTTTTACGCGCTCCA 222
   |||||||||||||||||||| ||||| |||||||||||| || |||||||| || || |||||
799TCCATTTGCAGCCTGCTTTCAATTGCAGTGGACAGGTACTTTACTATCTTCTATGCTCTCCA 860
```

B. Nucleotide sequence alignment of mouse MC4-R (end at extracellular loop 3) with that of human MC4-R

```
1    CACATTAAGAGGATTGCTGTCCTCCCAGGCACAGGGACCATCCGCCAGGGTACCAACATGAAGGGGGCGATTACCTTGAC
     |||||||||||||||||||||||||| ||||| ||  |||||||||| ||| |||| |||||||| ||||||||||||||
1057 CACATTAAGAGGATTGCTGTCCTCCCCGGCACTGGTGCCATCCGCCAAGGTGCCAATATGAAGGGAGCGATTACCTTGAC

81   CATCCTGATTGGAGTCTTTGTTGTCTGCTGGGCCCCGCTCTTTCTCCATTTACTGTTCTACATCTCTTGCCCTCAGAATC
     |||||||||||| |||||||||||||||||||||  |||| ||||| ||| | |||||||||||||| |||||||||
1137 CATCCTGATTGGCGTCTTTGTTGTCTGCTGGGCCCCATTCTTCCTCCACTTAATATTCTACATCTCTTGTCCTCAGAATC

161  CATACTGCGTGTGCTTCATGTCTCATTTTAATGTGTCTCTCATACTGATCATGTGTAACGCCGACATCGACCCT 234
     |||| || |||||||||||||||| |||||  ||| |||||||||||||||||||||  |   |||||| |||
1217 CATATTGTGTGTGCTTCATGTCTCACTTTAACTTGTATCTCATACTGATCATGTGTAATTCAATCATCGATCCT 1290
```

C. Nucleotide sequence alignment of mouse MC3-R (start from extracellular loop 1) with that of human MC3-R

```
2    CTGTGCAGCCTGGCTGCAGCCGACATGCTGGTGAGCCTGTCCAACTCCCTGGAGACCATCATGATCGCCGTGATCAACAG
     || |||||||||||  |  |||||||||||||  ||  |||||||  |||||||||||||||||||||||  |  ||  ||||
340  CTCTGCAGCCTGGCGGTGGCCGACATGCTGGTAAGTGTGTCCAATGCCCTGGAGACCATCATGATCGCCATCGTCCACAG

82   CGACTCCCTGACCTTGGAGGACCAGTTTATCCAGCACATGGATAATATCTTCGACTCTATGATTTGCATCTCCCTGGTGG
     |||||  |||||||||  |||||||||||||||||||||||||||  ||  ||||||||||  |||||  ||||||||||||||||
420  CGACTACCTGACCTTCGAGGACCAGTTTATCCAGCACATGGACAACATCTTCGACTCCATGATCTGCATCTCCCTGGTGG

162  CCTCCATCTGCAACCTCCTGGCCATTGCCATCGACAGGTACGTCACCATCTTCTATGCCCTTCGGTACCACAGCATCATG
     |||||||||||||||||||||||||  |||  ||||||||||||||||||||||  ||  ||  ||  ||||||||||||||||
500  CCTCCATCTGCAACCTCCTGGCCATCGCCGTCGACAGGTACGTCACCATCTTTTACGCGCTCCGCTACCACAGCATCATG
```

D. Nucleotide sequence alignment of mouse MC3-R (end at extracellular loop 3) with that of human MC3-R

```
3    GGCTCCACGTCCAGCGCATCGCAGTGCTGCCCCCTGCTGGCGTGGTGGCCCCACAGCAGCACTCCTGCATGAAGGGGGCT
     ||||  ||||||  |||||||  ||||  |||||  |||||  |  ||  ||||||||||||||  |||||  ||||||||||||||
755  GGCTGCACGTCAAGCGCATAGCAGCACTGCCACCTGCCGACGGGGTGGCCCCACAGCAACACTCATGCATGAAGGGGGCA

53   GTCACCATCACTATCCTGCTGGGTGTTTTCATCTTCTGCTGGGCGCCTTTCTTCCTCCACCTGGTCCTCATCATCACCTG
     ||||||||||  ||  ||  |||||  ||  ||||||||||||||||  ||  ||||||||||||||||||||||||||||||||
805  GTCACCATCACCATTCTCCTGGGCGTGTTCATCTTCTGCTGGGCCCCCTTCTTCCTCCACCTGGTCCTCATCATCACCTG

161  CCCCACCAATCCCTACTGCATCTGCTACACGGCCCATTTCAACACCTACCTGGTTCTCATCATGTGCAACTCCGTCATCG  242
     |||||||||  ||||||||||||||||||||  |||||  ||||||||||||||||  ||||||||||||||||||||||||
905  CCCCACCAACCCCTACTGCATCTGCTACACTGCCCACTTCAACACCTACCTGGTCCTCATCATGTGCAACTCCGTCATCG  984
```

E. Amino acid sequence alignment of mouse MC4-R (start from extracellular loop 1) with that of human MC4-R

```
 1  ICSLAVADMLVSVSNGSETIVITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYAL 73
    ||||||||||||||||||||:||||||||||||||||||||||||||||||||||||||||||||||||||||
83  ICSLAVADMLVSVSNGSETIIITLLNSTDTDAQSFTVNIDNVIDSVICSSLLASICSLLSIAVDRYFTIFYAL 155
```

F. Amino acid sequence alignment of mouse MC4-R (end at extracellular loop 3) with that of human MC4-R

```
  1 HIKRIAVLPGTGTIRQGTNMKGAITLTILIGVFVVCWAPLFLHLLFYISCPQNPYCVCFMSHFNVSLILIMCNADIDP 78
    |||||||||||| |||| ||||||||||||||||||| ||||:||||||||||||||||. |||||||. |||
222 HIKRIAVLPGTGAIRQGANMKGAITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFNLYLILIMCNSIIDP 299
```

G. Amino acid sequence alignment of mouse MC3-R (start from extracellular loop 1) with that of human MC3-R

```
  1 LCSLAAADMLVSLSNSLETIMIAVINSDSLTLEDQFIQHMDNIFDSMICISLVASICNLLAIAIDRYVTIFYALRYHSIM
    ||||| |||||||.||.|||||||::.|| || ||||||||||||||||||||||||||||:||||||||||||||||
114 LCSLAVADMLVSVSNALETIMIAIVHSDYLTFEDQFIQHMDNIFDSMICISLVASICNLLAIAVDRYVTIFYALRYHSIM
```

H. Amino acid sequence alignment of mouse MC3-R (end at extracellular loop 3) with that of human MC3-R

```
  1 RLHVQRIAVLPPAGVVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLVLIITCPTNPYCICYTAHFNTYLVLIMCNSVI
    ||||.||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
252 RLHVKRIAALPPADGVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLVLIITCPTNPYCICYTAHFNTYLVLIMCNSVI
```

Figure 2. Nucleotide and amino acid sequence comparison of mouse and human MC3-R and MC4-Rin the sequenced regions. Human MC3-R is according to Gantz et al. (Gantz et al., 1993) and Human MC4-R is from Mountjoy et al.(Mountjoy et al, 1994).

Figure 3. Expression of MC4-R in E16.5 mouse embryos.

A. brain, B, spinal cord. C, sites of teeth primordium, and D. dorsal root ganglia. CP, cortical plate; TH, thalamus; HY, hypothalamus; ME, the meddular of brain stem; PO, the pond of brain stem; SC, spinal cord, TP, teeth premordia, DRG, dorsal root ganglia.

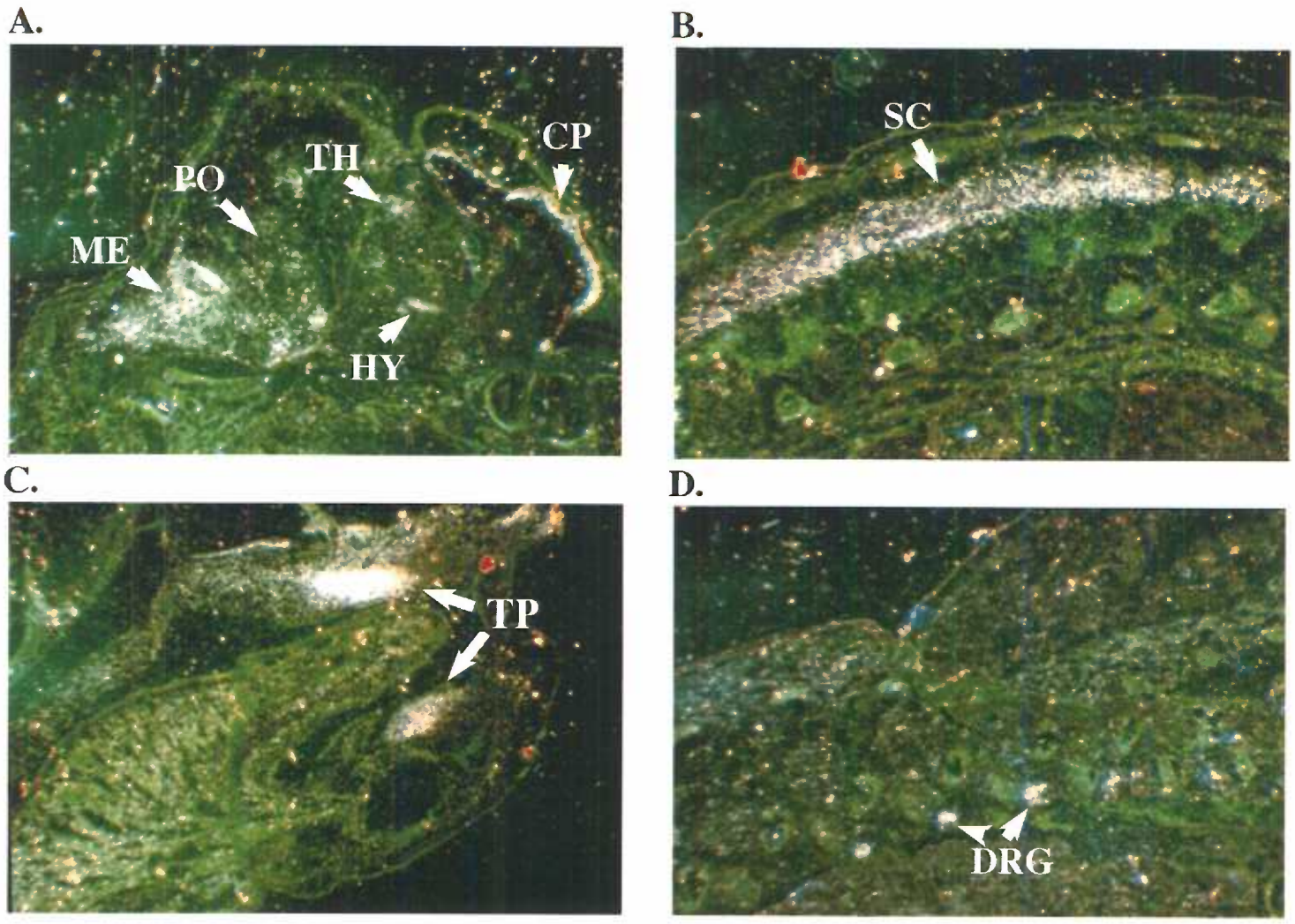

A.
PO
TH
CP
ME
HY
B.
SC
C.
TP
D.
DRG

Figure 4. Restriction enzyme map of MC5-R containing fragment.

A. λ clone. B. 9.0 kb Sac I fragment subcloned in pBKS(-). Shaded area in B represents the coding region of MC5-R. Arrow in the shaded box indicates orientation of transcription. Drawings are roughly in proportion to the scale bars.

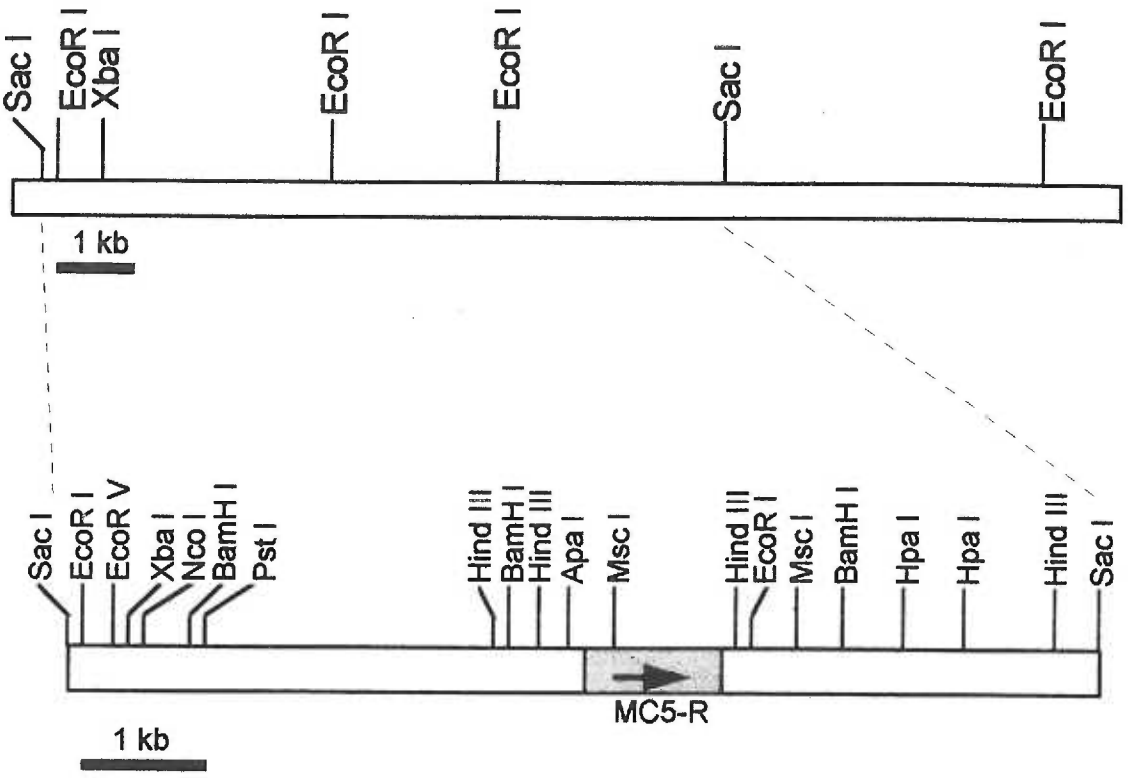

Sac I
EcoR I
Xba I
EcoR I
EcoR I
Sac I
EcoR I
1 kb
Sac I
EcoR I
EcoR V
Xba I
Nco I
BamH I
Pst I
Hind III
BamH I
Hind III
Apa I
Msc I
Hind III
EcoR I
Msc I
BamH I
Hpa I
Hpa I
Hind III
Sac I
MC5-R
1 kb

Figure 5. Schematic topology of mouse MC5-R according to Baldwin (Baldwin, 1993).

Red circles represent residues common to all GPCR based on Baldwin. Light blue circles are residues shared by most of melanocortin receptors. -P indicates possible phosphorylation site by protein kinase C. Y denotes possible N-glycosylation sites. Waves point to likely sites for myristylation on glycine residue and palmitoylation on cysteine residue. .

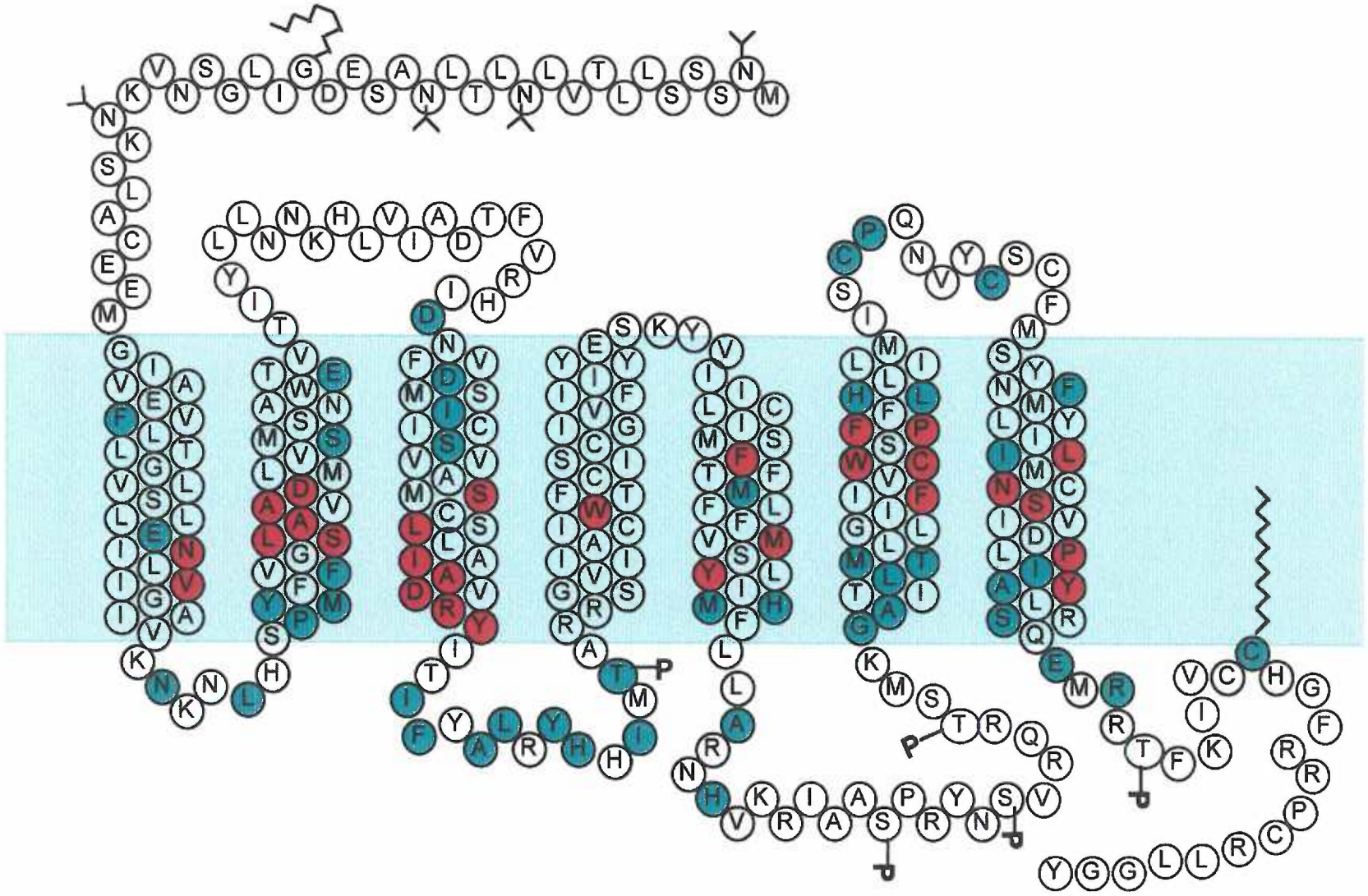
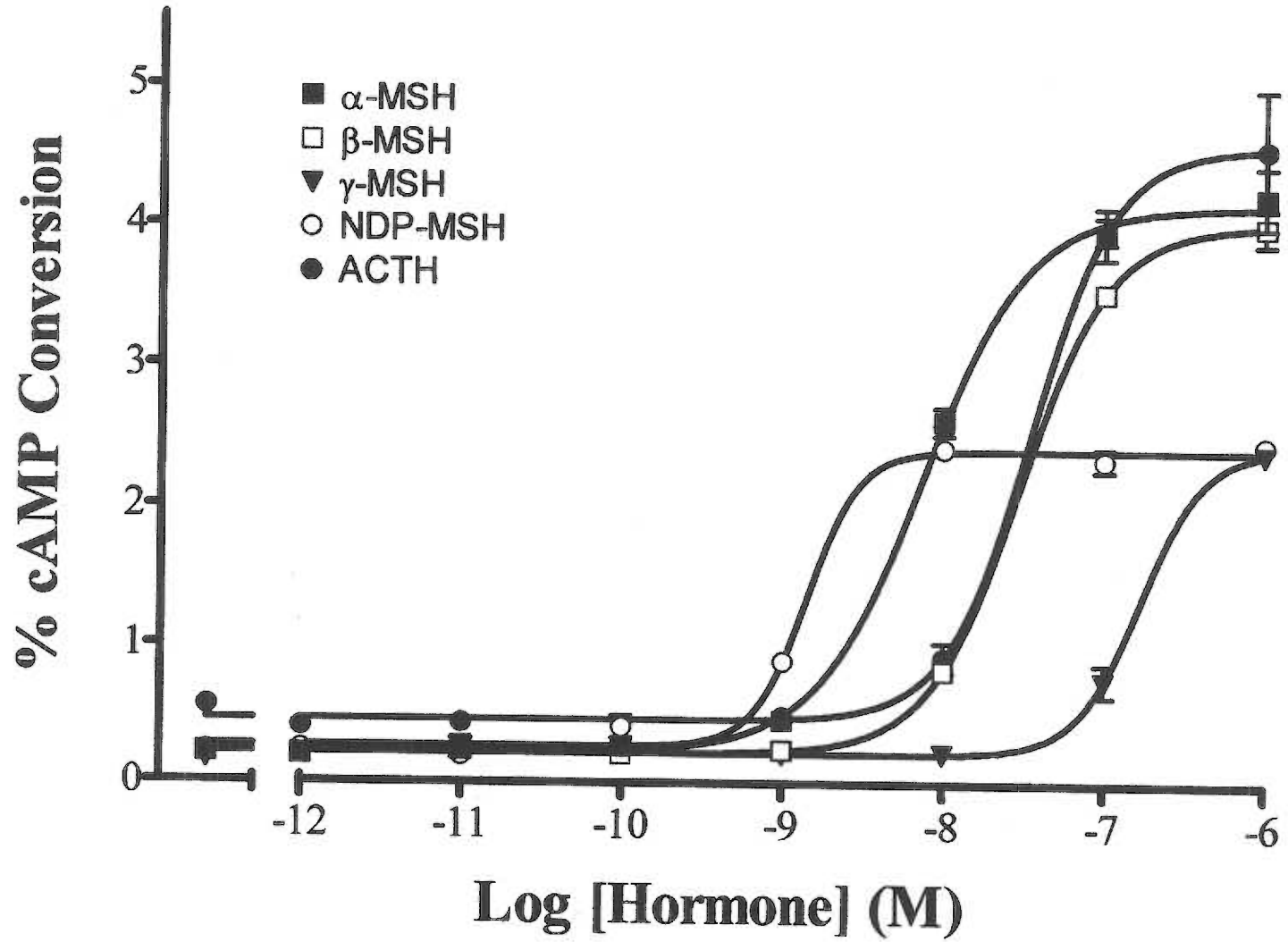


Figure 6. Pharmacological profiles of mouse MC5-R.

Data represent result from a typical experiment with triplicate measurement for each data point. EC_{50} are 1.4 ± 0.5 nM 7.3 ± 0.4 nM, 29 ± 3 nM, 35 ± 15 nM for NDP-α-MSH, α-MSH, β-MSH ,and $ACTH_{1\text{-}39}$, respectively.


% cAMP Conversion
5
4
3
2
1
0
α-MSH
β-MSH
γ-MSH
NDP-MSH
ACTH
-12
-11
-10
-9
-8
-7
-6
Log [Hormone] (M)

CHAPTER THREE

A COLORIMETRIC ASSAY FOR MEASURING ACTIVATION OF G_s AND G_q COUPLED SIGNALING PATHWAYS

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ABSTRACT

Current assays for functional activation of G_S coupled receptors usually involve quantitation of adenylyl cyclase, or measurement of cAMP concentration by radioimmunoassay. The activation of G_q coupled receptors is commonly assayed by measurement of the production of inositol triphosphate (IP_3) or diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate [PIP_2], or of changes in intracellular calcium. These assays generally require large numbers of cells (10^5-10^6) and/or the use of radioactive materials. We have developed a rapid non-radioactive colorimetric assay that utilizes a β-galactosidase (lacZ) gene fused to 5 copies of the cyclic AMP response element (CRE) to detect the activation of CREB (CRE binding protein) that results from an increase in intracellular cAMP or calcium. This assay can be performed using as few as 30,000 cells in a 96-well format with the end products measured simultaneously in a microplate reader. Consequently, a single individual can readily assay 1000 samples a day. Using this assay, the fold increase in β-galactosidase activity was similar in magnitude to increases in cAMP or adenylyl cyclase activity and was approximately linear from 0.01 to 0.27 fmole/cell of intracellular cAMP. Furthermore, pharmacological characterization of one of the melanocortin receptors, mMC5-R, using this assay resulted in a similar order of potency for several melanocortin peptides to that obtained with a commonly used adenylyl cyclase enzyme assay. This assay is also useful for the characterization of Gq-coupled receptors as is demonstrated here using cells transfected with the mouse bombesin receptor. The large-scale capacity of this assay makes it an excellent method for screening molecules of interest acting on G_S and G_q coupled receptors.

INTRODUCTION

G protein-coupled receptors are a superfamily of plasma membrane proteins with seven transmembrane domains. They respond to a vast variety of substances including photons, odorants, neurotransmitters, and peptide hormones (Clapham and Neer, 1993; Hepler and Gilman, 1992; Hille, 1992). The transmembrane signal resulting from ligand binding is mediated by peri-membrane heterotrimeric G-proteins. The dissociated $G\alpha$ and $G\beta\gamma$ subunits activate a wide variety of effectors, including adenylyl cyclases, phospholipases, and plasma membrane channels (Hepler and Gilman, 1992; Hille, 1992; Iniguez-Lluhi et al., 1993). The primary effectors of G_s and G_q coupled signaling pathways are adenylyl cyclase and phospholipase C, respectively.

Characterization of the pharmacological properties of G-protein coupled receptors has provided important information about the signal and potential metabolic effects mediated by each receptor. For measurement of G_s and Gq activation, researchers have assayed the enzymatic activities of adenylyl cyclase and phospholipase C (Johnson and Salomon, 1991; Reynolds et al., 1991). Activation of adenylyl cyclase results in production of cAMP, while phospholipase C stimulation increases the metabolism of membrane phosphatidylinositolbisphosphate (PIP_2) into inositol-triphosphate (IP_3) and diacylglycerol (DAG). Measurement of intracellular cAMP concentration by radioimmunoassay (RIA), or isolation and determination of accumulated cAMP following addition of a radioactively labeled substrate have been used previously to assay activation of the G_s mediated signaling pathway (Salomon, 1991). Chromatographic isolation of radioactive PIP_2 and IP_3 is used to monitor PLC activity (Imai and Gershengorn, 1987). As IP_3 can activate its membrane receptor in the endoplasmic reticulum and cause Ca^{++} release, a variety of Ca^{++} probes have also been used to detect the activation of G_q coupled signaling pathways [illegible]-Raaka and Gershengorn, 1987). Although the previous methods are sensitive, they

either employ radioactive materials or require expensive reagents/equipment, and may not be applicable for large-scale experiments.

Elevation of intracellular cAMP or Ca^{++} both result in the phosphorylation of the transcription factor CREB at serine 133 by protein kinase A or CaM kinase IV, respectively (Gonzalez and Montminy, 1989; Matthews et al., 1994; Sheng et al., 1990; Sheng et al., 1991) which in turn activates the transcription factor (Gonzalez and Montminy, 1989; Yamamoto et al., 1988). Therefore, it is possible to measure both Gs and Gq activation by monitoring CREB-mediated gene expression. Here we describe a rapid colorimetric method that measures CRE directed bacterial β-galactosidase expression. We used the mouse fifth melanocortin receptor (mMC5-R) (Labbe et al., 1994, Chen and Cone, unpublished result), a member of a new G_S-coupled receptor family (Mountjoy et al., 1992), and the murine bombesin/gastrin-releasing-peptide receptor, a G_q-coupled receptor (Spindel et al., 1990), as model receptors for these two signaling pathways using the CRE/β-galactosidase assay.

MATERIALS AND METHODS

Materials. Recombinant bacterial β-galactosidase, o-nitrophenyl-β-D-galactopyranoside (ONPG), Triton X-100, and bovine serum albumin (BSA) were from Sigma (St Louis, MO). Lipofectin reagents, DMEM, OPti MEM and newborn calf serum (NCS) were from Gibco BRL (Grand Island, NY). G250 protein reagent concentrate, 2-mercaptoethanol, Dowex and Alumina matrix were from Bio-Rad (Hercules, CA). cAMP Radioimmuno-assay (RIA) kits were purchased from Biomedical Technologies Inc. (Stoughton, MA). [8-^{3}H]-Adenine (888 GB_q/mmol) was from Amersham (Arlington Height, IL). Melanocortin

peptides were from Bachem (Torrance, CA). The 96-well plate spectrophotometer used for monitoring β-galactosidase activity was from Molecular Devices (Sunnyvale, CA).

Construction of pCRE/β-gal. A series of concatemers of the synthetic oligonucleotide, 5'-GAATTCGACGTCACAGTATGACGGCCATGG-3', containing the CRE (underlined) was obtained by self-annealing and ligation. A tandem tetramer obtained in this way was cloned upstream of a fragment (-93 to +152) of the human vasoactive intestinal peptide gene promoter (Fink et al., 1988). The resulting promoter was used to direct the expression β-galactosidase (β-gal) gene from E. coli. With the orginal CRE in the VIP fragment (-86 to -70), a total of 5 CREs are found in the synthetic promoter (Figure 1).

Cell Culture. If not specifically indicated, all cells were maintained in DMEM plus 10% NCS and 1% pen/strep in a 37 °C incubator with 5% CO_2.

Transfection. Stably transfected cell populations expressing mouse MC5-R or mouse bombesin receptor were produced using lipofectin reagents and G418 selection at 400 μg/ml for 2 weeks. Both receptors were expressed using the pcDNA I Neo expression vector (Invitrogen). Transient transfection of pCRE/β-gal plasmids was performed as follows. Cells at 40 to 60% confluence in a 6 cm plate (approximately 1.5 million cells) were washed once with Opti MEM, and maintained in 1 ml of Opti MEM until a lipofectin/DNA complex was ready. To prepare the lipofectin/DNA mixture, 3 μg of pCRE/β-gal DNA and 20 μl of lipofectin reagent were diluted separately in 0.5 ml of Opti MEM each and then mixed. After incubating at room temperature for 15 to 20 minutes, the mixture (1 ml) was added to the 6 cm plate. The medium was mixed by gentle swirling. Five to 24 hours later, Opti-MEM was replaced with normal medium, and all the cells split into a 96-well plate. Similar results could also be obtained using a calcium phosphate

transfection protocol. Two days later, the cells were stimulated with hormone in incubation medium [DMEM + 0.1 mg/ml of BSA + 0.1 mM of isobutylmethylxanthine (IBMX) (for G_S coupled receptors only)] for 6 hours. β-galactosidase enzyme assays were performed according to Felgner et al. with modifications (Felgner et al., 1994). Briefly, the medium was aspirated and 50 µl of lysis buffer (250 mM Tris.HCl, pH8.0, 0.1% Triton X-100) was added. The lysis was enhanced by one round of freeze and thaw. When the lysates were thawed, 10 µl aliquots were taken from each well and transferred to another 96-well plate for protein determination. To the remainder of the lysates, 40 µl of PBS with 0.5% BSA was added, followed by the addition of 150 µl of substrate buffer [60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 2 mg/ml ONPG]. The plate was incubated at 37°C for 1 hour and absorbance at 405 nM was measured in a 96-well plate reader. A series of 2-fold dilutions starting from 20 ng of recombinant β-galactosidase were carried along in parallel in each experiment to convert OD_{405} to a known quantity of β-galactosidase protein. The relative protein content in 10 µl of lysate was determined using Bio-Rad G250 Dye. To each well, 150 µl of deionized, distilled H_2O and 40 µl of dye concentrate were added and OD_{595} was measured in a 96-well plate reader.

Radioimmunoassay. Cells at confluence in a 24-well plate (about $1.5x10^5$ cells/well) were treated with hormone in incubation medium for 30 minutes. Cyclic AMP was extracted with 60% ethanol at 4 °C for an hour. Extracts were vacuum dried in a Savant SpeedVac Concentrator. cAMP RIA kits were used in all RIA assays. Non-acetylated samples were used following the manufacturer's instructions.

Adenylyl Cyclase Assay. Adenylyl cyclase assays were carried out essentially following the method of Salomon (Salomon, 1991). Briefly, cells at confluence in 24-well plates ($2.5x10^5$ cells/well) were labeled with ^{3}H-Adenine for 1 hour (5 µCi/well). Cells were then stimulated with hormones in incubation medium for 30 minutes. Cell lysates

were prepared by treatment with 2.5% PCA at 4°C for 30 minutes. Supernatants of a KOH precipitation were subjected to Dowex 50 and alumina chromatography to purify ^{3}H-cAMP. Results are described as % conversion of ^{3}H-adenine to ^{3}H cAMP, and are proportional to adenylyl cyclase activity in the absence of transport or degradation of cAMP.

Data Analysis. All data points are the means of triplicate determinations if not defined otherwise, and bars indicate standard deviation. Data was plotted using Prism Version 1.02 from GraphPad (San Diego, CA). Curves were fitted and EC_{50} values were determined by non-linear regression.

RESULTS

To test if CRE directed β-galactosidase gene expression can be used to monitor the activation of adenylyl cyclase, we transiently transfected pCRE/β-gal (Figure 1) into the human embryonic kidney 293 cell line commonly used for functional expression of G protein coupled receptors. Treatment of the cells with 10 μM forskolin resulted in the induction of β-galactosidase gene expression as demonstrated by increase of its enzymatic activity. We then tested many other cell lines to see if this method was generally applicable. To our surprise, 293 cells were much better than the other cells tested in both low basal and high inducible expression of β-galactosidase (Figure 2). We therefore chose 293 cells for all subsequent experiments.

The kinetics of the new assay were then examined using mMC5-R expressing 293 cells. Cells were stimulated with different concentrations of α-MSH for different lengths of time, and β-galactosidase assays performed (Figure 3). Ligand stimulated β-galactosidase

expression could be dectected at two hours, reached its peak at 6 hours and was sustained for at least 6 hours, and then declined. We chose 6 hours of hormone stimulation as a standard for all our experiments.

Next, the relationship between β-galactosidase activity and intracellular cAMP concentration was examined. Since elaboration of β-galactosidase activity requires both transcription and protein translation subsequent to elevation of intracellular cAMP, maximal β-galactosidase levels occur much later than those for intracellular cAMP. Consequently, the relationship between maximal levels of cAMP and β-galactosidase induced using a range of hormone concentrations was compared. Mouse MC5-R expressing 293 cells were transfected with pCRE/β-gal in a 10 cm plate and split into two 24-well plates. Cells were then stimulated with concentrations of α-MSH between $5x10^{-11}$ M and $1x10^{-8}$ M for 30 minutes and 6 hours, which are the peaks for cAMP production and β-galactosidase expression, respectively. The cAMP content in each well was measured by RIA and β-galactosidase activity was assayed as described. To convert the OD_{405} values to a quantity of β-galactosidase protein, we used 2-fold series dilutions of recombinant β-galactosidase protein starting from 20 ng in parallel in the assay. The results demonstrated that within a wide physiological range of peak intracellular cAMP concentrations (0.01 - 0.27 fmols/cell), the amount of β-galactosidase expressed several hours after stimulation increases in an approximately linear manner (Figure 4). β-galactosidase activity reached a plateau at intracellular cAMP levels above 0.27 fmols/cell.

To further validate the new assay, it was compared with a commonly used assay for activation of adenylyl cyclase. Figure 5 shows the pharmacological profiles of α-, β- and γ-MSH on mMC5-R obtained by the two assays. The two assays revealed the same order of potency for the three melanocortin peptides. In addition, the fold of induction of β-galactosidase and cAMP are also similar. In general, however, the CRE/β-galactosidase

assay is more sensitive; the EC_{50} values calculated using this assay were in general 3 to 5 times lower than those obtained with the adenylyl cyclase assay (MC5-R) or by measuring intracellular calcium (bombesin receptor).

As increase of both intracellular cAMP and intracellular Ca^{++} result in activation of CREB, we predicted that this method should be applicable to measurement of the activation of the G_q coupled signaling pathway. We generated 293 cells expressing mouse bombesin/gastrin-releasing peptide receptor, known to couple to Gq and elevate intracellular Ca^{++} (Schilling et al., 1991; Spindel et al., 1990). Using these cells and bombesin as agonist, β-galactosidase assays were performed (Figure 6). Bombesin stimulation produced a dose-dependent increase in β-galactosidase activity with no detectable increase in intracellular cAMP (Figure 6, inset). The pharmacological profile of murine bombesin receptor obtained by the β-galactosidase method results in a 3-fold lower EC_{50} than that obtained from a Xenopus oocyte expression assay (20 to 50 nM) using aequorin as a Ca^{++} indicator (Spindel et al., 1990).

DISCUSSION

We have designed a rapid CRE/β-galactosidase based colorimetric assay for Gs and Gq coupled receptors. This assay is approximately 10 fold faster and several times more sensitive than the existing assays, at a fraction of the cost. In our experience, we can assay 1000 samples a day, whereas the adenylyl cyclase assay can only measure 100 samples within the same time. In addition, our method employs no radioactive materials.

This assay measures CRE directed β-galactosidase expression, most likely as a result of activation of the transcription factor CREB. The CRE element from human vasoactive intestinal peptide has been shown to be necessary and sufficient to confer cAMP responsiveness (Fink et al., 1988). cAMP activates CREB by stimulation of PKA, and subsequently phosphorylation of CREB at serine 133 (Gonzalez and Montminy, 1989; Yamamoto et al., 1988). Our data demonstrate that β-galactosidase activity measured in this assay increases in a linear relationship to intracellular cAMP across more than a 20-fold range of cAMP concentrations (0.01 to 0.27 fmole/cell) (Figure 4). We do not yet know why 293 cells are so much more effective than other cells for this assay, although it is unlikely that differences in transfection efficiency are the only reason for this phenomenon. 293 cells are derived from E1A transformed human embryonic kidney cells, however, the products of 12S and 13S E1A early genes seem to play little, if any, role in the sensitivity of 293 cells to cAMP, as transfection of the other cells with either of the adenoviral genes does little to improve the responsiveness (data not shown).

The expression of β-galactosidase reaches its peak after 6 hours of hormone stimulation (Figure 3), similar to observations made by Meinkoth *et al* . in rat-2 fibroblast cells (Meinkoth et al., 1991). Using a similar construct and X-gal histochemical staining, this group detected β-galactosidase expression at 4 hours and found maximal expression at six hours after treatment of the cells with 8-bromo-cAMP. This may reflect the time required for cAMP elevation, activation of PKA, phosphorylation of CREB, and transcription, translation and accumulation of β-galactosidase. The decline in β-galactosidase activity after 12 hours of hormone treatment may be due to the action of protein phosphatase on CREB (Hagiwara et al., 1993) or repression of the catalytic subunit of PKA by chronic agonist treatment. This decline in β-galactosidase expression after 12 hours could also involve downregulation of the receptor by receptor internalization, and/or transcription repression.

This assay can also be used for analysis of cells transiently expressing a G_s or G_q coupled receptor (data not shown). This feature is potentially useful for rapid characterization of new receptors or receptor mutants. A rapid and large-scale assay of this type may also be useful for screening various compound banks (Houghten et al., 1991; Jayawickreme et al., 1994). Using mouse MC5-R and bombesin receptors as examples of G_s and G_q coupled receptors, respectively, we have demonstrated that the CRE/β-galactosidase assay is very sensitive to agonist activity (Figures 5 and 6). This assay has also been successfully used to detect compounds with antagonist activity (Lu and Cone, unpublished data). The other feature of this assay is the requirement of very small amount of ligand. We routinely use 50 μl of incubation medium in our assay, therefore extending the use of compound libraries.

A cell line stably transfected with a c-fos/β-galactosidase reporter construct has been used previously to characterize stimulus-transcription coupling of endogenous G_q and G_s coupled receptors (α_1 and β_2-adrenergic receptors, respectively) (Schilling et al., 1991). However, the sensitivity of the fos promoter to a wide variety of stimuli might make this system susceptible to false positives in large scale compound screening. For example, the fos promoter is activated by a number of mitogenic stimuli, while the CRE/β-galactosidase construct did not respond to EGF, insulin, or TPA in 293 cells (data not shown). CRE-containing promoters have also been fused to reporter genes encoding chloramphenicol acetyltransferase (CAT) or luciferase and these constructs used for expression cloning (Spengler et al., 1993) or pharmacological characterization of Gs coupled receptors (Himmler et al., 1993; Migeon and Nathanson, 1994; Pepperl and Regan, 1993). For rapid pharmacological screening, however, CAT assays do not appear to have the sensitivity or speed of the CRE/β-galactosidase assay, requiring over 10^6 cells/assay and the use of a radioactive substrate, [^{3}H]-chloramphenicol (Pepperl and Regan, 1993). In one report detection of luciferase activity was reported to require approximately 10^5 cells in

a 15mm well format and involved transferring cell lysates to luminometer cuvettes (Migeon and Nathanson, 1994). In another report, however, a CRE-luciferase construct was successfully used in a 96 well format for assaying induction of adenylyl cyclase by the D_1 and D_5 dopamine receptors (Himmler et al., 1993). The use of luciferase as a marker, while also extremely sensitive, requires the use of more specialized equipment (96 well luminometer).

The mechanism for the induction of CRE/β-gal expression by G_S coupled receptor activation is very likely through the PKA pathway. However, the mechanism by which a G_q coupled receptor induces CRE/β-gal expression is uncertain. There is no increase in cAMP production after bombesin treatment of bombesin receptor expressing 293 cells (data not shown), thus excluding the possibility that bombesin receptor may activate G_S. In addition, down-regulation of PKC by chronic pre-treatment of the cells with 10mM TPA had no effect on bombesin stimulated β-galactosidase expression (data not shown), suggesting that bombesin induces CRE/β-gal expression through an IP_3 pathway. Ca/calmodulin dependent kinases have been shown to phosphorylate CREB at Ser-133 *in vitro* and *in vivo* (Matthews et al., 1994; Sheng et al., 1990; Sheng et al., 1991), and this might be the mechanism by which activated Gq coupled receptors induce the expression of CRE/β-galactosidase reporter.

ACKNOWLEDGEMENTS

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Figure 1. Structure of the pCRE/β-gal Plasmid.

The bacterial β-galactosidase gene (lacZ) is under the control of a promoter containing five CREs. The promoter was made by inserting a synthetic oligonucleotide encoding 4 tandem CRE sequences upstream of the human vasoactive intestinal peptide (VIP) promoter (-94 to +152). With the original CRE in the VIP fragment (-70 to -86), there are a total of five CREs in the promoter.

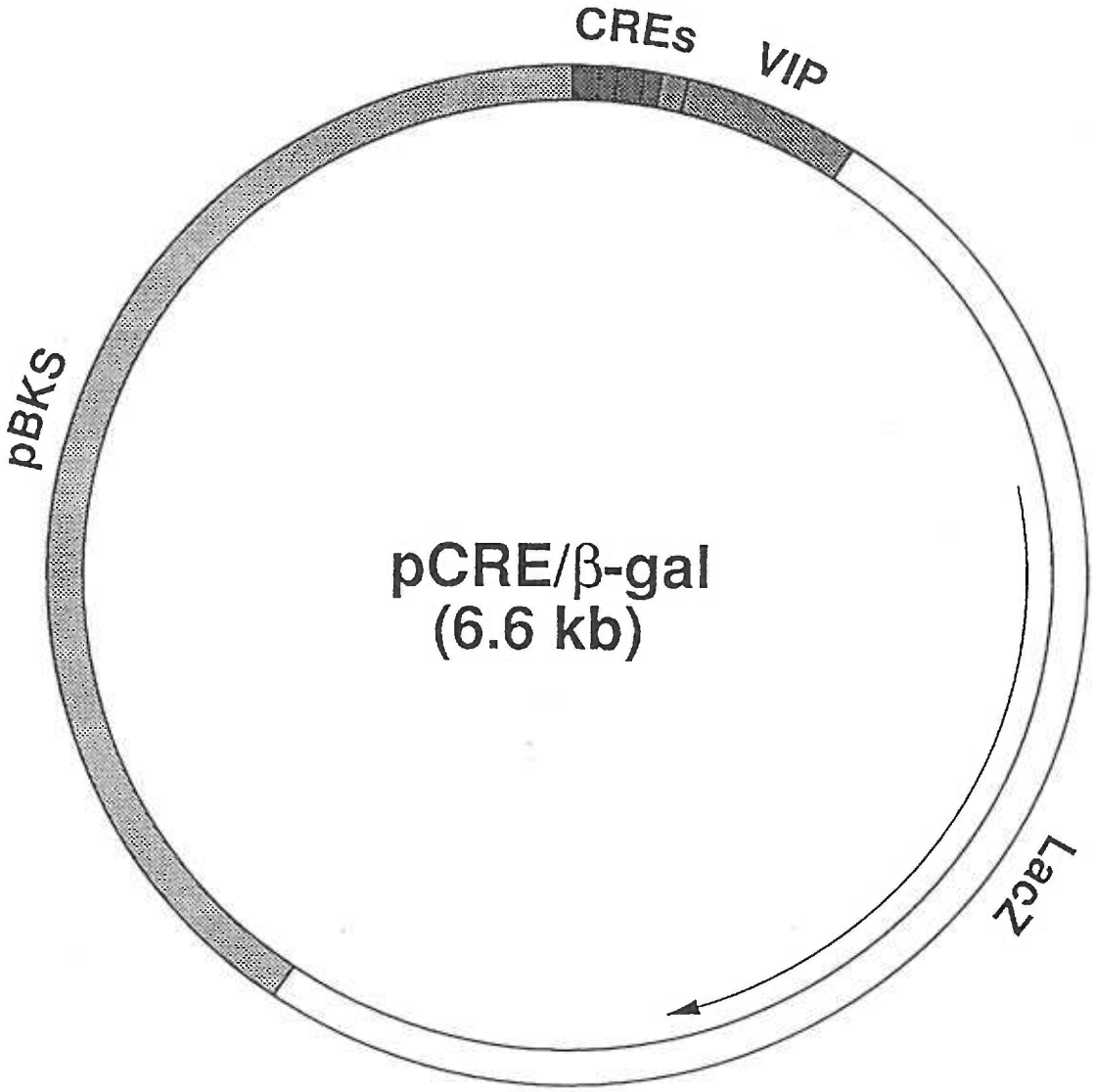

CREs
VIP
pBKS
pCRE/β-gal
(6.6 kb)
lacZ

Figure 2. Identification of 293 cells as the optimal cell line for the CRE/β-galactosidase assay.

Cells were transfected with pCRE/β-gal plasmid and stimulated with 10 μM forskolin in incubation medium for 6 hours and β-galactosidase activity was measured. Controls were cells treated in incubation medium without forskolin.

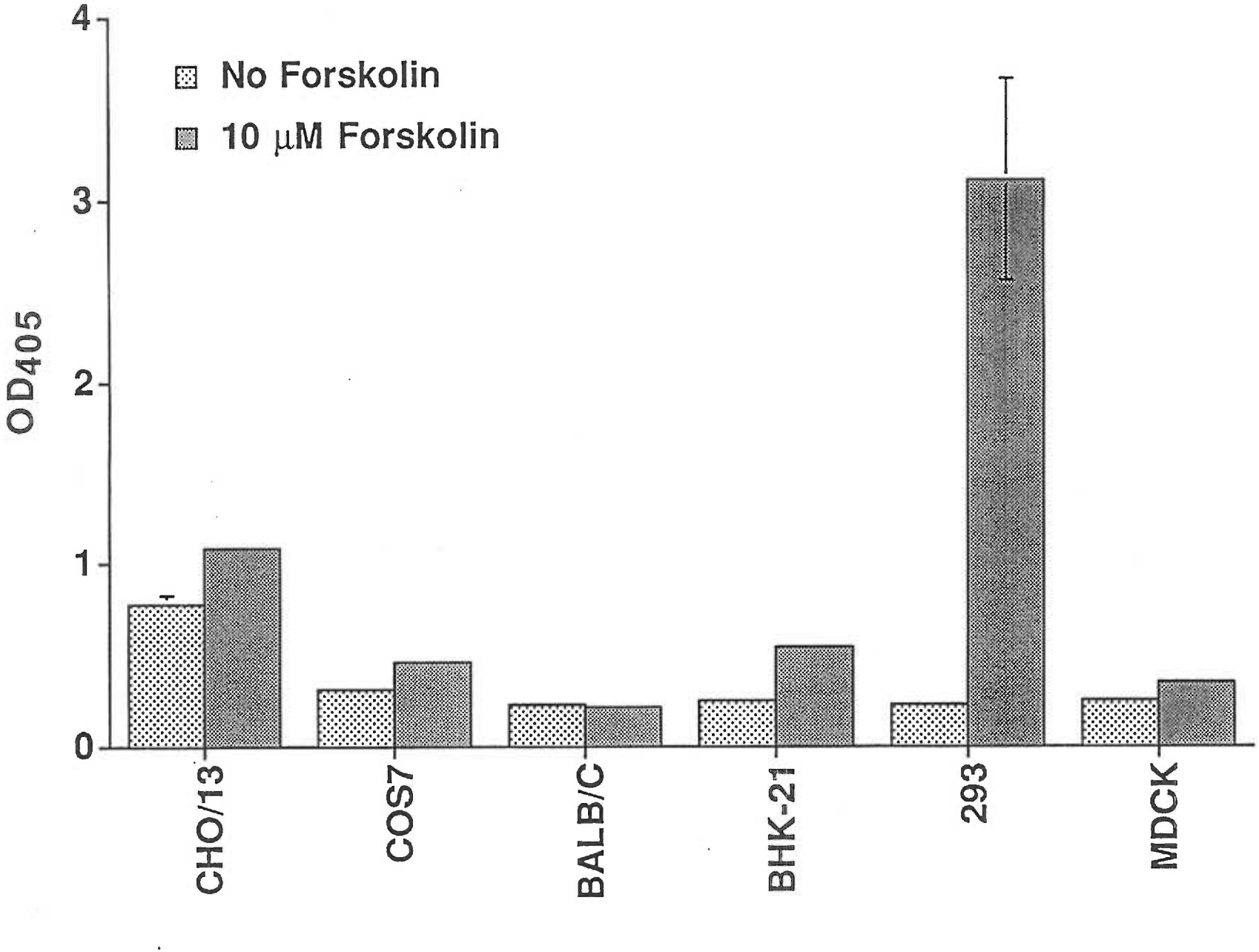

No Forskolin
10 μM Forskolin
OD405
4
3
2
1
0
CHO/13
COS7
BALB/C
BHK-21
293
MDCK

Figure 3. Time Course of CRE/β-galactosidase Assay.

An Apa I/HindIII fragment containing the entire coding region of the fifth melanocortin receptor (MC5-R) from a mouse genomic clone was inserted into pcDNA I Neo in the correct orientation. The resulting construct was used to transfect 293 cells and a stable transfected cell population was obtained after continuous G418 selection. These cells were transfected with pCRE/β-gal plasmid and stimulated with different concentrations of α-MSH for 2, 6, 12, 24 hours. Cyclase activity is presented in the form of OD_{405}. Insert is a plot of OD_{405} against the length of stimulation using 10^{-8} M α-MSH.

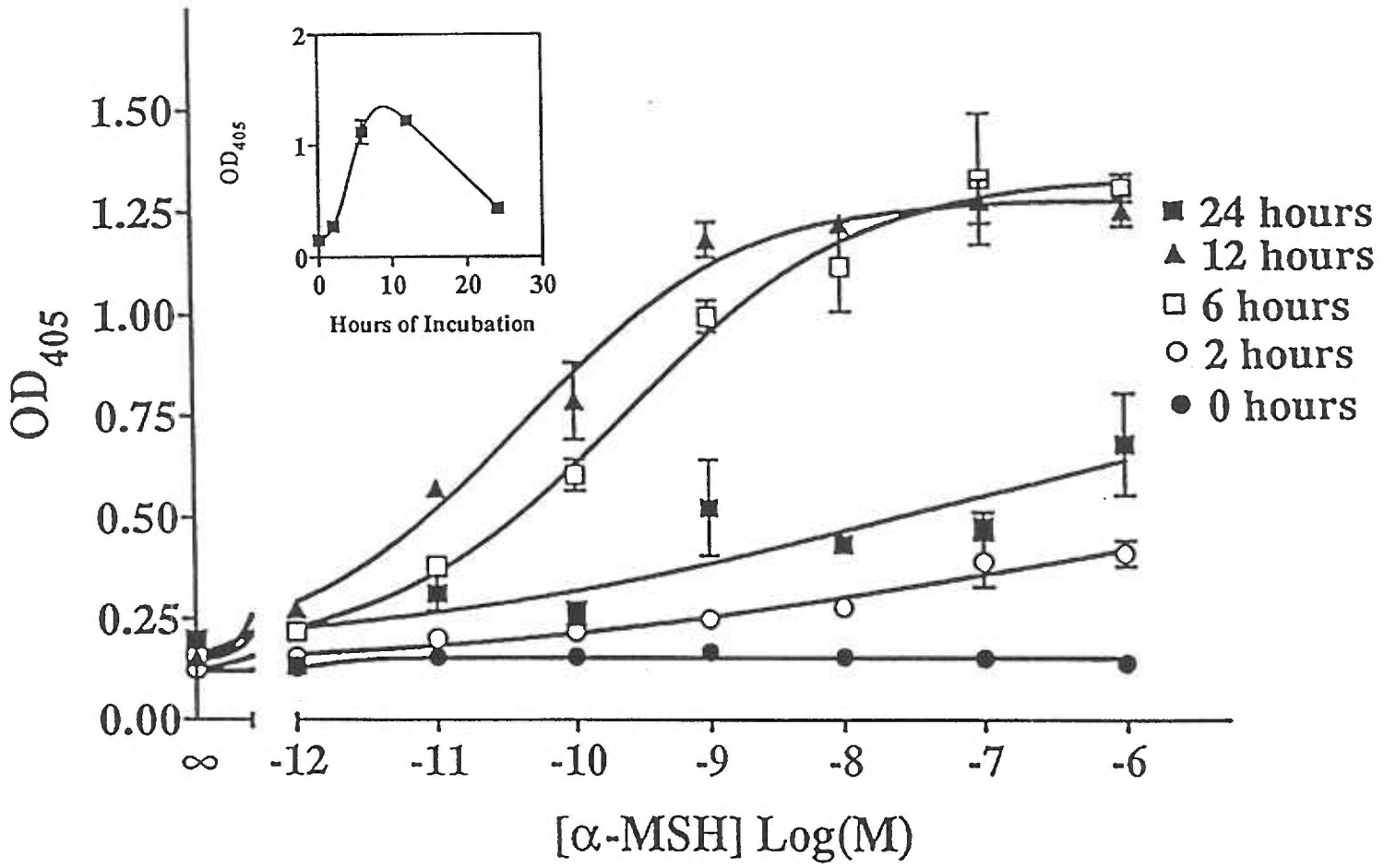

1.50
1.25
1.00
0.75
0.50
0.25
0.00
OD405
∞
-12
-11
-10
-9
-8
-7
-6
[α-MSH] Log(M)
24 hours
12 hours
6 hours
2 hours
0 hours
OD405
2
1
0
0
10
20
30
Hours of Incubation

Figure 4. The Relationship Between Intracellular cAMP Concentration and β-galactosidase Activity.

HEK293 cells expressing MC5-R were transiently transfected with a pCRE/β-gal construct and split into 24-well plates. Cells were stimulated with different concentrations of α-MSH (5×10^{-11} to 1×10^{-8} M). β-galactosidase activity and cAMP content were measured as described. OD_{405} of each well was converted to nanograms of β-galactosidase using the standard curve obtained in parallel with the assay.

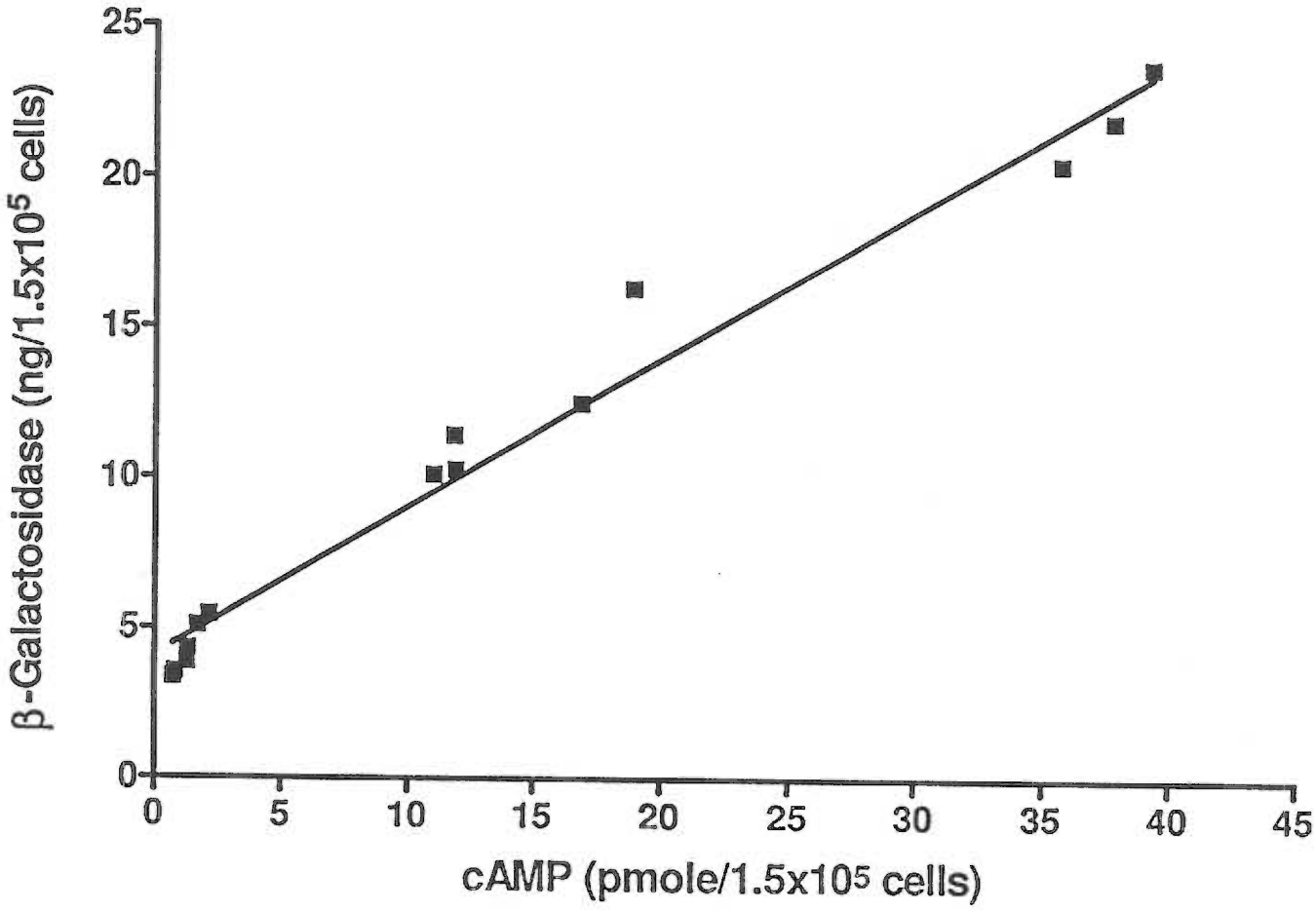

β-Galactosidase (ng/1.5x10⁵ cells)
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
45
cAMP (pmole/1.5x10⁵ cells)

Figure 5. A Comparison of the β-galactosidase Method with An Adenylyl Cyclase Assay.

The dose response of mouse MC5-R expressing 293 cells to α-, β-, and γ-MSH was determined using the CRE/β-galactosidase assay and a commonly used adenylyl cyclase assay. The OD_{405} value of each sample was converted to amount of β-galactosidase as described. The fold induction is the ratio of the amount of β-galactosidase protein or adenylyl cyclase activity in the sample to the mean of unstimulated controls. Solid and dashed lines represent results from β-galactosidase and adenylyl cyclase assays, respectively. EC_{50} values for α-, and β-MSH obtained from the adenylyl cyclase assay are 7.2×10^{-9}M, and 3.0×10^{-8}M, and that obtained from the CRE/β-galactosidase assay are 1.7×10^{-9}M, and 5.0×10^{-9}M, respectively. As the highest concentration of γ-MSH examined (1 μM) did not produce full activation, the true EC_{50} values for γ-MSH could not be determined.

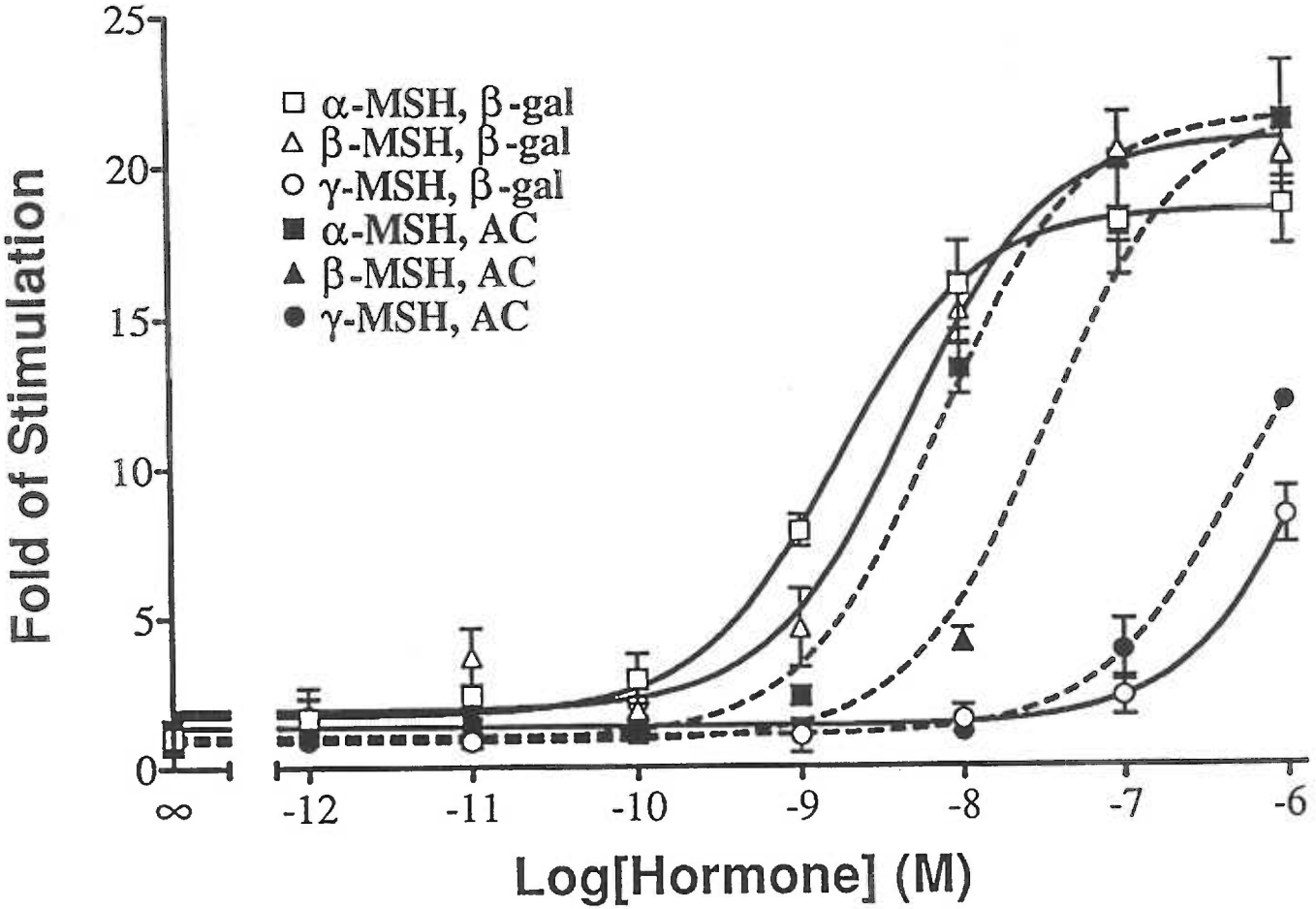
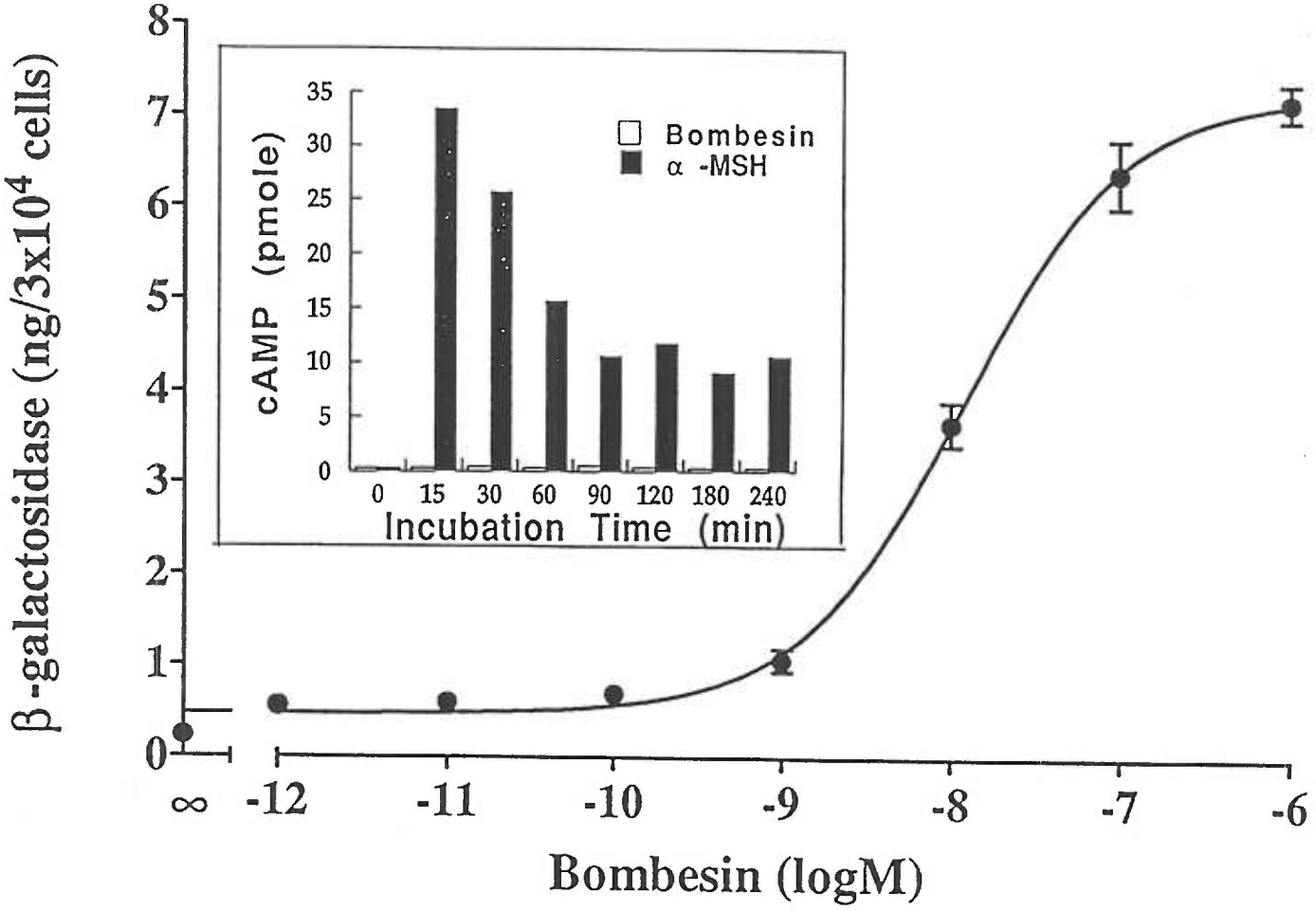

Fold of Stimulation
25
20
15
10
5
0
∞
-12
-11
-10
-9
-8
-7
-6
Log[Hormone] (M)
□ α-MSH, β-gal
△ β-MSH, β-gal
○ γ-MSH, β-gal
■ α-MSH, AC
▲ β-MSH, AC
● γ-MSH, AC

Figure 6. Dose Response Curve of Mouse Bombesin Receptor to Bombesin Measured by CRE/β-galactosidase Assay.

A full-length cDNA clone of murine bombesin receptor was inserted into pcDNA I Neo in the sense orientation. Stably transfected 293 cell population was obtained as described. Cells were stimulated with concentrations of bombesin shown for 6 hours and β-galactosidase activity determined as described. The EC_{50} is about 10 nM. Bombesin receptor expressing cells were then transiently transfected with the MC5-R and independently stimulated with α-MSH (5×10^{-8}M, solid bars) or bombesin (5×10^{-8}M, open bars) indicating that bombesin did not elevate intracellular cAMP under these conditions (inset). Intracellular cAMP concentrations were determined by RIA. Values represent the mean of 6 determinations.


β-galactosidase (ng/3x10⁴ cells)
8
7
6
5
4
3
2
1
0
∞
-12
-11
-10
-9
-8
-7
-6
Bombesin (logM)
cAMP (pmole)
35
30
25
20
15
10
5
0
0 15 30 60 90 120 180 240
Incubation Time (min)
Bombesin
α -MSH

CHAPTER FOUR

EXOCRINE GLAND DYSFUNCTION IN MC5-R DEFICIENT MICE:

EVIDENCE FOR COORDINATED REGULATION OF EXOCRINE GLAND FUNCTION BY MELANOCORTIN PEPTIDES

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SUMMARY

The systemic effects of pituitary-derived melanocortin peptides are primarily attributed to ACTH-mediated adrenocortical glucocorticoid production. The identification of a widely-distributed peripheral receptor for ACTH/MSH peptides, called the melanocortin-5 receptor (MC5-R), raised the possibility of non-steroidally mediated systemic effects of these peptides. Targeted disruption of the MC5-R produced a mouse with a severe defect in water repulsion and thermoregulation due to decreased production of sebaceous lipids. Further investigation demonstrated high levels of MC5-R in multiple exocrine tissues, including the Harderian, preputial, lacrimal, and sebaceous glands. The MC5-R was also shown to be required for the production of porphyrins by the Harderian gland, and physiological concentrations of ACTH were demonstrated to regulate protein secretion by the lacrimal gland via the MC5-R. The data show a requirement for the MC5-R in multiple exocrine glands for the production of a diverse set of products, including lipids, proteins, and porphyrins, and suggest the existence of a coordinated system for the regulation of exocrine gland function by melanocortin peptides.

INTRODUCTION

The cloning of genes encoding receptors has often led to the identification of more receptor isoforms in each receptor family than could be discriminated previously by pharmacological or physiological methods. For example, the five cloned dopamine receptors were originally classsified into two pharmacological sites, D_1 and D_2 (Kebabian, 1979; Spano, 1978). Melanocortin peptides (ACTH, α-MSH, β-MSH, γ-MSH) derived from the proopiomelanocortin (POMC) gene are found as pituitary-derived peptide hormones in the serum, as neuropeptides, largely in the forebrain and brainstem (Bronstein et al., 1992; Jacobowitz and O'Donohue, 1978; Nilaver et al., 1979; Watson and Akil, 1979), and at a growing number of peripheral sites, such as keratinacytes (Schauer et al., 1994). Two receptors for the MSH and ACTH peptides were defined by classical pharmacological methods on melanocytes (MSH-R) and adrenocortical cells (ACTH-R) and demonstrated to regulate pigmentation (Pawelek, 1976) , and adrenocortical steroidogenesis (Buckley and Ramachandran, 1981), respectively. Thus far, the biological activities of the melanocortin peptides appear to be mediated by a family of five G protein coupled receptors (Cone, 1996).

The primary role of serum-derived ACTH is the regulation of adrenocortical glucocorticoid production. In response to physical or psychological stress, hypothalamic corticotropin releasing hormone stimulates the production of ACTH by anterior pituitary cells. Serum ACTH is elevated 3-5 fold, producing a subsequent 10-100 fold elevation in circulating cortisol or corticosterone. Glucocorticoids then support the response to stress, serving to stimulate hepatic gluconeogenesis and elevate blood glucose, and mobilize amino acid stores from muscle and fatty acids from adipose tissue. Glucocorticoids also have an important role in the resolution of immune responses, acting on numerous cell types to reduce inflammation.

Pigmentation in most mammals is primarily regulated by MSH. Although α-MSH derived from the intermediate lobe is found in the serum of most mammals, basal pigmentation in mouse and man is not dependent on the pituitary, and may instead be regulated by α-MSH secreted as a paracrine factor by keratinocytes.

In addition to the well-defined pigmentation and adrenocortical pathways, numerous activities of the melanocortin peptides have been reported both in the brain and the periphery. Central effects of melanocortins have been demonstrated on cardiovascular tone (Gruber and Callahan, 1989), thermoregulation (Feng et al., 1987), and learning and memory (De Wied, 1982), however, the receptor site(s) mediating these effects were not well characterized pharmacologically. Additionally, numerous peripheral effects have been reported. Removal of the neurointermediate lobe of the pituitary was demonstrated to decrease sebaceous lipid production (Thody and Shuster, 1973). The reduction was fully restored by concomitant α-MSH and androgen administration (Ebling et al., 1975; Thody et al., 1976), possibly through the stimulation of lipogenesis (Thody et al., 1976). The preputial gland is a specialized sebaceous gland implicated in pheromone production in rodent (Bronson and and Caroom, 1971; Chipman and and Alberecht, 1974; Orsulak, 1972), and the lipid content of this gland can also be stimulated by α-MSH. Injection of α-MSH has been shown to elicit several behavioral changes in the conspecific animals, including altered sexual attraction in male rats (Thody and Wilson, 1983), and modified aggression in male mice due to olfactory cues presumably from the preputial gland (Nowell et al., 1980).

MSH and ACTH have also been demonstrated to stimulate nerve regeneration following sciatic nerve crush (Bijlsma, 1983; Strand, 1980), and to stimulate myoblast proliferation (De Angelis et al., 1992). High affinity ACTH and MSH binding sites have also been reported to regulate lipolysis in adipocytes (Oelofsen and Ramachandran, 1983; Ramachandran, 1976) and protein secretion in the lacrimal gland (Jahn, 1982; Tatro and Reichlin, 1987)

The distribution of expression of the cloned melanocortin receptors has largely fit expectations regarding the known biological activities of the melanocortin peptide ligands encoded by the POMC gene. The MC1-R, or classical MSH receptor, is expressed almost exclusively in melanocytes (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992), where it regulates melanin synthesis. The MC2-R, or classical ACTH recepor, is expressed primarily in the adrenal cortex (Mountjoy et al., 1992), where it regulates adrenocortical steroidogenesis, although this receptor is also expressed in adipocytes, explaining the ability of ACTH to stimulate lipolysis. The MC3-R and MC4-R are expressed mainly in the CNS in regions that correlate well with presumptive terminal fields originating from the two groups of POMC cell bodies in the arcuate nucleus of the hypothalamus and the nucleus of the solitary tract of the brainstem (Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993; Tatro, 1990). Recent data suggest that MC3-R and MC4-R regulate feeding behavior and metabolism (Fan et al., 1997; Huszar et al., 1997), grooming behavior (Adan et al., 1994), body temperature (Tatro, 1997), and cardiovascular tone (Li et al., 1996).

Since the major systemic effects of ACTH can be explained by ACTH binding to the adrenocortical MC2-R, it was thus surprising when a fifth melanocortin receptor, the MC5-R, with high affinity for ACTH was cloned and found to be expressed, albeit at low levels, in a wide variety of peripheral tissues, including muscle, skin, lung, adrenal, thyroid, and in brain as well (Barrett et al., 1994; Chhajlani et al., 1993; Fathi et al., 1995; Gantz et al.,

1994; Griffon et al., 1994; Labbe et al., 1994). This receptor has been cloned from human, mouse, rat, and sheep, and is highly conserved, being approximately 80% identical amongst these mammals. Furthermore, EC_{50} values determined for elevation of intracellular cAMP, or activation of adenylyl cyclase demonstrate that the MC5-R is highly responsive to both α-MSH (EC_{50} = 0.1 - 57nM) and ACTH (EC_{50} = 3-30nM) . We report here a physiological role for the melanocortin peptides in the coordinate control of exocrine gland function mediated by the MC5-R, as determined by targeted disruption of the MC5-R gene in the mouse.

MATERIALS AND METHODS

Cloning of murine MC5-R

The entire rat MC3-R coding sequence and coding region of TM3 to TM6 of the rat MC4R were mix equally and labelled with a random labeling kit from Boehringer Mannheim. These probes were then used to screen a lambda Fix II genomic library from the 129 Sv mouse strain (Stratagene). Sequences spanning TM2 to TM7 were amplified from resulting clones by PCR using degenerate primers designed based upon all known melanocortin receptors as of 1993. The following are the sequence for the primers.

primer1:5íC/T/CGT/C/A/CGT/GT/C/ACG/C/C/CG/A/T/G/T/A/CT/T/AGT/CT/T/T.

Primer2: 5í-T/C/CT/AGT/G/AG/C/T/CG/CT/G/AG/A/AG/AGT/G/C/AG/T/A/AGT/A/T

PCR products were cloned into pGEM-T (Promega) and identity of each clone determined by nucleotide sequencing. The complete sequence of the cloned MC5-R is identical to the previous reported sequences (Fathi et al., 1995; Gantz et al., 1994; Labbe et al., 1994).

Targeting Vector

The purified MC5-R lambda clone contains the whole coding sequence, plus 5 kb 5' noncoding sequence, as well as 7.8 kb 3' noncoding sequence. A 9 kb SacI fragment was subcloned for subsequent manipulations. To make a knock-out construct, a 650 bp Apa I/MscI fragment was replaced with a PGK-Neo cassette (Rudnicki et al., 1992). This fragment encompasses the coding sequence from the iniation codon to the middle of TM3. The PGK-TK cassette (Rudnicki et al., 1992) was placed to MC5-R coding sequence. The vector pMC5-RKO thus contains 4.5 kb and 1.2 kb identical sequences for homologous recombination. The targeting construct can be linearized with Xho I.

Transfection of ES cells and blastocyst injection

Twenty-five μg of linearized pMC5-RKO DNA was electrophorated into 10^7 AK7 ES cells. The cells were selected with G418 and Ganciclovir 24 hour after transfection. Individual colonies were picked up one week after selection and expanded in 96 well plates. DNA from individual clones was screened by PCR for homologous recombinants, using one primer outside of pMC5-RKO (5'-CTAGGATAGGGGAACTGTAGT) and one primer in the PGKNeo cassette (5'-GAGGATTGGGAAGACAATAGCA). The positive clones were confirmed by Southern analysis. Selected clones were injected into blastocysts from C57BL/6 mice. Chimeric male mice were then breed with C57BL/6J or 129SvEv Tac mice. Germline transmission was identified using PCR as before plus one wild-type specific primer (5'- ATGAACTCCTCCTCCACCCTG), and Southern analysis. Heterozygotes males and females were breed to generate homozygous muatant mice. Continuous backcrossing with C57BL/6 was carried out to obtain C57BL/6J-like congenic lines.

Characterization of functional membrane receptor

To make crude membrane, tissues were minced and homogenized with a Polytron. The 500 x g supernatant of tissue homogenate was centrifuged at 100k x g for 40 minutes at 4 oC. The pellet was rinsed twice with PBS and protein content determined using the method of Bradford (Bradford, 1976). Specific ^{125}I-NDP-a-MSH binding by membrane containing 100 μg of protein was determined as described (Vage, 1997). To monitor ligand induced cAMP production, excised tissues of interest were minced and incubated in DMEM containing 0.1 mg/ml of BSA in the presence or absence of ligand for 20 minutes before being frozen in liquid nitrogen. cAMP was extracted with 60% ethanol and measured by RIA as described (Chen et al., 1995). Protein content in ethanol extracted pellets was determined by the method of Bradford.

Water retention assay and temperature measurement

Core temperature was measured by inserting a rectal thermoprobe 2.5 cm inside the mice. Five to 10 minutes prior to swim, the core temperature of each mouse was read 3 times to obtain the baseline. Mice were then weighed and immediately let swim in 32 °C water for 3 minutes. They were taken out and put on paper towels for about 5 seconds to eliminate excessive water. Mice were weighed, their temperature recorded, and then put into an empty plex glass cage. Weight and temperature was measured every five minutes for half an hour. The weight of absorbed water was calcualted by subtracting pre-swim weight.

Hair lipids extraction and analysis

Hair lipids was extracted as descibed with modifications (Ebling, 1974). Seventy to 100 mg of hair from each mouse was extracted with 20 ml of acetone for 15 minutes. The extractants were filtered and the hair was then washed with 20 additional ml acetone. The pooled filtrant was let evaporate to about 5 ml in a chemical hood. The acetone was then transfered to a tared aluminum foil boat and evaporated to dryness. The aluminum foil was then weighed again. The amount of hair lipids was calculated by subtracting the pre-determined weight of the foil. Hair lipids (100-150 μg) were loaded on a Silica Gel 60 plate (Aldrich) and resolved by Haxane/Benzene (55:45 v/v). The plate was then sprayed with sulfuric acid/ethanol (1:1) and charred in a 150 °C oven until approporiate color development occured (Downing, 1985).

Measurement of lacrimal gland protein discharge.

Protein discharge from lacrimal glands was determined as decribed by Jahn (Jahn, 1982). Lacrimal glands were dissected and each cut into four pieces. The explants were incubated in 10 ml of Kreb-Ringer bicarbonate buffer (KRB) in the presence of 25 μCi of ^{3}H-leucine

for 20 minutes in a 37 °C chamber gased with 5% CO_2 and 95% O_2. The tissues were rinsed three times with KRB and further incubated in KRB for 60 minutes to allow incorporation of radioactivity into protein. After rinse with KRB, 8 pieces of labeled tissue (2 glands) were put into one well of a 12-well plate, each well containing 2 ml of KRB. Buffer (0.5 ml) was taken from each well before returning the plate into the chamber. Fifteen minute later, another 0.5 ml of buffer was removed from each well. After addition of hormones to 50 nM, the plate was further incubated in the chamber for 30 minutes and 0.5 ml of buffer removed from each well again. The radioactivity in each sample was measured in a liquid scintillation counter. The rate of protein discharge for each sample during the last 30 minutes was the net increase of radioactivity in the period divided by that in the previous 15 minutes. The relative secretion rate was computed by setting the rate of wild-type control as 1.

Measurement of Harderian Porphyrins

Porphyrins in the Harderian gland were extracted as described (Wetterberg et al., 1970). Briefly, the glands were removed from individual mice and homogenized by a motorized micro pestle in 0.5 ml acetic acid/ether mixture (1:4). Supernatant of a 5 min, 3000 x g spin was transfered to a new tube, and the pellet was similarily extracted two more times. The extractants were pooled and concentrated in a speed-vac (Savant). The samples were disolved in 50 µl chloroform. After addition of 0.95 ml 0.25 N HCl, samples were characterized by scanning spectrophotometry and spectrofluoremetry excised at 402 nm.

RESULTS

Generation of MC5-R Deficient Mice

MC5-R was isolated from a 129 Sv mouse genomic library using a mixture of rat MC3-R and MC4-R coding regions as probes at low stringency. Its nucleotide sequence is identical to the reported murine MC5-R (Gantz et al, 1994) (data not shown). A 650 bp Apa I/ Msc I fragment that extends from -200 bp (starting from the initiation codon) to the middle of TM3 of the receptor was replaced by a PGKneo expression cassette (Rudnicki et al., 1992) (Figure 1a). A PGKTK expression cassette (Rudnicki et al., 1992) was also included in the construct to enrich homologous recombinants by negative selection against the thymidine kinase from human simplex virus (Capecchi, 1989). After linearization, the DNA was electroporated into AK7 ES cells and selected with G418 and Gancyclovir. Genomic DNA from individual ES cell clone was tested by PCR for homologous recombination using primers specific for the mutant MC5-R allele (Figure 1A). Positive clones were further confirmed by Southern analysis. About 20% of clones obtained were homologous recombinants. Positive ES cells were microinjected into blastocysts and several chimera produced. Three independent chimeric lines were transmitted through germline. One clone was bred with 129SvEv Tac to produce inbred offspring (Note: a recent study indicated that there are differeces among 129 strains, therefore the 129 mice used for this study are not truly inbred, see Simpson et al., 1997), and the other two were backcrossed 7 - 9 generations with C57BL/6J mice to make congenic strains. These mice were all named MC5-RKO mice.

The deficiency of MC5-R was confirmed by Southern hybridization, northern analysis and ^{125}I-Nle^{4}, D-Phe^{7}-α-MSH (NDP-α-MSH) binding on crude plasma membranes from

skeletal muscle (Figure 1B and 1C). MC5-R-null mice reproduce and thrive normally. There was no obvious anatomic, or behavior abnormalities. Therefore, MC5-R is not essential for normal development and daily life under laboratory conditions. The anti-inflammatory action of α-MSH is preserved in these mice, indicating MC5-R is not essential for this function (data not shown). The mutant mice also have an apparently intact hypothalamic-pituitary-adrenal axis, suggesting MC5-R in the adrenal cortex is not essential for the stress response (data not shown). Mutant mice also were also indistinguishable from wild-type mice in swim-induced anagelsia, exluding the involvement of MC5-R in the proposed inhibition of morphine-induced anagelsia by ACTH (Smock, 1980).

Defective Water Repulsion and Thermoregulation in MC5-R Deficient Mice

During a stress-induced analgesia test involving 3 minutes swim, it was noticed that MC5-RKO mice needed more time for their fur to dry than their wild-type counterparts (Figure 2A, 2B). In a cage without bedding, wild-type mice dried their hair in about 25 minutes on average after a 3 minute swim at 32 °C. In contrast, it took MC5-RKO mice more than 40 minutes to dry (Figure 2B).

The longer drying time in the mutant mice is due to impaired water repulsion. MC5-RKO mice absorbed almost twice as much water as the wild type controls (figure 2B). The water absorbed by MC5-RKO mice totalled about 5% of their body mass, while that absorbed by controls only 2.5%. The rate of evaporation, however, was comparable.

This defect in water repulsion appeared to be related to surface lipids. Removal of lipids by a 5% SDS wash increased water absorption to 9% of body weight in wild type mice (Figure 2B).

The initial observation that MC5-RKO mice a needed longer time to dry than their wild-type counterparts prompted investigations on thermoregulation in the mice. The MC5-RKO and wild type animals had the same core body temperature at an ambient temperature at 26 °C. Indeed, the colonic temperature decreased 2 °C during the 3 minute swim at 32 °C in mutant mice, compared to 0.7 °C in the controls. In addition, colonic temperature dropped another 0.5 °C before recovering, whereas no further decline was seen in wild-type mice (Figure 2C). The core body temperature returned to baseline in 20 minutes in wild type mice, at which time the colonic temperature in MC5-RKO mice was still 1.5 °C below normal. Again, lipid removal in wild-type mice led to more severe and longer lasting hypothermia (Figure 2C).

Lipids in the mammalian coat may also be important for optimal regulation in cold air as well. To test this, mice were challenged with cold air (5-6 °C cold room), and again, mutant and wild-type exhibited remarkable difference in their colonic temperature. Wild-type mice increased core temperature slightly at the beginning, and maintained above normal temperature for at least 3 hours. In contrast, MC5-RKO mice underwent a mild hypothermia(Figure 2D). Removal of surface lipids with 5% SDS made normal mice more sensitive to cold exposure (Figure 2D) as was seen in MC5-RKO mice. All together, these results suggested an impairment in water repulsion as well as a defect in the insulating properties of the coat in the MC5-RKO mice due to some sort of deficiency in hair lipids.

Hair lipid content in MC5-RKO mice was examined, and a 15-20% reduction of acetone extractable materials, both in male and female mutants, was observed (figure 2E). It is not unexpected to see less sebum made by females as sebaceous gland activity is upregulated by androgens (Thody, 1975). To determine whether there is a general or specific deficiency, surface lipids were analysed by thin layer chromatography (TLC). A dramatic

reduction of sterol esters in both male and female mutants was observed (Figure 2F). Sterol esters constitute more than 26% of the total acetone extractable lipids in wild-type mice, but only about 13% in the mutants (Figure 2F). There was no other significant defference in other sebum components. As sterol esters are the most hydrophobic species of sebaceous lipids, their deficiency could very likely explain in the impaired water repulsion seen in MC5-RKO mice.

The MC5-R is Expressed at High Levels in Multiple Exocrine Glands

Expression of the MC5-R in sebaceous or other exocrine glands had not previously reported. The defect seen in sebum production suggests a direct role for MC5-R in sebaceous gland function. To test this hypothesis, in situ hybridization was performed on skin sections. High abundancy of MC5-R mRNA was detected in hair follicle associated sebaceous glands of wild-type skin (Figure 3A1, 3A3), but not in skin sections of MC5-RKO mice (Figure 3A2). No hybridization was found in wild type sections probed with sense probe (Figure 3A4), further confirming the specificity of the hybridization signal.

Given earlier data suggesting actions of MSH on sebum production, the finding of MC5-R mRNA in sebaceous gland inspired a comprehensive search for its expression in other exocrine tissues including preputial gland, a specialized sebaceous gland, lacrimal gland and Harderian gland. In agreement with previous studies, MC5-R mRNA was detected at moderate levels in muscle and skin, and was present at very low levels in spinal cord, brain stem, and adipose tissues (Figure 3B). Strikingly, MC5-R mRNA was found to be extremely abundant in the Harderian gland, lacrimal gland and preputial gland (Figure 3C). The level of MC5-R in preputial gland is approximately 30 times higher than that in the skin (Figure 3D).

Functional MC5 Receptor Protein is Found in Multiple Exocrine Glands and in Spinal Cord

To determine if functional receptors are present in these exocrine glands, radio-ligand binding studies were performed. As in skeletal muscle membrane, there was strong specific ^{125}I-NDP-α-MSH binding in crude plasma membranes prepared from Harderian gland, preputial gland, and lacrimal gland of wild-type mice (Figure 4A). When binding was conducted in membrane from heterozygous MC5-RKO mice, intermediate levels of specific binding was found (data not shown). Specific binding was absent in membranes from MC5-RKO mice, indicating the absence of significant levels of expression of the MC1-R, MC3-R and MC4-R in these tissues (Figure 4A).

Specific ^{125}I-NDP-α-MSH binding was also seen in the spinal cord. The decreased binding in the heterozygotes and mutant mice indicates that MC5-R is the major melanocortin receptor in spinal cord (Figure 4B). The residual binding may be due to MC4-R in this tissue.

To further examine the functionality of the receptor, exocrine glands were excised and cultured in vitro. Application of physiological levels of α-MSH and/or NDP-α-MSH markedly stimulated cAMP synthesis in the cultures, further demonstrating the presence of functional receptor protein (Figure 4C, 4D). There was less stimulation of cAMP synthesis by the synthetic ligand NDP-α-MSH than α-MSH, suggesting that NDP-α-MSH may be a partial agonist at the MC5-R. This is consistent with data obtained from MC5-R expressed in HEK293 cells (Chen, unpublished data). The inhibition of α-MSH induced cAMP

production by NDP-α-MSH suggests the compound may act as a mixed agonist/antagonist.

MC5 Receptor Regulates Protein Secretion by the Lacrimal Gland

The lacrimal gland is the major source for the protein-rich aqueous layer of tear film. Its secretion is under neuronal and hormonal control. To assess the consequence of MC5-R ablation on lacrimal gland secretion, we measured melanocortin-stimulated protein secretion in the lacrimal gland fragments in culture. Lacrimal gland acini were pulsed with ^{3}H-leucine, and then allowed further incubation to incorporate the radioacivity into newly synthesized proteins. The rate of protein secretion was determined by monitoring the rate of radioactivity discharge from the cells. Physiological levels of α-MSH and ACTH increased protein secretion about 80% in the gland of wild-type mice, but not that of MC5-RKO mice (Figure 5C). The rate of melanocortin stimulated protein discharge increased in a dose dependent fashion, with an EC_{50} of 4 nM for ACTH (Figure 5B).

MC5 Receptor is Required for Porphyrin Production in the Harderian Gland

The Harderian gland is a bilobular retro-orbital structure that secrets primarily two products, lipids and prophyrins, into the eyes. These products are spreaded the the body surface by grooming. Most vertebrates, with the exception of man, have Harderian glands, although their functional role is not well understood. In rodents, the lipids components are distributed along the coat of the animal by grooming behaviors, and play an important thermoregulatory role. The porphyrins absorb UV light, and coat the cornea, where they could play some role in phototransduction. The porphyrins are co-secreted in abundance with lipids and thus an excellent marker of Harderian function. To characterize the role of the MC5-R, we examined porphyrin content in Harderian glands from wild type and

mutant mice. Under UV, bright fluorescence was seen in organic extractants from Harderian glands of wild-type and heterozygous males. In contrast, no fluorescence was visible in those from mutant males (Figure 6A). That the extracted substances displayed two peak absorbance at 402 and 560 nm confirmed the presence of porphyrins in wild type Harderian glands. There was almost no visible absorbance at the two peaks in extracts from mutant Harderian glands, suggesting a nearly complete porphyrin deficiency in these animals (Figure 6B). Moreover, when excited at 402 nm, very little fluorescence at 602 nm was emmitted from the mutant samples than those from wild-types or heterozygotes (Figure 6C).

To investigate if MC5-R has a prosecretory role in Harderian gland, the following experiment was conducted. Ten μg of $ACTH_{1-24}$ was injected into each wild-type 129 mice. Porphyrins were extracted from Harderian glands of individual mice and semi-quantitated by UV illumination. As shown in figure 6D, 15 minutes after ACTH injection, no detectable porphyrins were found in Harderian gland extracts as indicated by the lack of fluorescence, while strong fluorescence was seen in Harderian gland extracts from control mice and mice injected with ACTH 5 hours earlier. These data suggest that activation of MC5-R may also stimulate exocytosis in Harderian gland.

DISCUSSION

No readily visible phenotype was apparent in mice bred to contain a homozygous deletion of the MC5-R, in either the C57Bl/6J or 129Sv strain backgrounds. Appearance, behavior, growth, muscle mass, adipose mass, reproduction, and basal and stress-induced corticosterone, glucose, and insulin levels in these animals were indistinguishable from heterozygous or wild-type littermates (data not shown). Crossing the dominant *agouti* allele, A^Y into the homozygous MC5-R deficient background had no effect on agouti peptide action in pigmentation or induction of the agouti obesity syndrome (data not shown). In order to identify more subtle physiological phenotypes of the knockout, we proceeded to examine these animals for their response to exogenous melanocortin peptides in a number of adrenocortical-independent biological assays. Melanocortin peptide activities examined included anti-inflammatory activity of α-MSH in carageenan-induced ear-swelling (Macaluso et al., 1994), enhanced recovery from sciatic nerve crush by α-MSH (Bijlsma, 1983; Strand et al., 1993), α-MSH induced inhibition of stress-induced analgesia (Belcher et al., 1982; Smock and Fields, 1981). None of these assays produced identifiable differences between the wild type and knockout animals.

Remarkably, identification of the phenotype described here occurred fortuitously as a result of an accidental cage flooding caused by a defective automatic watering device. One of us (W.C) observed that the knockout animals seemed less able to climb out of the flooded cage floor. Later, during a stress-induced analgesia assay in which the mice are made to swim for three minutes to activate the hypothalamic-pituitary-adrenal axis (Mogil, 1996), it was observed that the knockout animals had adsorbed more water, and remained wet for a longer period of time than littermate controls. This effect was then quantitated,

and the absence of the MC5-R demonstrated to nearly double water retention in the coat (Fig 2b), resulting in severe thermoregulatory defects in the animal as well (Fig 2c, 2d).

The MC5-R is essential for normal mammalian thermoregulation

Thermoregulation is a complex process involving many physiological responses including basal metabolic rate, vasodilation and constriction, shivering, non-shivering thermogenesis mediated by brown fat stores, sweating, panting, and lastly, insulation via the skin and coat. In addition to their obvious role in repelling water, dermal lipids produced by the sebaceous and Harderian glands are critical for supporting the optimal insulating capabilities of the mammalian coat. Removal of the Harderian gland, a large bi-lobed gland found in the retroorbital region in most vertebrates, results in approximately 40-50% reduction in lipids extractable from the coat (Thiessen and and Kittrell, 1980). This, in turn, results in a dramatic thermoregulatory defect in the gerbil (Thiessen and and Kittrell, 1980), reducing core body temperature 4.6^{o} in response to a cold water bath in the Harderianectomized animal compared to 1.6^{o} in the sham operated control. Likewise, sebaceous lipids play an important thermoregulatory role, as has been demonstrated in the muskrats (Harlow, 1984).

We demonstrate here that the MC5-R is expressed in both Harderian and sebaceous glands (Fig 3b, 3c, 3d), and when absent results in a 15 - 20% decrease in total acetone-extractable lipids from the murine coat (Figure 2e). In particular, thin-layer chromotography demonstrated a 50% or greater reduction in sterol ester type lipids, extracted from the coat as described (Figure 2f). No differences in the composition of Harderian lipids was detected by this method, consequently it is most likely that the defect in lipid production results from the absence of the MC5-R specifically in the sebaceous glands. The specific defect in sterol esters identifies this class of lipids as being

particularly important in water repulsion and the insulating capability of the murine coat. Shampooing the wild type 129Sv animals was observed to remove approximately 50-80% of the acetone extractable lipids, and this treatment was found to produce an even greater increase in water aborption and loss in thermoregulatory capability relative to that resulting from MC5-R knockout.

As is seen in Fig 2d, the thermoregulatory defect resulting from a deficit in sterol ester lipids also occurs in cold air, demonstrating a role for these lipids in the insulating properties of the mammalian coat, outside of the role in water repulsion. Moreover, the knockout animal was still slightly more sensitive to cold than the wild type even after both animals had previously been shampooed (data not shown). This could result from a defect in a class of lipid that is not readily removed by shampooing, or may indicate the existence of yet a third mechanism, in addition to water repulsion and insulation, by which the MC5-R participates in thermoregulation.

The MC5-R encodes the primary MSH binding site in exocrine gland, skeletal muscle, and spinal cord

The creation of the MC5-R knockout mouse allowed us to examine the role of this receptor in the expression of MSH binding sites, as assessed by the binding of radiolabelled ^{125}I-NDP-α-MSH (Figs 1e, 3f, 3g). Deletion of the MC5-R resulted in the loss of detectable ^{125}I-NDP-α-MSH binding to Harderian gland, lacrimal gland, preputial gland, spinal cord, and skeletal muscle. Consequently, in addition to the phenotypes described here, other biological activities of melanocortin peptides acting at these tissues are likely to be mediated by the MC5-R. Particularly striking was the high level of MC5-R binding sites expressed in spinal cord and skeletal muscle (Fig. 1e, 3g). Roles for these receptors

might include effects of melanocortin peptides on nerve regeneration (Bijlsma, 1983; Strand, 1980), muscle satellite cell proliferation (Cossu, 1989; De Angelis et al., 1992), and muscle deuse deconditioning..

The binding sites demonstrated here were also shown to be effectively coupled to adenylyl cyclase (Fig 4a, 4b) in Harderian, lacrimal, and preputial glands. In some cases, as much as a twenty-fold increase in intracellular cAMP could be seen following stimulation with 50 nM α-MSH.

The MC5-R regulates Harderian and lacrimal gland function

Since we were able to demonstrate the existence of a functional MC5-R in several exocrine glands, we next wished to identify potential exocrine gland products that might be regulated via the MC5-R. The production of sebaceous and preputial lipids has previously been demonstrated to be regulatable by exogenous α-MSH (Cooper et al., 1976; Cooper et al., 1974; Thody et al., 1976). The work presented here demonstrates a pharmacological rationale for these observations, with the MC5-R expressed at high levels on these glands strongly implying a role for endogenous α-MSH or ACTH in the regulation of sebaceous and Harderian lipid production, Harderian porphyrin production, and perhaps Harderian and preputial pheromones as well.

The lacrimal gland is known to secrete both electrolytes and proteins, largely under parasympathtic control (Dartt, 1994). Like many other glands, however, cAMP is also a secretagogue for the lacrimal gland (Bothelo, 1973). Furthermore, both ACTH and α-MSH have been demonstrated to increase total protein discharge 3-4 fold from lacrimal

glands in culture (Jahn, 1982; Leiba et al., 1990). The demonstration of high affinity melanocortin binding sites in lacrimal glands has previously been demonstrated (Leiba et al., 1990; Tatro and Reichlin, 1987). Furthermore, α-MSH stimulation of peroxidase secretion by the lacrimal gland was as robust as that seen with epinephine and carbamylcholine, and was not blocked by atropine, propranolol, or phentolamine, suggesting that a-MSH is an independent secretagogue (Leiba et al., 1990). We demonstrate here that the receptor mediating these effects is the MC5-R, and that ACTH can stimulate total protein secretion from the lacrimal gland with an EC_{50} of 4nM (Fig. 5B). While the regulated secretion of lacrimal gland protein by melanocortin peptides is absent in the MC5-R knockout mouse, the size and appearance of the gland was normal, suggesting that the receptor does not play a role in the development of the gland.

Following the administration of ^{125}I-NDP-α-MSH in rats, Tatro and Reichlin found the highest concentration of radiolabel accumulated in the Harderian gland (Tatro and Reichlin, 1987). While we found no alteration in the production of Harderian lipids in the MC5-R deficient animals, this gland is also known to produce and secrete large amounts of porphyrins (Payne, 1994). Remarkably, in some rodents the gland contains higher concentrations of 5-amino-laevulinate synthase, the rate limiting enzyme for heme biosynthesis, than are found in liver (Payne, 1994). The role of porphyrin secretion by the Harderian gland is unknown, however since these compounds absorb UV light and appear to be spread over the surface of the cornea they may be involved in some aspect of phototransduction (Spike et al., 1992). We demonstrate here that the MC5-R is essential for maintenance of Harderian gland porphyrin synthesis, with little or no porphyrin detected by absorption spectrophotometry in the MC5-R knockout mouse. As was true for lacrimal, preputial, and sebaceous glands, development of the Harderian gland appeared

normal, thus implicating the MC5-R specifically in the regulated synthesis of certain Harderian gland products.

Is the MC5-R Part of a Hypothalamic-Pituitary-Exocrine Gland Axis?

Exocrine gland function is known to be coordinately controlled by the parasympathetic and sympathetic nervous systems, with the former exerting a stimulatory effect in most cases. However, hormonal regulation of exocrine gland function is also well characterized, such as the stimulation of sebaceous gland function by androgens involved in acne (Ebling et al., 1975; Thody et al., 1976). However, the discovery of the regulation of the synthesis of lipids, proteins, and porphyrins in a variety of exocrine glands by the MC5-R suggests the existence of a coordinated system for hormonal control of exocrine gland function by melanocortin peptides. What is the nature of this system?

Previous data on sebaceous gland function shows that testosterone and α-MSH are synergistic in their control of sebum production (Ebling et al., 1975; Thody et al., 1976). Hypophysectomy in mice (Ebling et al., 1969), and hypopituitarism in man (Goolamali et al., 1974) decreases sebum production. The MC5-R is approximately five fold more sensitive to α-MSH than ACTH , and furthermore, ablation of the neurointermediate lobe, the source of circulating α-MSH, decreases sebum production as much as a total hypophysectomy, without decreasing testosterone levels (Thody and Shuster, 1973). These data tend to suggest that pituitary α-MSH regulates sebaceous gland function, and led one group to refer to this peptide as a sebotropic factor (Thody and Shuster, 1973).

On the other hand, the MC5-R remains very sensitive to ACTH, with EC_{50} values reported in the low nM range (Fathi et al., 1995; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994), comparable to the 1nM EC_{50} reported for activation of adenylyl cyclase by the adrenocortical ACTH receptor, MC2-R (Buckley and Ramachandran, 1981). While the affinity of the MC5-R for ACTH is somewhat lower than the MC2-R, it is important to keep in mind that activation of steroidogenic gene expression by the ACTH-R can be detected at ACTH levels as low as 10^{-11}M, several logs below half-maximal receptor occupancy (Simpson, 1988). Furthermore, since circulating α-MSH is generally not detectable in man, a pituitary-derived melanotropin peptide involved in the regulation of sebaceous glands would, by necessity, have to be ACTH. Consequently, the existence of a hypothalamic-pituitary-exocrine axis would suggest the possibility of exocrine gland regulation by the stress axis.

Stress-mediated regulation of exocrine gland function via elevated levels of ACTH acting via the MC5-R might be particularly interesting in regard to pheromonally-mediated mammalian behaviors. If this axis is functional, it would provide a pathway for effects of stress on conspecific mammalian behavior via the regulation of olfactory cues, in short, a mechanism for animals to "smell" stress. Preputial, Harderian, and sebaceous glands are all known to produce pheromones, and all express high levels of functional MC5-R (Figs 4a and 4b). Numerous reports suggest a variety of elicited behaviors in the rodent in response to exogenously administered ACTH and MSH. α-MSH has been demonstrated to stimulate the release of a preputial odorant into the urine which stimulates aggressive attacks (Nowell et al., 1980). The preputial gland is also known to produce pheromones that function as sexual attractants (Bronson and and Caroom, 1971; Chipman and and Alberecht, 1974; Orsulak, 1972), as does the Harderian gland (Thiessen and Harriman, 1986). In preliminary experiments, Dr. John Lepri has demonstrated that MSH, when

administered to wild type but not MC5-R knockout animals, induces expression of a preputial odorant that stimulates aggressive attacks . Future experiments will be directed towards identifying the source of melanocortin peptides involved in the regulation of exocrine gland function, and determining if stress-induced levels of pituitary-derived ACTH can induce exocrine gland function via the MC5-R.

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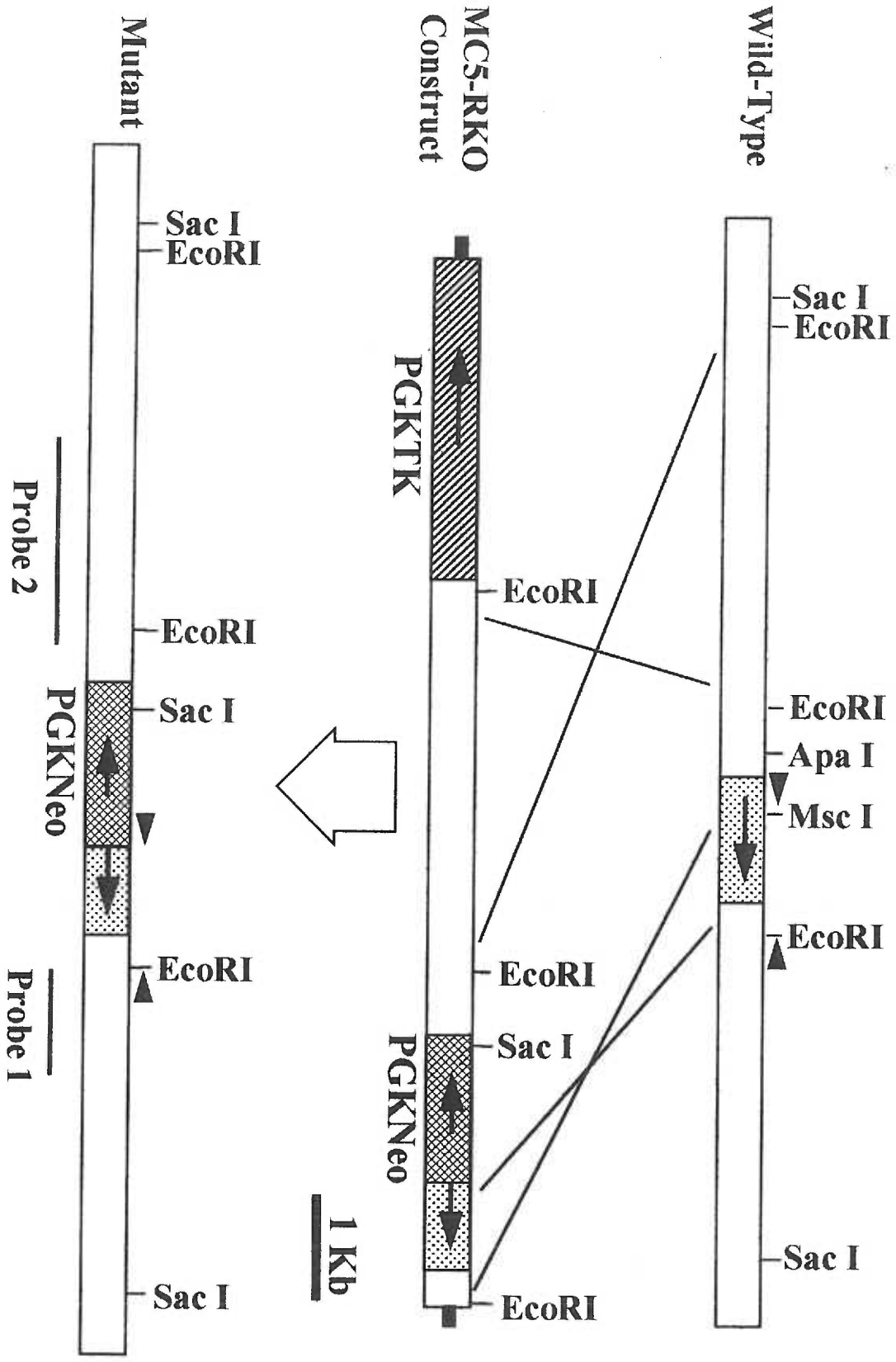
Figure 1. Generation of MC5-RKO mice.

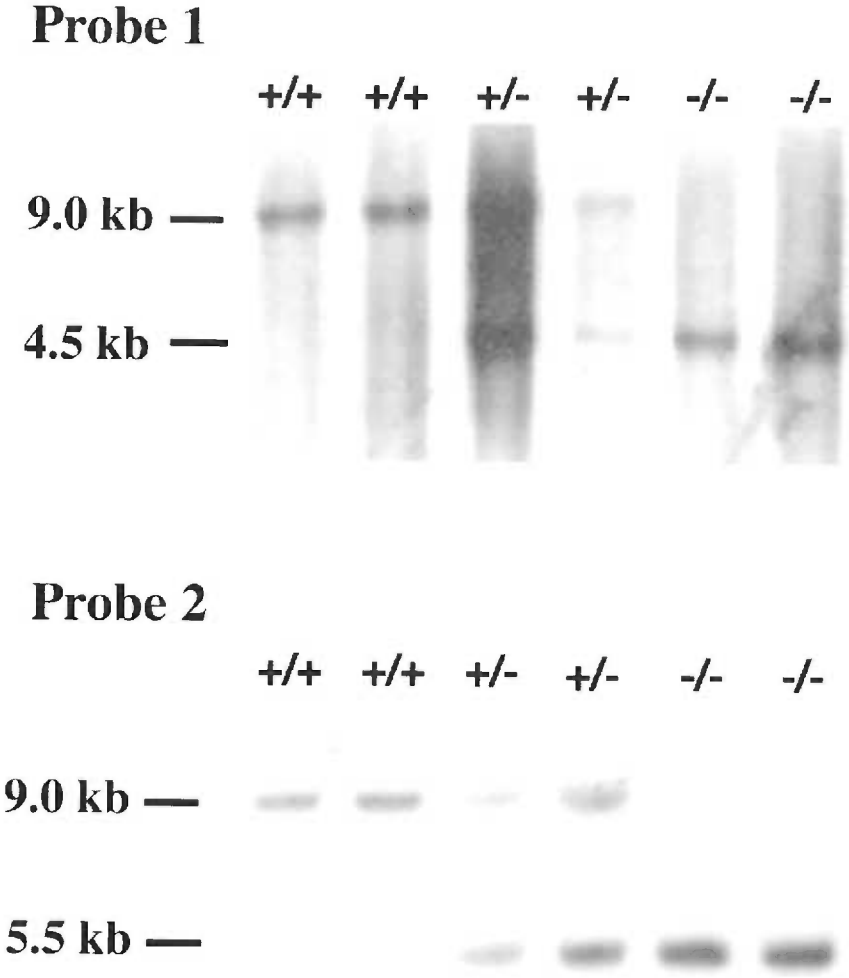
1A. Schematic drawing of the knock-out construct.

The shaded box in the wild-type allele represents the single coding exon of the murine MC5-R. The arrows in the boxes indicate the orientation of transcription. Small arrows above the boxes in the wild-type and mutant alleles stand for the PCR primers for genotyping.

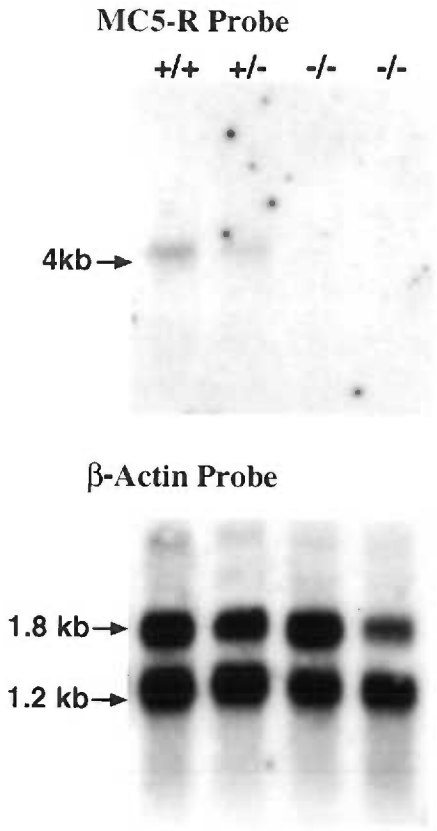

Mutant
MC5-RKO Construct
Wild-Type
Sac I
EcoRI
PGKTK
EcoRI
Sac I
EcoRI
Probe 2
EcoRI
PGKNeo
Sac I
Apa I
Msc I
EcoRI
EcoRI
Probe 1
EcoRI
Sac I
PGKNeo
1 Kb
Sac I
EcoRI

1B. Southern analysis of genomic DNA from different genotypes of F1 offsprings with probe 1 (a) and probe 2 (b).

The genomic DNA of 21-day old progenies were isolated and their genotypes were determined using the mixture of three primers as indicated in the drawing, and as described in Methods section. Ten μg of DNA from putative wild-type, heterozygous and homozygous mutant mice was digested with Sac I for Southern analysis with probe 1 and probe 2. The 4.5 kb band in (a) and 5.5 kb band in (b) represent the mutant allele.


Probe 1
+/+ +/+ +/- +/- -/- -/-
9.0 kb
4.5 kb
Probe 2
+/+ +/+ +/- +/- -/- -/-
9.0 kb
5.5 kb

1C. Northern analysis of MC5-R expression in skeletal muscles. Poly A+ mRNA from 250 μg of total RNA was loaded in each lane. After electrophoresis and transfer, the membrane was probed with the radioactive 650 bp Apa I/Msc I fragment.


MC5-R Probe
+/+ +/- -/- -/-
4kb
β-Actin Probe
1.8 kb
1.2 kb

1D. Radioligand binding to skeletal muscle membranes.

Fresh skeletal muscles of the hind limbs from individual mice of each genotypes were minced, homogenized, and crude plasma membranes isolated as described. Total and non-specific binding was measured after incubation of the membranes with ^{125}I-NDP-α-MSH (10,000 cpm/sample) in the presence or absence of 1 μM α-MSH. After extensive washing, specific binding was calculated and normalized to total protein.

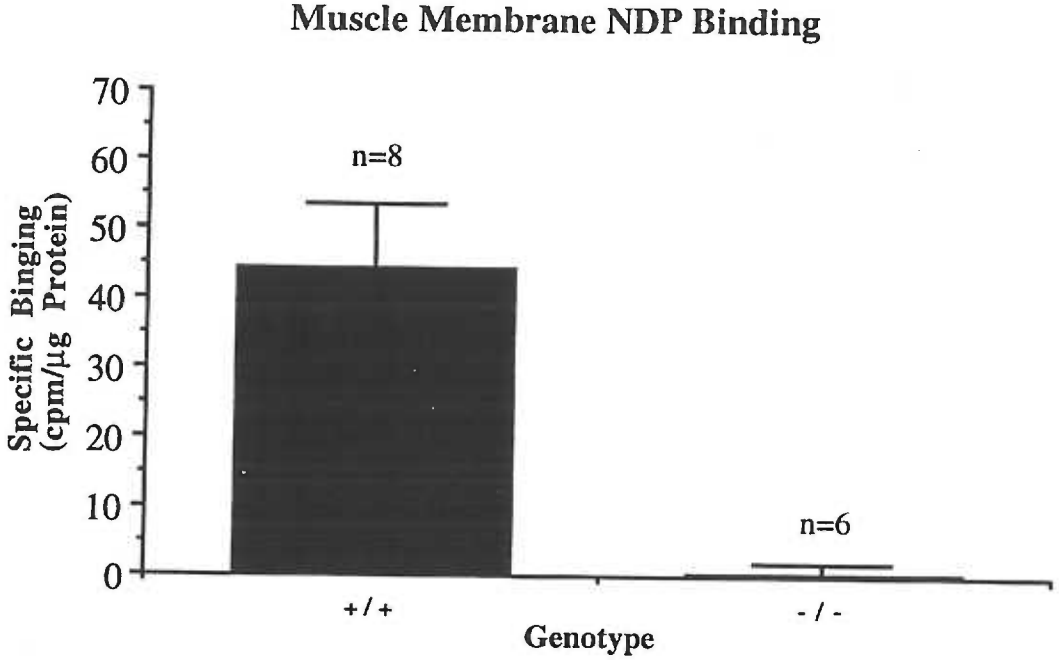

Muscle Membrane NDP Binding
Specific Binging (cpm/µg Protein)
70
60
50
40
30
20
10
0
n=8
n=6
+/+
-/-
Genotype

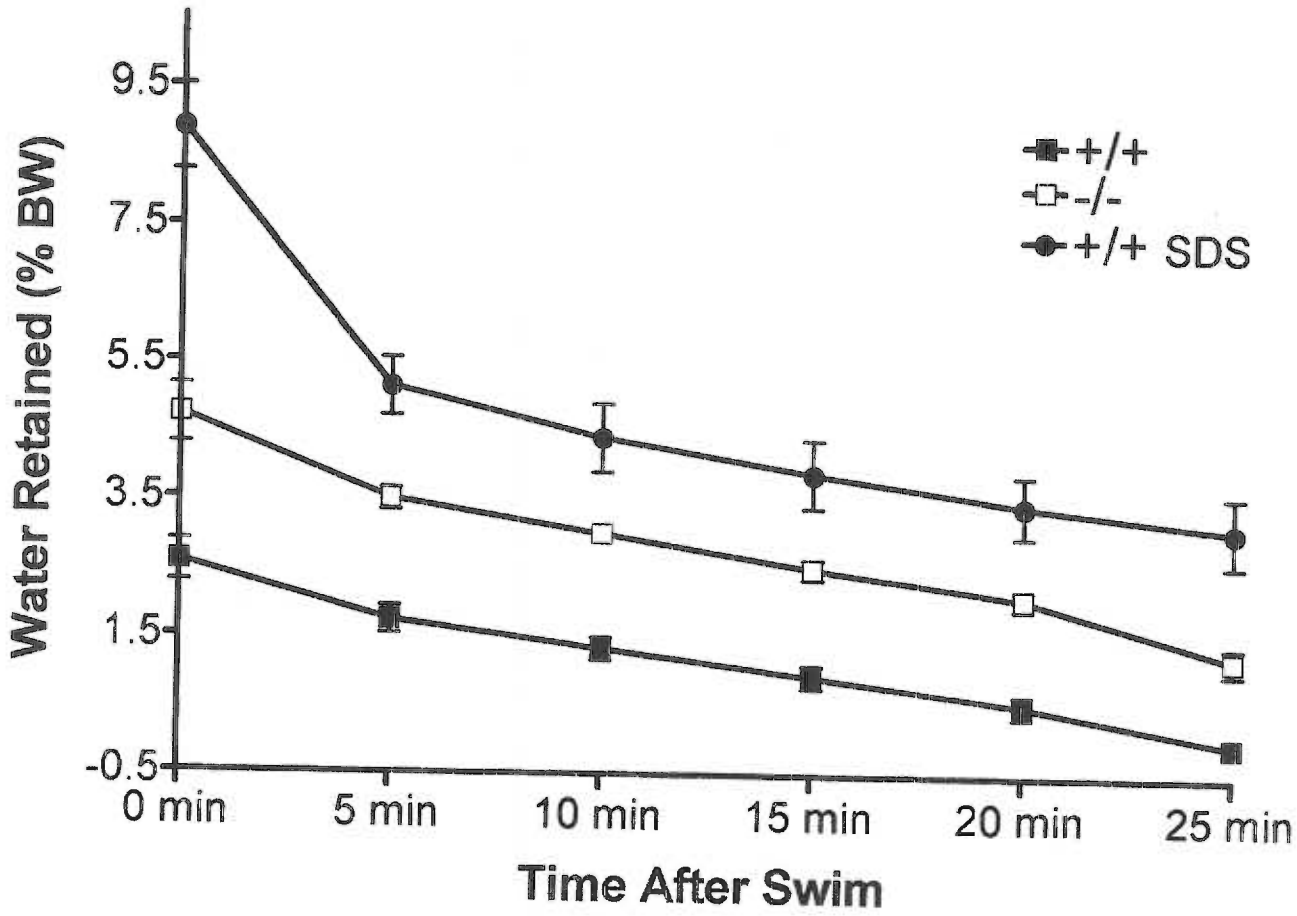
Figure 2. Defects in water repulsion and thermoregulation in MC5-RKO mice.

2A. MC5-RKO mice dry more slowly after a swim.

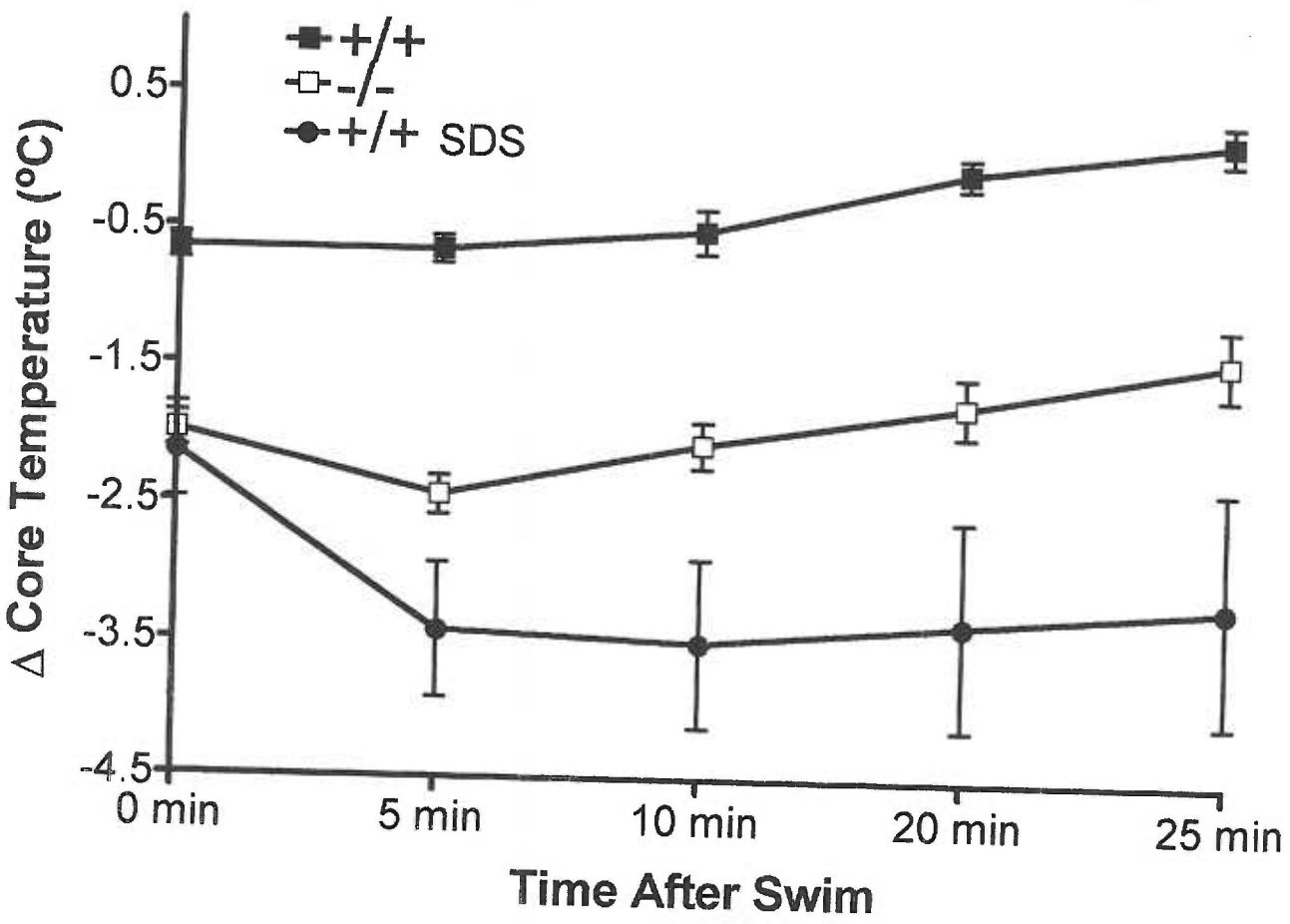
Picture taken about 15 minutes after a 3 minute swim in 32 °C water. The two wet mice one the left are MC5-RKO mice. The other two are wild-types.

2B. Impaired water repulsion in MC5-RKO mice.

MC5-RKO mice absorb more water during the swim than wild-type controls. Removal of hair lipids with 5% SDS wash increases water adsorption in wild-type mice.

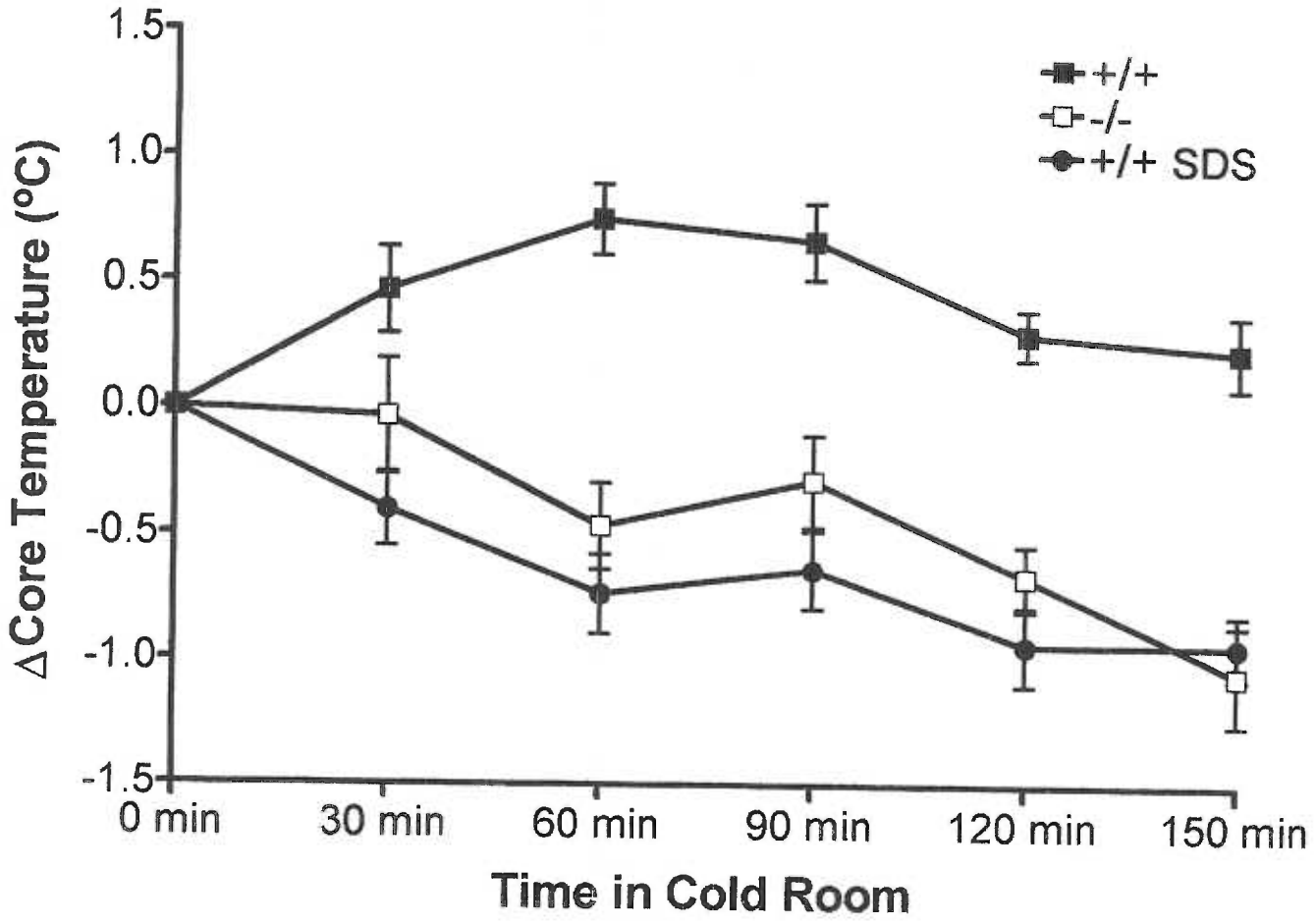

9.5
7.5
5.5
3.5
1.5
-0.5
Water Retained (% BW)
+/+
-/-
+/+ SDS
0 min
5 min
10 min
15 min
20 min
25 min
Time After Swim

2C. Increased water absorption induces hypothermia in MC5-RKO mice and in shampooed wild-type mice.

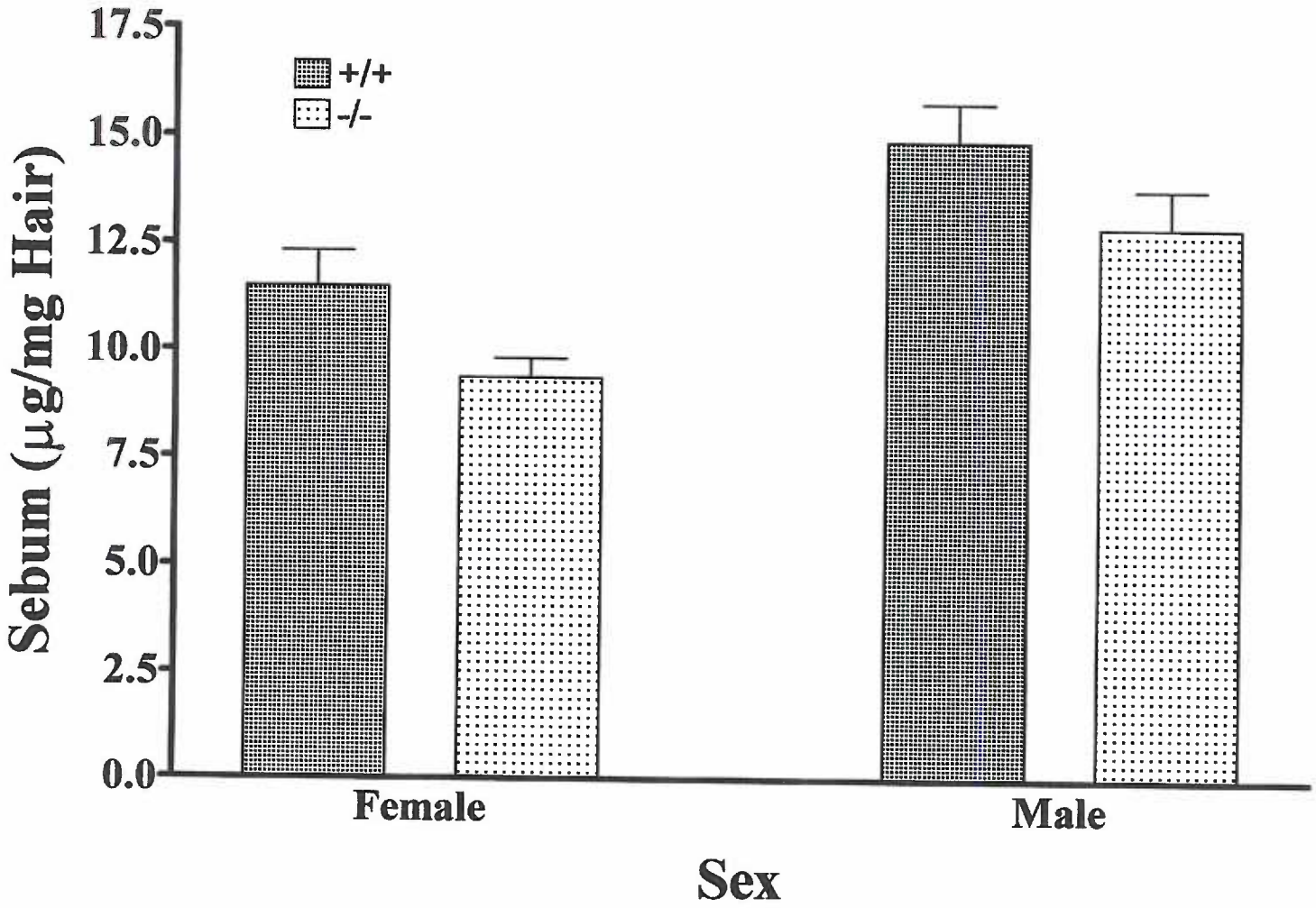

+/+
-/-
+/+ SDS
0.5
-0.5
-1.5
-2.5
-3.5
-4.5
Δ Core Temperature (°C)
0 min
5 min
10 min
20 min
25 min
Time After Swim

2D. MC5-RKO and shampooed wild-type mice exhibit hypothermia in cold air.

Mice were put in 5-6 °C cold room without bedding in a Plexiglas cage. Colonic temperature was measured every 30 minutes immediately.


1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
ΔCore Temperature (°C)
+/+
-/-
+/+ SDS
0 min
30 min
60 min
90 min
120 min
150 min
Time in Cold Room

2E. Sebum production is reduced 15-20% in MC5-RKO mice.


17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
+/+
-/-
Sebum (µg/mg Hair)
Female
Male
Sex

2F. Significant deficit in sterol ester lipids in the MC5-RKO mouse.

Hair lipids are extracted as described. Lipids were resolved in Silica Gel 60 plate (20 x 20 cm) with hexanes/benzenes (55:45, v/v). Each lane contained 150 μg of total lipids.

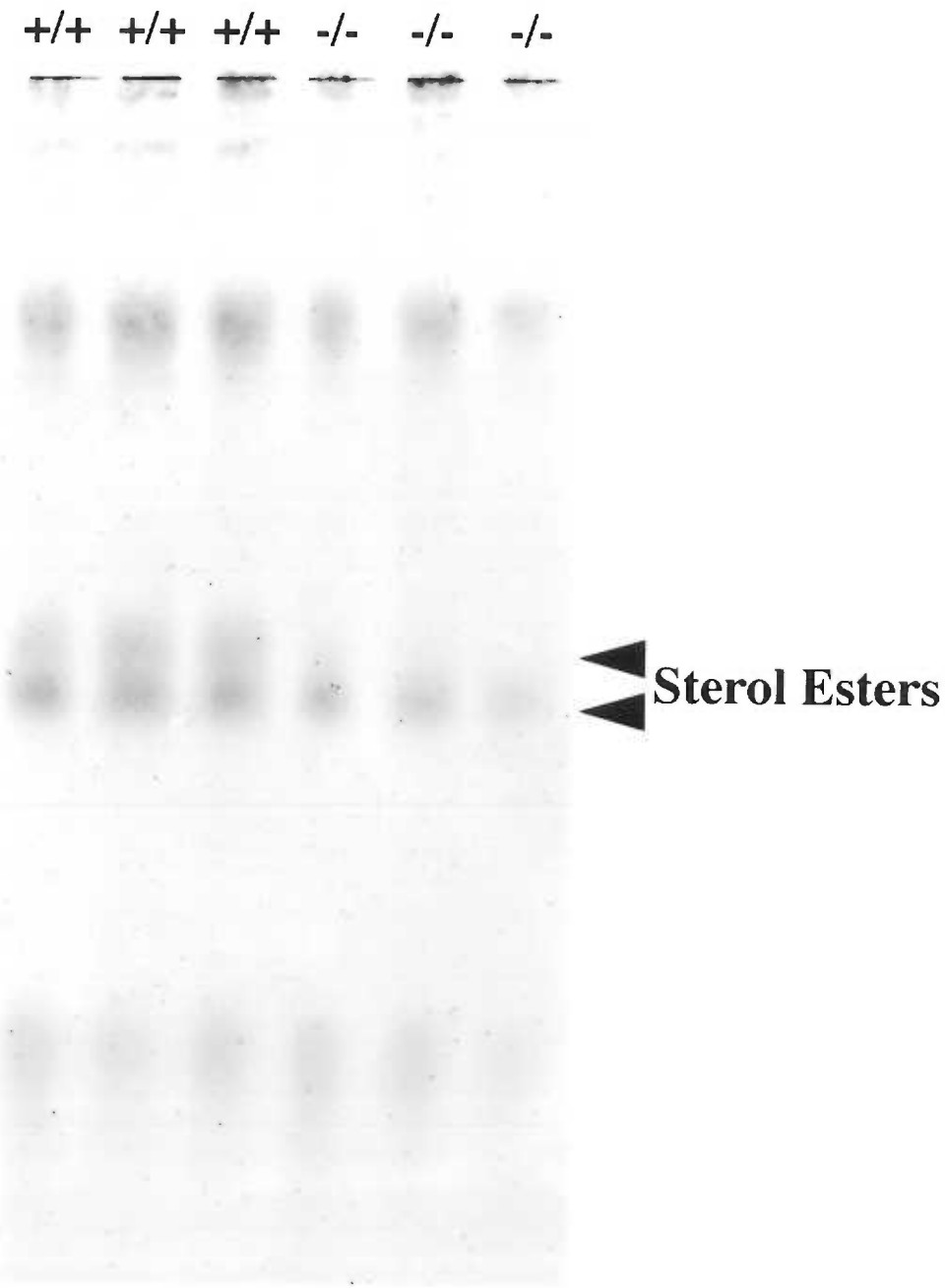
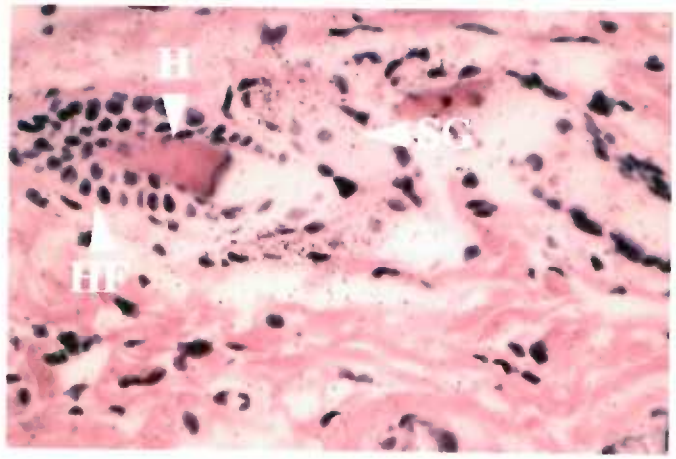

+/+ +/+ +/+ -/- -/- -/-
Sterol Esters

Figure 3. MC5-R is highly abundant in exocrine glands and present at low levels in a number of other tissues.

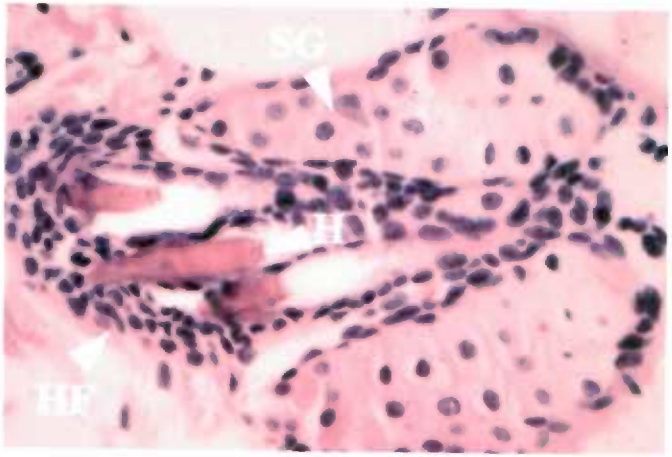
3A. MC5R is specifically expressed in sebaceous gland in the skin.

Five μM sections were made from paraffin embeded skin tissues. After proteinase K digestion and acetylation, the sections were probed with antisense (A,B,C) or sense(D) riboprobe of the deleted region in MC5RKO mice. Hybridization of MC5R was found in wild-type skin (A,C) but not in MC5RKO skin (B). No hybridization was detected by sense probe of the same sequence in mild-type skin (D). H, Hair; HF, Hair Folicle; SG, Sebaceous gland.

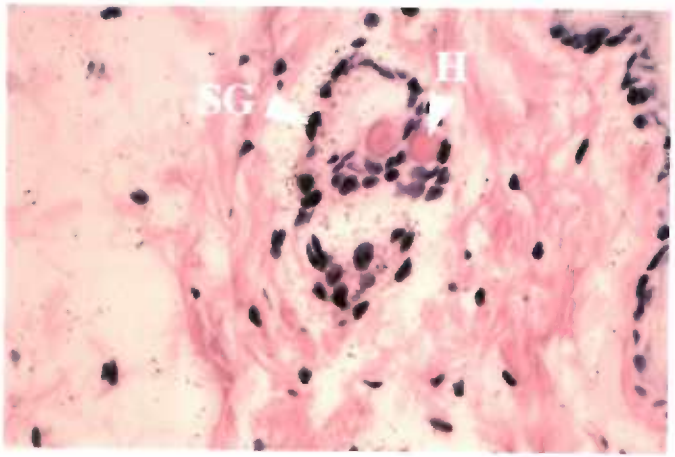
A.



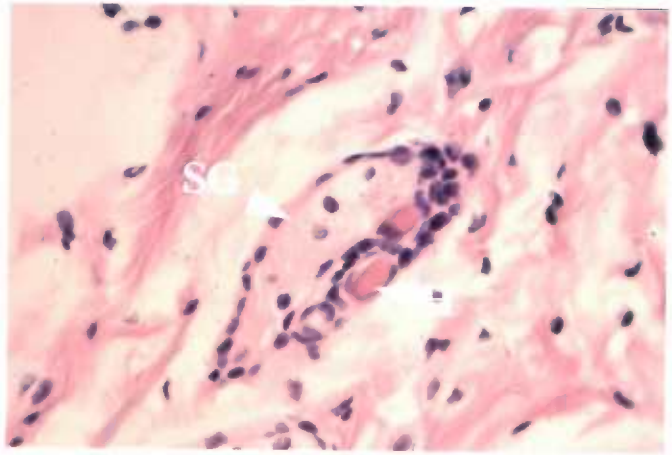
B.



C.



D.



3B. MC5-R mRNA is expressed at low levels in a number of neuronal and non-neuronal tissues.

Forty μg of total RNA was loaded in each lane (10 μg for pituitary, thyroid and adrenal).

3C. MC5R is highly expressed in preputial, Harderian and lacrimal glands.

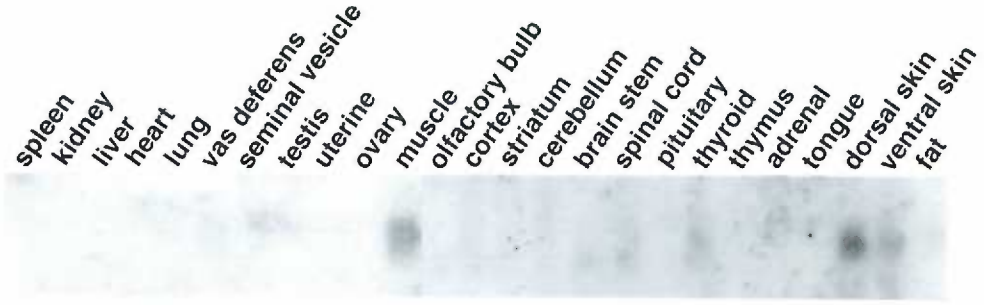
Ten μg of total RNA is loaded in each lane.

3D. The level of MC5R in preputial gland is much higher than in the skin. Twenty ug of total RNA was loaded in each lane.

3E. MC5R is not present in preputial and Hardrian gland of MC5-RKO mice.

Ten μg of total RNA was loaded in each lane. The membrane-bound RNA was probed with the deleted fragment in MC5-RKO mice.

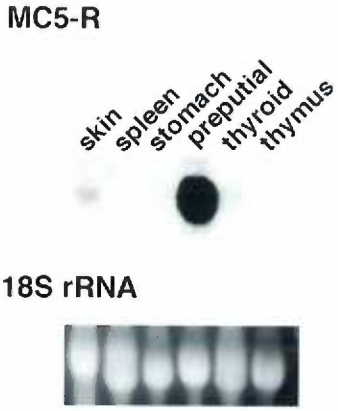
3B


spleen
kidney
liver
heart
lung
vas deferens
seminal vesicle
testis
uterine
ovary
muscle
olfactory bulb
cortex
striatum
cerebellum
brain stem
spinal cord
pituitary
thyroid
thymus
adrenal
tongue
dorsal skin
ventral skin
fat

3C


MC5R
colon
duodenum
Harderian
lacrimal
parotid
preputial
preputial
ovary
uterus
seminal vesicle
testis
testis
vas deferens
β-actin

3D


MC5-R
skin
spleen
stomach
preputial
thyroid
thymus
18S rRNA

3E

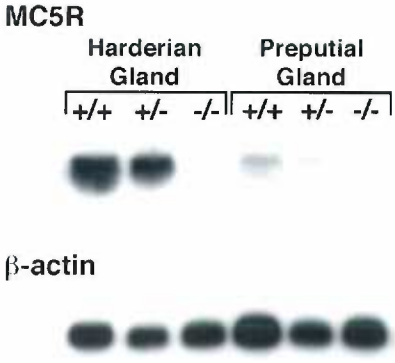
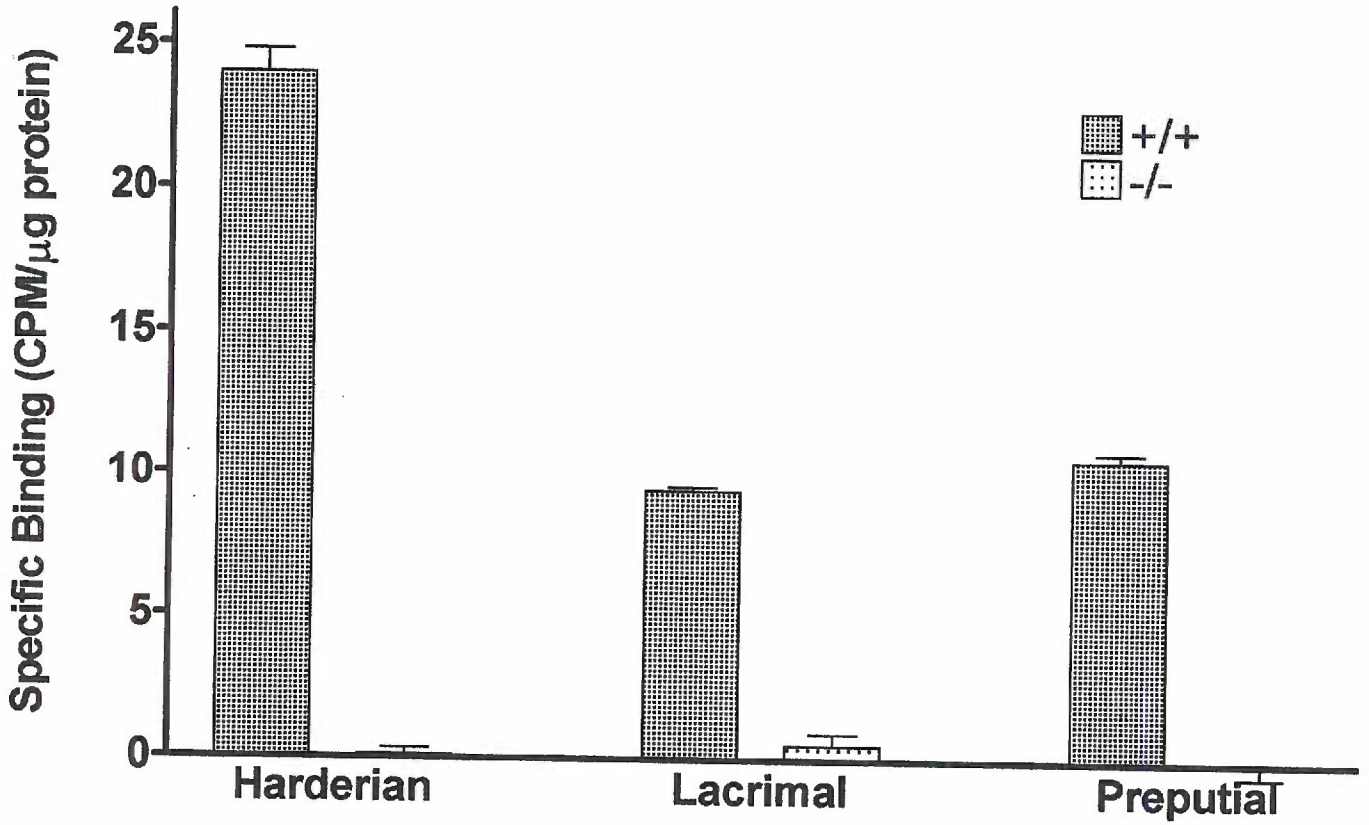
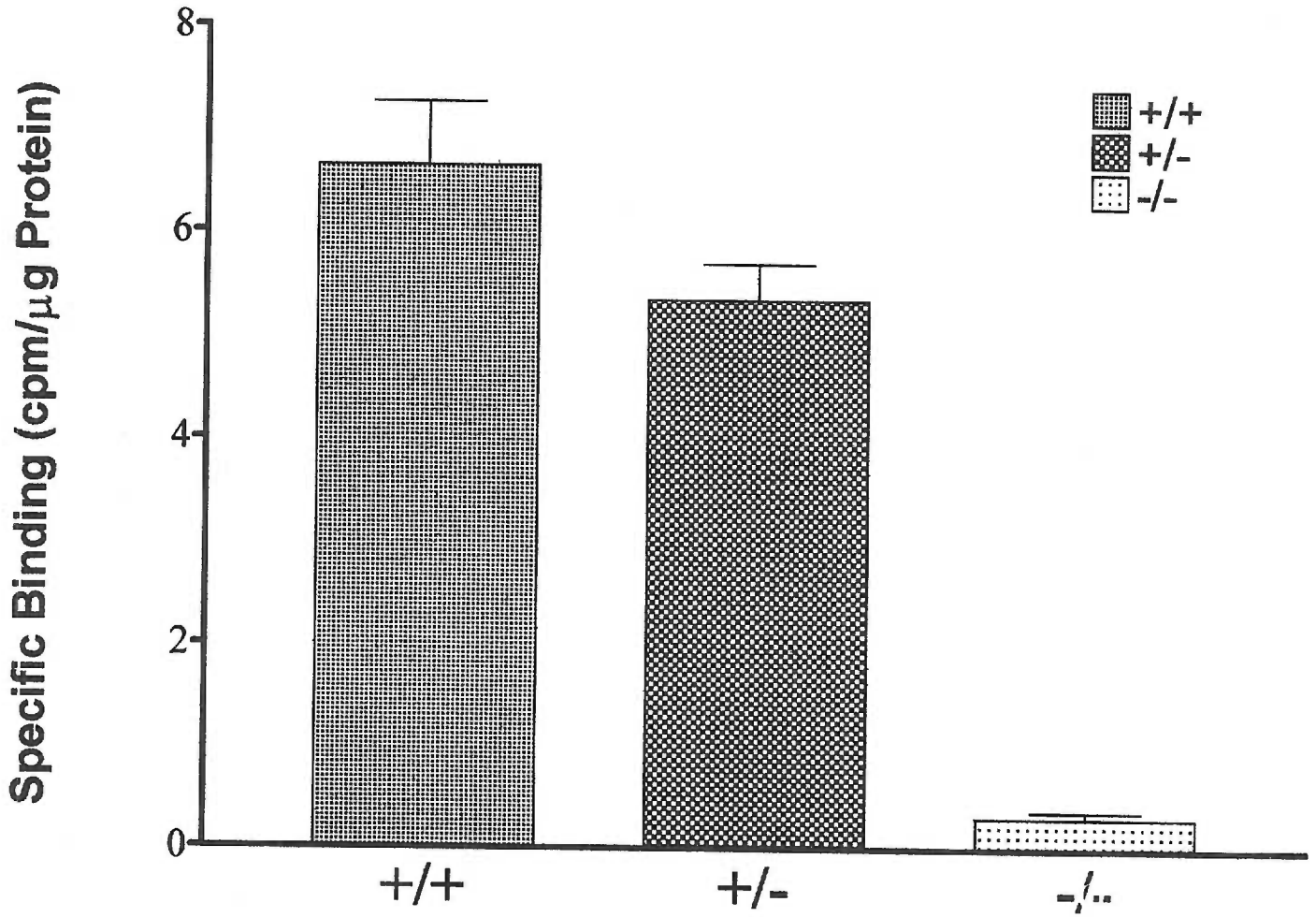

MC5R
Harderian Gland
Preputial Gland
+/+ +/- -/- +/+ +/- -/-
β-actin

Figure 4. MC5-R is the only functional melanocortin receptor in several exocrine glands, and the primary melanocortin receptor in the spinal cord. 4A. Specific binding sites are present in plasma membrane of Harderian gland, preputial gland and lacrimal gland.

The crude membranes were prepared as described. The specific binding activity in different tissues does not necessarily represent the levels of expression, as the purity of the membrane preparation may be different between samples from different tissue.

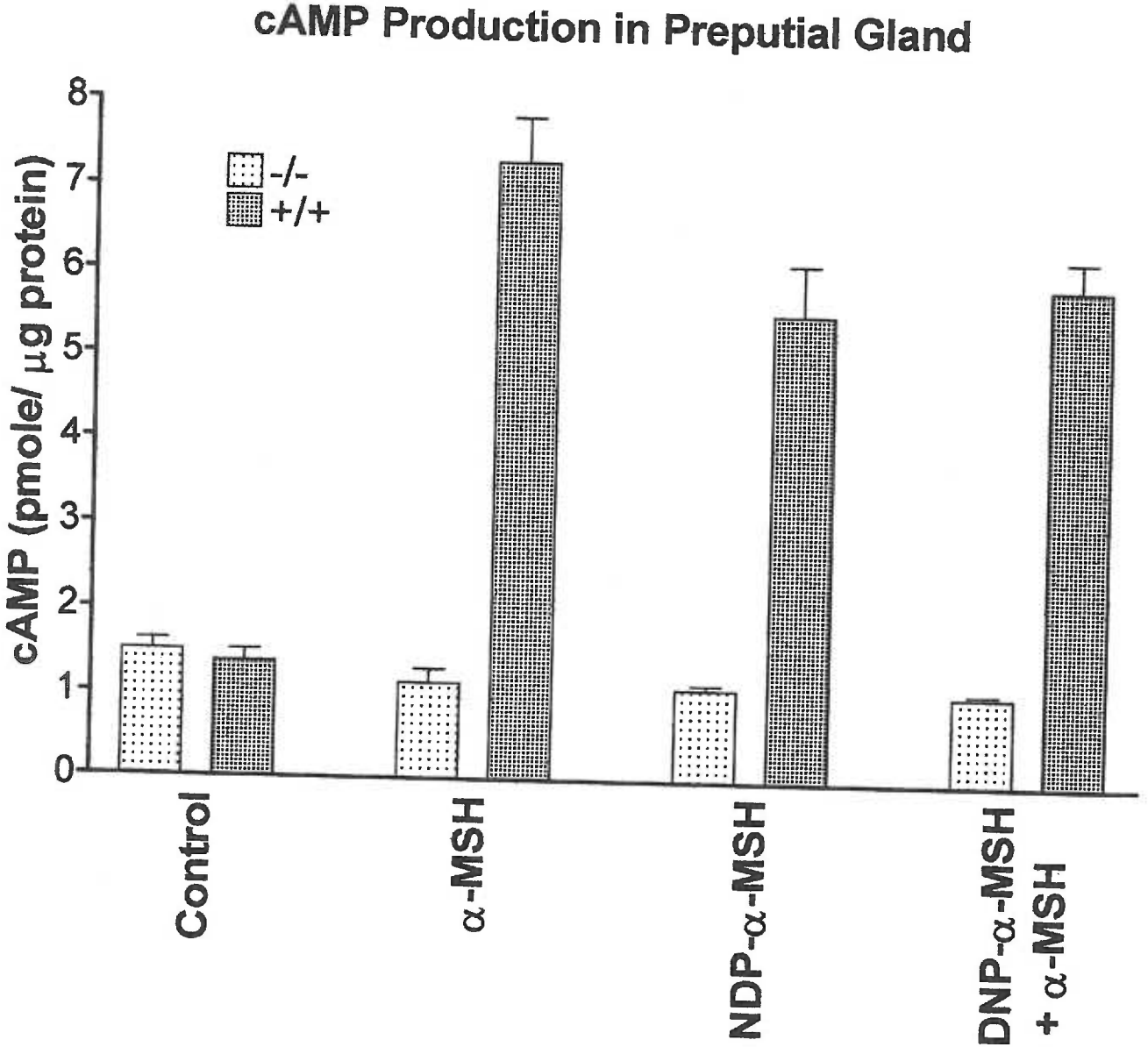

Specific Binding (CPM/μg protein)
25
20
15
10
5
0
+/+
-/-
Harderian
Lacrimal
Preputial

4B. NDP-α-MSH binding is markedly decreased in the spinal cord of MC5-RKO mice.


Specific Binding (cpm/μg Protein)
8
6
4
2
0
+/+
+/-
-/-
+/+
+/-
-/-

4C. Lack of α-MSH and NDP-α-MSH regulated cAMP production in preputial glands from MC5-RKO mice.

Glands were excised and incubated with DMEM containing α-MSH (50 μM), NDP-α-MSH (100 μM), or the two combined. Twenty minutes later, the glands were snap frozen in liquid nitrogen and subsequently homogenized in 60% ethanol. After centrifugation, the cAMP supernatant was vaccuum dried. The quanity of cAMP in each sample was determined by a cAMP RIA kit purchased from NEN.


cAMP Production in Preputial Gland
cAMP (pmole/ µg protein)
8
7
6
5
4
3
2
1
0
-/-
+/+
Control
α-MSH
NDP-α-MSH
DNP-α-MSH
+ α-MSH

4D. Lack of α-MSH and NDP-α-MSH regulated cAMP production in Harderian glands from MC5-RKO mice.

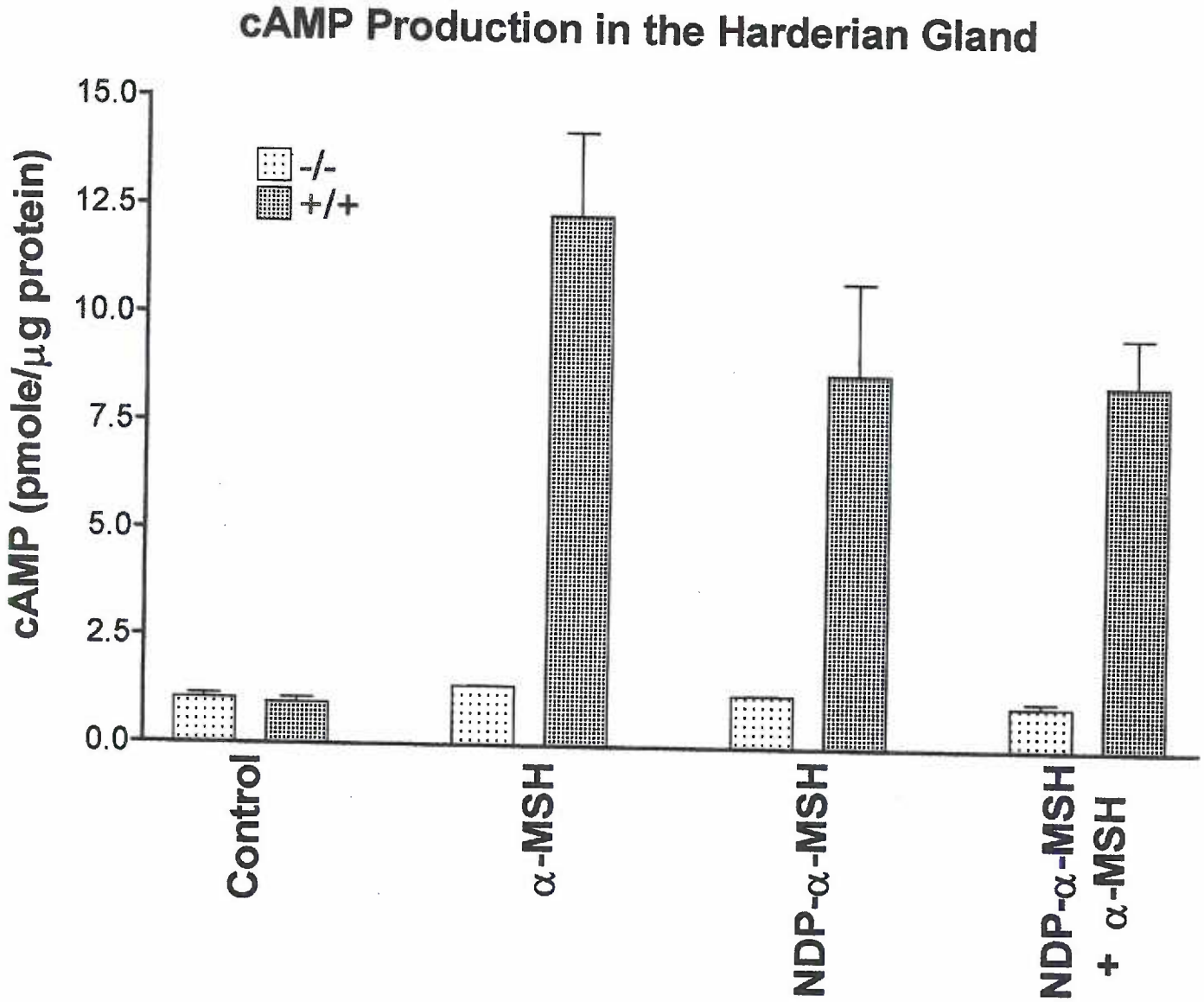
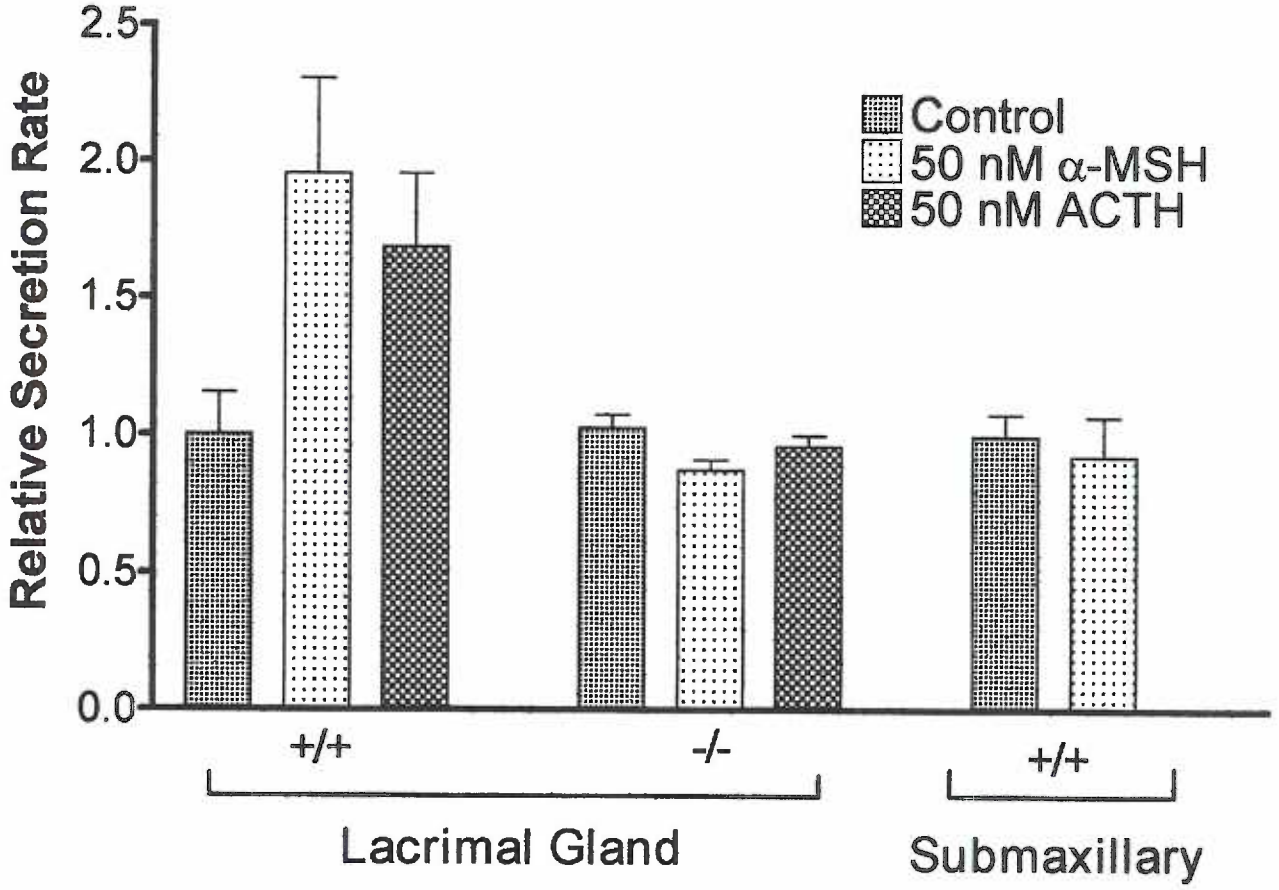

cAMP Production in the Harderian Gland
cAMP (pmole/μg protein)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
-/-
+/+
Control
α-MSH
NDP-α-MSH
NDP-α-MSH + α-MSH

Figure 5. MC5R deficiency results in lacrimal gland dysfunction.

5A. Lack of melanocortin-stimulated protein secretion in lacrimal gland from MC5-RKO mice.


Relative Secretion Rate
2.5
2.0
1.5
1.0
0.5
0.0
Control
50 nM α-MSH
50 nM ACTH
+/+
-/-
+/+
Lacrimal Gland
Submaxillary

5B. Dose response curve of ACTH stimulated protein secretion in lacrimal gland of C57bl/6J mice.

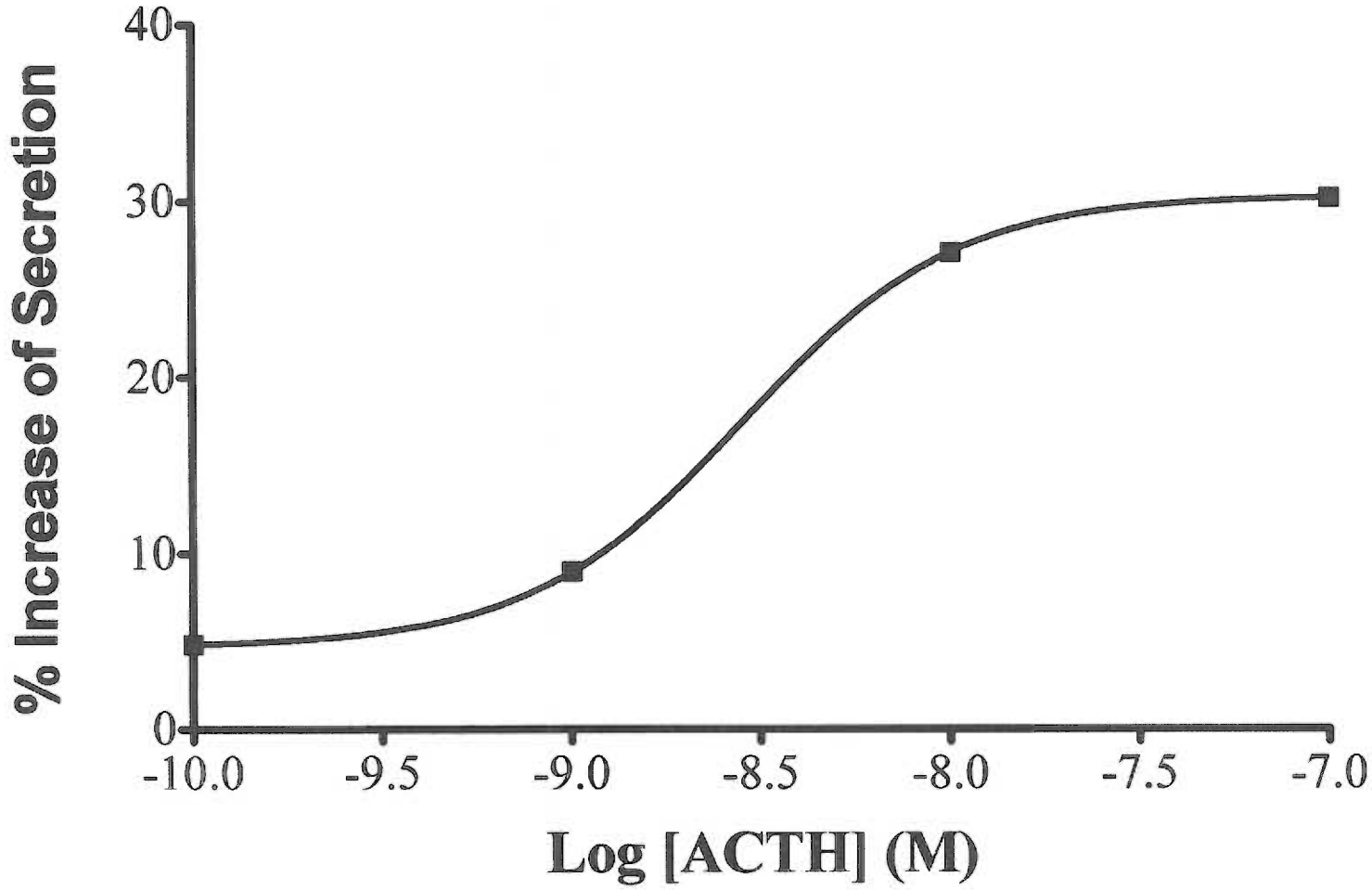
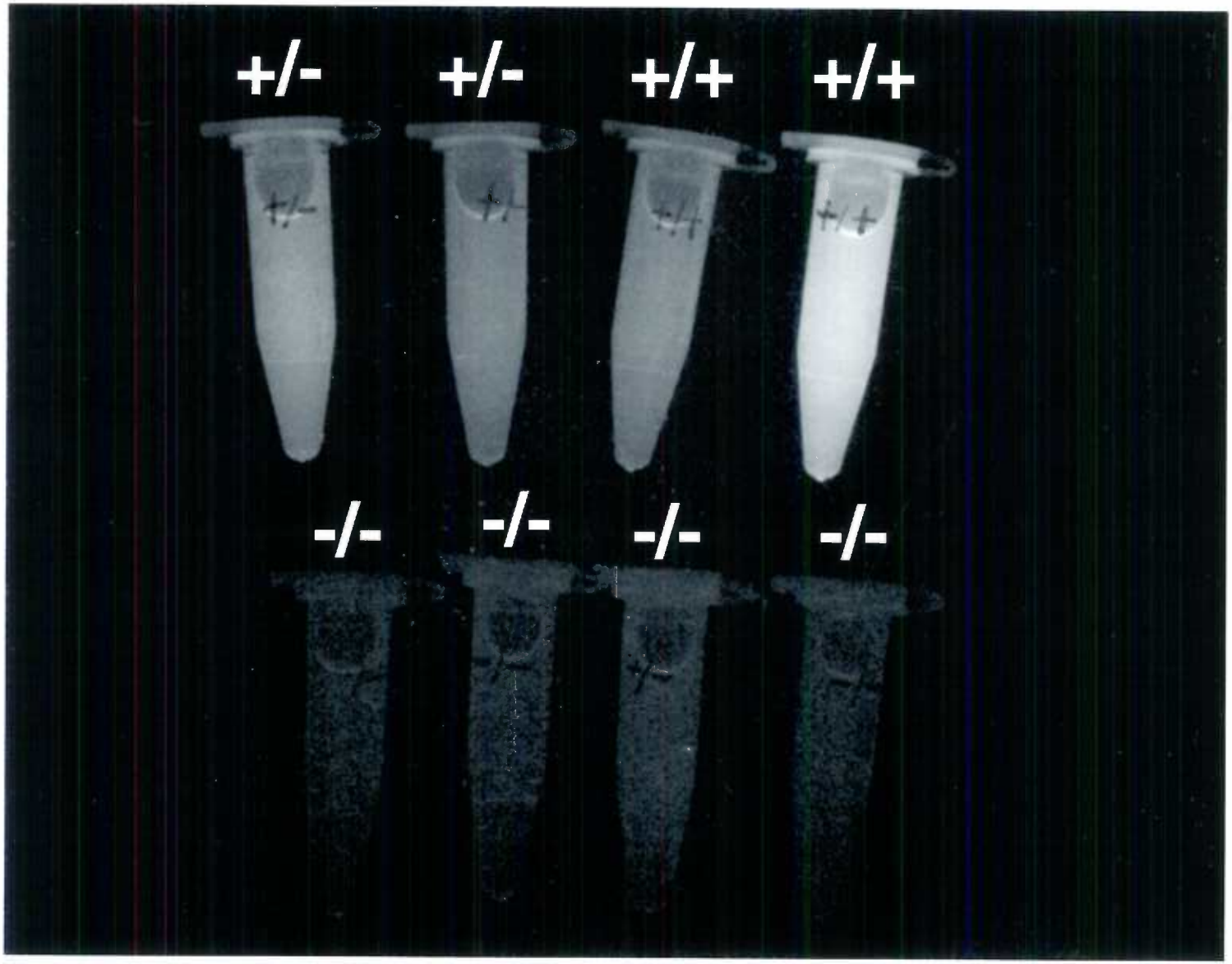

% Increase of Secretion
40
30
20
10
0
-10.0
-9.5
-9.0
-8.5
-8.0
-7.5
-7.0
Log [ACTH] (M)

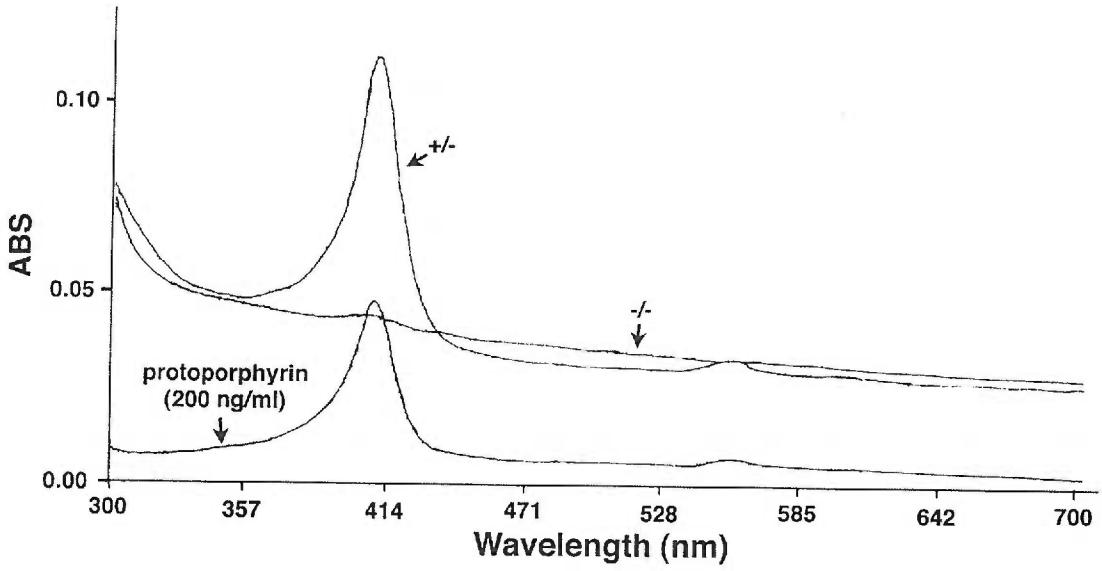
Figure 6. MC5R deficiency result in markedly reduced porphyrin content in the Harderian gland.

a. Comparison of UV illuminated fluorescence between extracts from Harderian gland of individual MC5-RKO mice or controls (wild-type or heterozygous).

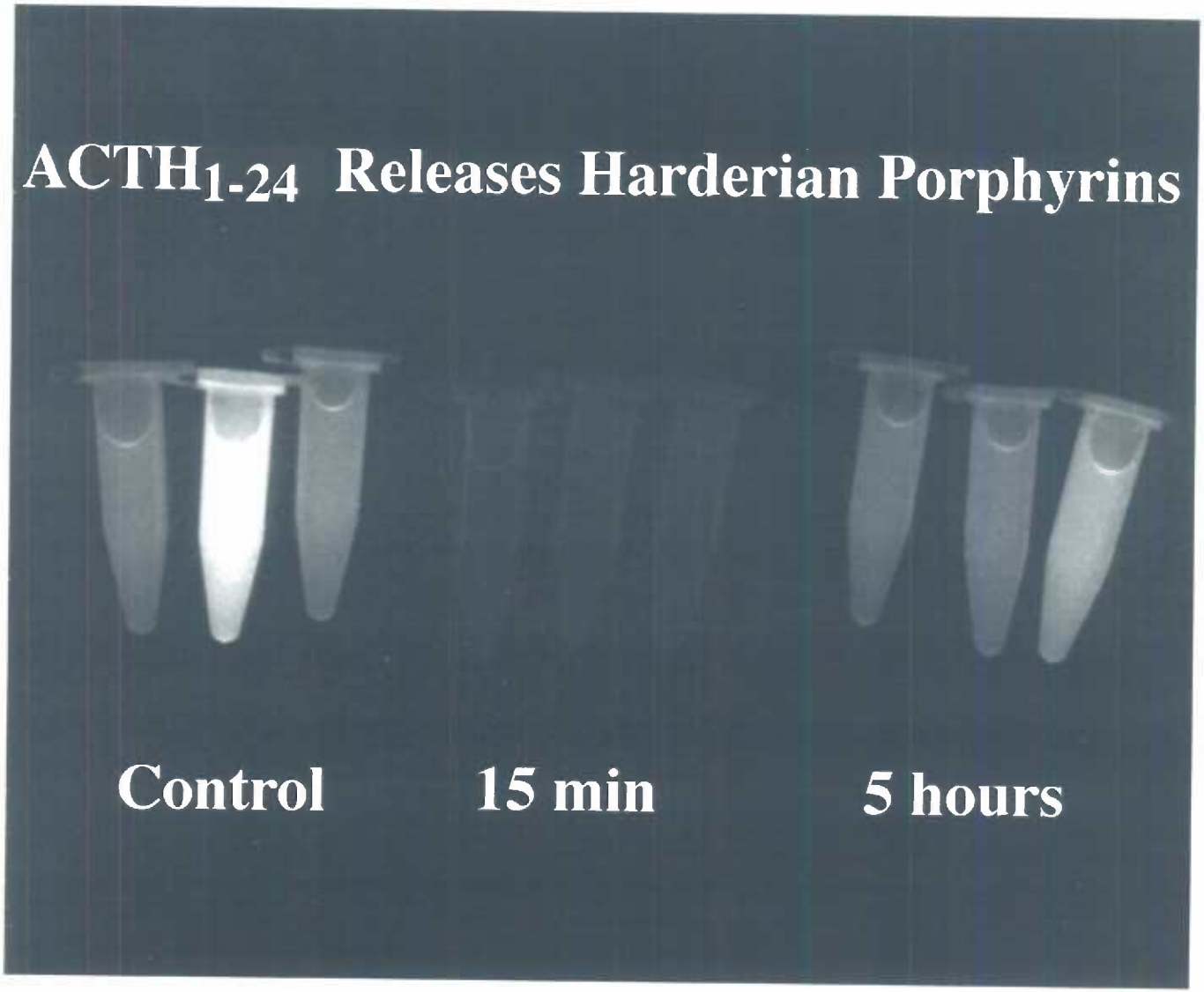


6B. Comparison of porphyrins from a pair of Harderain gland by scanning spectrophotometry.

One quarter of the total extracts from individual pairs of glands in 0.5 ml 0.25 N HCl was scanned. The two absorbance peaks at 402 and 560 nm are characteristics of porphyrins.


0.10
0.05
0.00
ABS
+/-
-/-
protoporphyrin
(200 ng/ml)
300
357
414
471
528
585
642
700
Wavelength (nm)

6C. $ACTH_{1-24}$ stimulate porphyrin release from Harderian gland. Wild-type 129 mice received 10 μg $ACTH_{1-24}$ i.p.. Mice were killed at the indicated time points and Harderian porphyrins of individual mice were extracted and semi-quantitated as described. Control mice did not receive any injection.


ACTH1-24 Releases Harderian Porphyrins
Control
15 min
5 hours

CHAPTER FIVE

DEVELOPMENT OF A CELL CULTURE SYSTEM FOR THE IDENTIFICATION OF ACTIVATING MUTATIONS IN Gs- AND Gq- SIGNALING PATHWAYS

SUMMARY

Clonal HEK293 cell lines have been engineered that are dependent upon CREB activation for survival in the presence of hygromycin B. After transfection with a plasmid (C_5HyTK) that expresses a hybrid gene of hygromycin-phosphate-transferase (Hpt) and herplex virus thymidine kinase (HyTK) under the control of five copies of cAMP responsive element (CRE), several clones were selected for undectable basal but robust cAMP-inducible transgene expression. Under hygromycin selection, cells transfected with activating mutations of MSH receptor (MC1-R^{som}), Gsα, or CaM Kinase IV formed a number of clones, while their wild-type counterparts yielded none. The viability of cells that express wild-type MSH receptor under selection was increased in the presence of NDP-MSH, a potent long lasting agonist, in a dose dependent manner. Morever, when MC1R^{som} expression plasmid was diluted 1000-fold with wild-type MC1-R expression plasmid before transfection, only cells that expresses MC1-R^{som} survived. Some of the clones have been further tailored to express nuclear antigen of EBV virus (EBNA-1) to support episomal replication of oriP-containing plasmids. As CREB activation is a common target for multiple signal transduction pathways, these cells should be useful in isolating new members/regulators of these pathways, and identifing activating mutations of the known components from randomly mutagenized pools of clones.

INTRODUCTION

The cyclic AMP-responsive element binding protein (CREB) is a transcription factor that regulates expression of numerous genes (Gonzalez et al., 1989; Montminy et al., 1990). Phosphorylation of ser-133 plays a key role in the transcriptional activity of CREB by recruiting CREB binding protein (CBP) to stimulates the general transcription machinery (Chrivia et al., 1993; Gonzalez and Montminy, 1989; Kwok et al., 1994; Parker et al., 1996). Several serine/threonine protein kinases phosphorylate CREB at serine 133. These include protein kinase A (PKA) (Gonzalez and Montminy, 1989), Calcium/Calmodulin dependent protein kinase IV (CaM KIV) (Matthews et al., 1994; Sheng et al., 1991; Sun et al., 1994) and the recently identified RSK-2 (Xing et al., 1996). The kinases are effectors of distinct signaling pathways elicited by different extracellular signals. RSK-2 is activated by multiple environmental cues such as UVC irradiation, neurotrophins, and mitogens (Ginty et al., 1994; Iordanov, 1997; Tan, 1996; Xing et al., 1996). CaM kinases are down stream effectors of Gq coupled receptors (Hunter, 1995), and also mediates gene expression induced by Ca influx upon neurotransmitter stimulation (Sheng et al., 1991; Thompson et al., 1995). PKA is the classic CREB kinase regulated by cAMP, a second messenger regulated by Gs and Gi coupled receptors. It is conceivable that there may be other factors that regulate CREB activity directly or indirectly, and their identities are yet unknown. CREB activity is required for a number of important biological process such as synapase formation and long-term memory (Bourtchuladze et al., 1994; Deisseroth et al., 1996; Martin and Kandel, 1996; Yin and Tully, 1996).

Gs, Gq, and Gi coupled receptors all belong to a superfamily of membrane proteins called G protein coupled receptors (GPCR) (Gudermann, 1997). GPCRs all possess seven transmembrane helices and this hydrophobic property makes structural analysis of these receptors extremely difficult. Molecular modelling in conjunction with site-specific

mutagenesis has been widely used to predict the structure of GPCR based on the bacterial rhodopsin coordinates. The packing and arrangement of the helices may be different between the GPCR and bacterial rhodopsin (Baldwin, 1993; Elling and Schwartz, 1996). Morever, molecular modelling provides very little information about the mechanisms by which ligand binding activates downstream signalling.

A number of constitutively active mutations in GPCRs have been found both in in vitro mutagenesis studies as well as in a variety of human diseases (Clapham, 1993; Lefkowitz et al., 1993). These are all point mutations that transmit signals in the absence of ligand. Thus, change of one amino acid in the receptor results in a conformational mimicry of the active receptor state, R*. Therefore, these types of mutations may shed lights on the molecular mechanism of receptor activation and the structure of the GPCR (Lefkowitz et al., 1993; Robbins et al., 1993; Robinson et al., 1992; Samama et al., 1993). Activating mutations are distributed all of the GPCR domains, suggesting that it is possible to induce an activated conformation by alterations in multiple locations in a receptor. Unfortunately, often only limited activating mutations have been identified in any one receptor. Thus, available information is insufficient to form a consensus on what sorts of structural alterations lead to receptor activation. Such consensus may be deduced if a large number of activating mutations could be found in one receptor.

Random mutagenesis is a powerful approach to determine functionally important residues in a protein (Black and Loeb, 1996; Kuipers, 1996). Random mutagenesis can be easily achieved chemically or by PCR. The mutation rate can be controlled by alterating the experimental conditions. For gene sized random mutagenesis, PCR is the method of choice. To make use of the mutagenized library, a selection scheme is needed to identify the characteristics of interest. Such a selection system can be easily established in yeast, where activating mutations were successfully isolated in the third intracellular loop of the

yeast pheromene receptor (Boone et al., 1993). Unlike yeast, few genetic selection schemes exist for screening mammalian cells for activated GPCR signaling.

In this chapter, I descibe the development of a somatic genetic system potentially useful for identification of novel CREB regulators by expression cloning, and for screening for activating mutations in Gs and Gq coupled receptors.

MATERIALS AND METHODS

Materials

Plasmid pCEP4, pREP9, pZeoSV, and pcDNA3 were products of InvitroGen. pBluescript KS(-) and pBK-CMV were from Strategene. Bbs I was purchased from NEB. Gsα alleles were obtained from Dr. Mike Forte (Vollum Institute), and CaM KIV expression plasmids were provided by Dr. Tom Soderling (Vollum Institute).

Construction of plasmids

The plasmid pC_5HyTK was made by replacing the lacZ gene of p5xCREβ/gal (Chen et al., 1995) with a Nar I/BamH I fragment from pHyTKCx (Lupton et al., 1991). A Bam HI/Xba I fragment from pCEP4 (Invitrogen) containing the polyadenylation sequence of SV40 was subsequently added to the plasmid. Both versions of pC_5HyTK were used for transfection. To construct pBKCMVP, a Nsi I/Xba I fragment from pREP9 (Invitrogen) that harbors the intact EBV oriP was inserted into pBK-CMV (Stratagene) at the Mlu I site. Plasmid pZeoSVEBNA-1 was built by placing a 2kb Hga I fragment into pZeoSV (Invitrogen) at the blunted Xho I site, and selecting for clone of the correct orientation.

Transfection and screening.

HEK293 cells were transfected with lipofectin reagents from BRL as described (Chen et al., 1995). To obtain stable transfectants of pC_5HyTK, transfected cells were selected in hygromycin (200 μg/ml) in the presence of forskolin (5 μM) for one week. Cells that express high levels of transgene were eliminated by selecting in gancyclovir without forskolin for one week. Individual clones were further evaluated as outlined in Figure 1. EBNA-1 expressing cells were produced by selecting pZeoEBNA-1 transfected cells with 200 μg/ml of Zeocin for 3 weeks.

Colony formation assay

Clone 5.0 and clone 2.0 was used for colony formation assays under hygromycin selection. Two days after transfection with the DNA of interest, 90% of the cells were selected with hygromycin (150 μg/ml for clone 5.0 and 200 μg/ml for 2.0) for 7 days. The remaining 10% were selected with 400 μg/ml G418 for 3 weeks to monitor transfection efficiency. The cells were then cultured in hygromycin-free medium containing 400 μg/ml G418 for 2 weeks. The plates were stained with crystal violet for documentation (Figure 2 and 3).

$MC1R^{WT}$ and $MC1R^{Som\text{-}3j}$ Genotyping

The point mutation conferring the constitutively active E92K change in $MC1R^{Som\text{-}3j}$ creates a Bbs I reconginition site (Robbins et al., 1993). This was used to discriminate between the wild-type and mutatant mouse MC1R alleles. Cells of individual clone were scraped off the plate and transferred into a PCR tube. After addition of 50 μl PCR reaction cocktail (1 x Taq polymerase buffer from Promega, plus 3 mM $MgCl_2$, 200 μM dNTP, 1 μM of each

primer), the tubes were placd in a MJ mini-cycler. The genomic DNA was released and denatured at 100 °C for 10 minutes. The MC1R alleles were amplified by 35 cycles immediately following addition of 2 units of Taq polymerase. Each cycle consists of 1 minute at 92 °C , 1 minute at 60 °C, and 1 minute at 72 °C. Sequence of the two primers are: 5'-TTCCTGACAAGACTATGTCCA-3' and 5'-GGGGCCCCAGCACAGGAAGAA-3'. PCR products were purified by Qiagen columns and analysized by Bbs I digestion.

Other Methods

Hirt extraction of episomal plasmid DNA was performed accordig to Hirt (Hirt, 1967). RNA isolation and northern analysis procedures were those of Chomczynski (Chomczynski, 1992; Chomczynski and Sacchi, 1987). Cells were stained in 5% crystal violet in ethanol for documentation of olony formation assays. All other procedures were according to standard protocols (Sambrook, 1989).

RESULTS

Establishment of the system.

Both positive and negative selections were employed to obtain optimal clones of cells. Hygromycin selection in the presence of 5 μM forskolin eliminated cells that did not express sufficient levels of Hpt, if any. However, it did not distinguish induced expression from basal expression. In the absense of cAMP-inducing agents, 5 μM gancirclovir killed all the pCMV/HyTK (Lupton et al., 1991) transfected cells (data not shown), and should have excluded cells with high basal expression of the transgene. In fact, an average of 50% of clones from the positive selection were killed by the negative selection (Table 1). Therefore, individual clones obtained from the first round of selection

were subjected to another screen, as shown in Figure 1. Only 1-2% of the clones qualified after the final selection. Northern analysis showed that transgene expression in all four clones so obtained is highly inducible, and three out four clones demonstrated very little basal expression (Figure 2). The size difference of HyTK transcript in clone 1.0 and 5.0 was due to the lack of poly-adenylation signal in the construct. These clones were used for further experimentation.

CREB activation dependent survival of the C5HyTK cells.

To test if activation of Gs or Gq linked pathways is sufficient to confer cell survival under hygromycin selection, the following experiments were conducted. When 10^6 C5HyTK cells (Clone 5.0) were transfected with pCDNA3 expressing wild-type (MC1-R^{wt}), or constitutively active murine MC1-R (MC1-R^{som}) (Robbins et al., 1993), and subsequently selected in 150 μg/ml of Hygromycin for 1 week, no clone was found in the wild-type transfected cells, whereas more than 100 clones were formed by cells transfected with MC1-R^{som} upon subsequent culture (Figure 3a). However, in the presence of 100 nM NDP-α-MSH, a potent long lasting MC1-R agonist (Hruby et al., 1993), a large number of colonies appeared in the wild-type MC1-R transfected cells (Figure 3b). Furthermore, the number of NDP-α-MSH induced colonies increased in a dose dependent manner(Figure 3c). α-MSH was not able to induce colony formation in a similar experiment, probably due to its instablity in the medium (data not shown). These data suggested that prolonged activation of CREB by either a constitutively active Gs-linked receptor, or activation of a Gs-linked receptor by an stable agonist, induced transgene expression sufficient enough to permit survival in 150μg/ml of hygromycin B in a large number of independent transfectants. Furthermore, comparison of wild-type and constitutively active Gsα (Lyons et al., 1990) also demonstrated the dependence of CREB

activation for survival (Figure 3d). This is also true for CaM KIV, a known activator of CREB. Constitutively active CaM KIV (Tokumitsu et al., 1994) produced a number of colonies under hygromycin selection, while its wild-type counterpart produced none (Figure 3e). In every case, transfection efficiency was similar as suggested by the number of colony formed with G418 selection. However, G418 selection consistently yielded 20-30 times more clones than hygromycin selection (data not shown).

Rescue of MC1-R^{som} from a 1000-fold diluted background.

To evaluate the possibility of using the system to isolate activating mutations from a large pool, we mixed expression plasmid of MC1-R^{som} and MC1-R^{wt} at a ratio of 1:1000. The two alleles can be differentiated by Bbs I digestion (Figure 4a). Ten μg of the mixture was then transfected into clone 5.0 cells. After selection with 150 μg/ml of hygromycin for one week, three clones were formed in the plate. These cells were collected for PCR amplification. The identity of the PCR products was diagnosed by Bbs I digestion. Figure 4b demonstrated that all three clones harbor MC1-R^{som}, although at different ratios to wild-type receptor. This result clearly demonstrated that it is possible to use this cell culture system to identify activating mutations from a complex background.

Functional expression of EBNA-1 in C_5HyTK cells

The system needs further improvement for identifying novel point mutations from a library representing randomly mutagenized clones of one gene. The stable transfection system was not suitable for identification of novel single nucleotide alterations, because multiple integrations of transfected DNA usually occur in a single cell, making the identification of the causative mutation formidable.

Expression of EBNA-1 in primate cells confers episomal replication of plasmids containing oriP (Reisman et al., 1985). C5HyTK cells of clone 2.0 were transfected with EBNA-1 expression plasmid pZeoSVEBNA-1 (Figure 5a). Individual clones were tested for the expression of full-length EBNA-1 mRNA (Figure 5b) and the basal and induced expression of HyTK (data not shown). Resultant clones were termed C5HyTKE cells. Next, pBK-CMV was modified by inserting an intact oriP (pBKCP, Figure 5c), thus making this versatile expression vector suitable for episomal maintainence in C5HyTK-E cells. To test if EBNA-1 is functional, the cells were transfected with pBKCP or pcDNA3, and selected with 400 μg/ml of G418. Four weeks after selection, the cells were lysed to isolated episomal DNA (Hirt, 1967). A portion of the resulted DNA were transformed into E. coli. More than one hundred kanamycin resistant clones were formed by bacteria receiving Hirt material from pBKCP transfected cells, whereas no ampicillin resistant clones were obtained from cells transfected with pcDNA3. Of the 20 random kanamycin resistant clones analysed, only 2 of them showed gross deletions (Figure 5d).

DISCUSSION

In this chapter I have described the development of a somatic cell genetic system that is potentially useful to isolate a variety of signaling molecules linked to CREB activation, and to identify mutations in known signaling molecules along these pathways.

The most important criteria in chosing candidate cells are low basal and high inducible expression of the selectible marker. As the expression of transgene is influenced by the flanking environment, and the insertion is more or less random, it is very difficult to obtain cells that satisfy this criteria. This is manifested by the number of colonies formed in the absence of any stimulation (Table 1). In fact, in earlier experiments aimed at the isolation of stable p5xCRE/β-gal expressing clones, more than 200 clones were screened and all

found to contain high basal β-galactosidase activity. The successful development of C_5HyTK cells is likely due to the employment of both positive and negative selection. Even after the selection scheme described (Figure 1), only about 1% of the resultant clones were satisfactory.

The three C_5HyTK cell lines described all display very low basal and high inducible expression of HyTK gene (Figure 2). Although the basal levels are slightly different among the clones, the inducibility is very similar. The basal level difference is reflected by the variation in the minimum lethal hygromycin concentration. While 150 μg/ml of hygromycin for one week is sufficient to completely kill clone 5.0 cells, 170 μg/ml and 200 μg/ml are needed to achieve the same result for clone 4.0 and clone 2.0 cells, respectively. The basal expression seen in clone 1.0 is able to sustain more than 250 μg/ml of hygromycin for one week. Expression of activating mutations in this clone produced variable results after selection. The clone was thus discarded.

One pitfall of thecAMP pathway is down regulation after prolonged activation (Armstrong et al., 1995; Hagiwara et al., 1992). Surprisingly, down regulation is not pronounced in the three lines. Activation of the cAMP pathway by forskolin for four days did not significantly reduce the expression of the HyTK gene (Figure 2). This is in contrast to the observation that there are refractory periods of cAMP induced gene expression followed by forskolin stimulation in several cell lines such as FRTL-5 and PC12 (Armstrong et al., 1995; Hagiwara et al., 1992). This probably owes to the use of different cell types, or may be due to unique properties of the cAMP pathway in clones 2.0, 4.0, and 5.0.

The dependence on CREB activation for the survival of C_5HyTK cells under hygromycin selection was clearly demonstrated. Along the Gs pathway, activation of CREB by a receptor ligand, by a constitutively active receptor, or by a mutant Gs that lacks GTPase activity, all induced the formation of 50 to 100 colonies per transfection (figure 3). The CaM kinase IV mutant was equally effective in colony induction. Unfortunately, the recovery rate of selection is only 3-5%, and this may be an inherent drawback of the system. Although the cells are clonally derived, their susceptibility to hygromycin differs. The levels of hygromycin used will inevitably kill many cells that contain activating mutations. Nevertheless, it is demonstrated in this chapter that an activating mutation of MC1-R could be recovered after 1000 fold dilution in a wild-type background. By limiting the number of species included in each transfection, one should be able to isolate any activating mutant in the pool. Other strategies may also imposed for selection. For instance, one may use lower concentration of hygromycin or shorter selection time to enrich activating mutations in the pool. The enriched pool is then rescued and transformed into E. coli for amplification. The degree of enrichment may be adapted accordingly by varying the selection condition or the number of enriching steps. This strategy may be suitable for screening high complexity libraries.

One advantage of this system over the one-hybrid and two-hybrid schemes in yeast is its broader application. The yeast systems are powerful tools to isolate genes that directly interact with a DNA element or a target protein (Fields and Song, 1989; Wang and Reed, 1993). However, it is not suitable for identification of indirect interacting genes. Although yeast has been engineered by extensive modifications to detect mammalian G protein coupled receptors (Price et al., 1995; Price et al., 1996), its application is limited for certain components of a single pathway. In contrast, the system described in this chapter makes use of the intact endogeneous signal transduction pathways. It monitors perturbation of the

pathway caused by any exogenous DNA. Thus the mammalian cell culture system promises a much broader application.

The modification of the system to support episomal replication improves the selection system for several reasons, in particular for selecting point mutations from a mutagenized pool of clones. First, it omits any further subcloning steps, thus limiting the introduction of mutation by subsequent PCR. Second, The pBKCP plasmid enables one to test the function of rescued plasmid directly by transient cotransfection with 5xCRE/β-gal plasmid (Chen et al., 1995). Finally, continuous replication under selection may enrich plasmids that confer drug resistance, further facilitate identification of causal mutations, and perhaps enhancing the efficiency of recovery by allowing amplification of plasmids that confer growth advantage under selection.

In summary, this system is designed for easier isolation and characterization of genes/mutations in proteins that acting along the signalling pathways coupled to CREB. Similar concepts may extend to other signaling pathways with known cis-acting elements.

SUMMARY

A mammalian cell culture system has been developed for isolation of genes/mutations that activate CREB by translating CREB activation into hygromycin resistance. The system has been evaluated under several experimental conditions.

1. Activation of CREB by a ligand, an activating mutation in a Gs coupled receptor, or by a constitutively active Gsα led to colony formation under hygromycin selection.

2. Constitutively active CaM kinase IV, a target of stimulated Gq coupled receptor and of Ca influx, also result in hygromycin resistance.

3. It is possible to rescue an activating Gs-coupled receptor mutant that was diluted in 1000 fold in wild type counterparts.

Application of this system will potentially identify activating mutations from a randomly mutated receptor pool, and thus may provide an important method for the study of the mechanism of GPCR activation. The broad use of the system may potential identify novel genes that regulate signal transduction pathways converging on CREB activation.

Table 1. Colony formation under different selective conditions.

Forskolin (5 μM)	Hygromycin B (200 μg/ml)	Gancyclovir (5 μM)	Number of Clones
-	+	+	520
-	+	-	950
+	+	-	2080
+	+	+	1250

HEK-293 cells ($2x10^6$) were transfected with pC_5HyTK and aliquoted into four 100 mm plates. Cells were then cultured in conditions as listed, and number of colony counted after crystal violet stain.

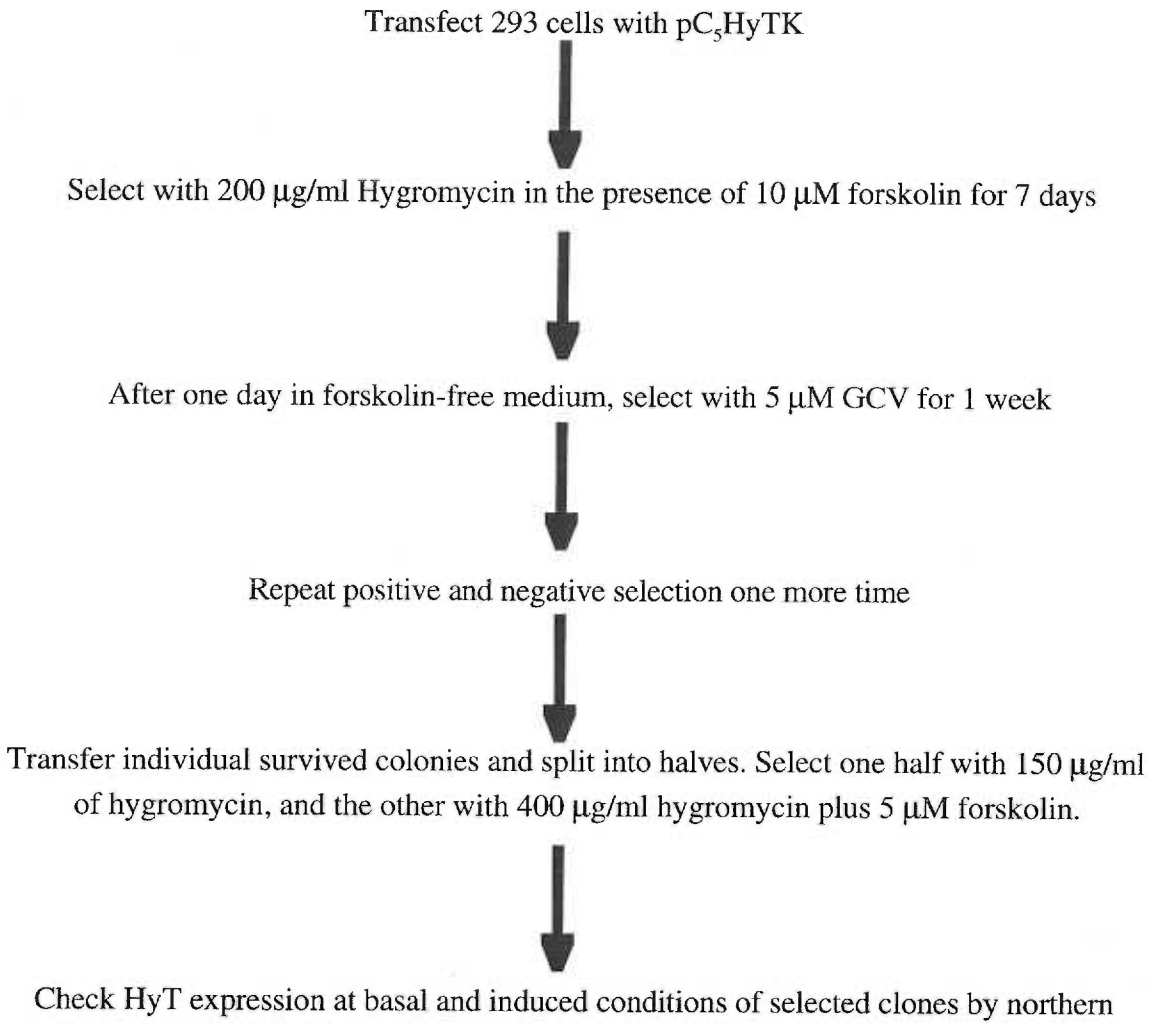


Figure 1. Selection scheme.

Figure 2. Northern analysis of transgene expression under basal and stimulated conditions. Cells were treated with 5 μM forskolin for indicated length of time. RNA was isolated as described. Ten μg of total RNA was loaded in each lane. After electrophesis and downward alkaline transfer, the membrane was probed with the coding region of hygromycin phospate transferase. The membrane was striped, and re-hybridized with an mouse β-actin probe to ensure similar amount of RNAs were used.

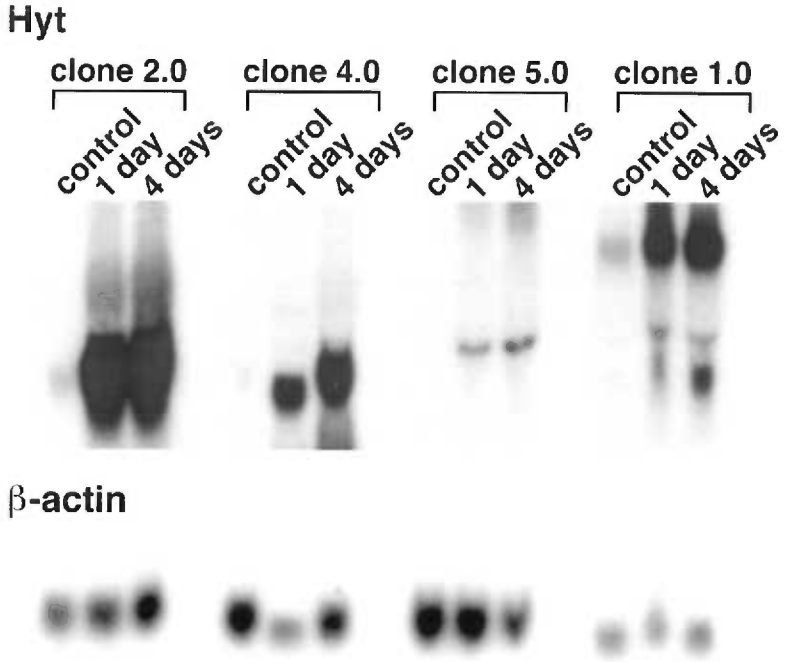
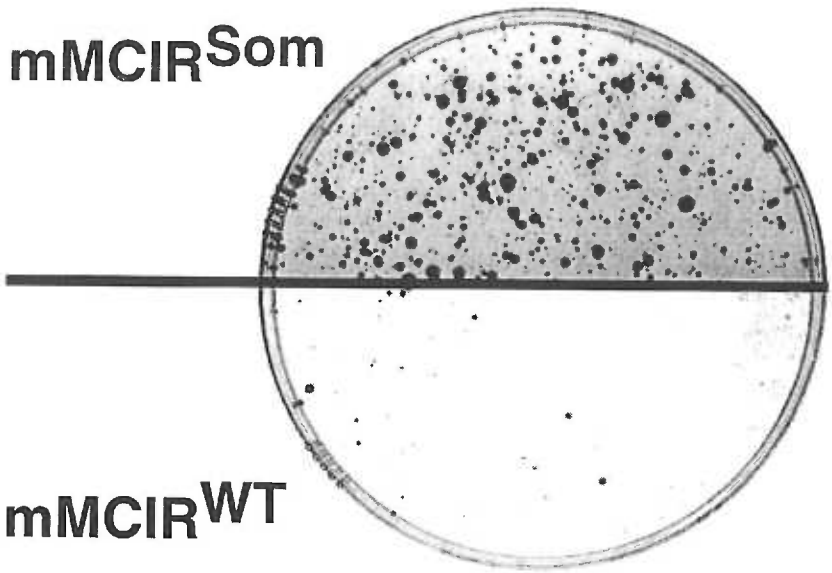

Hyt
clone 2.0
clone 4.0
clone 5.0
clone 1.0
control
1 day
4 days
control
1 day
4 days
control
1 day
4 days
control
1 day
4 days
β-actin

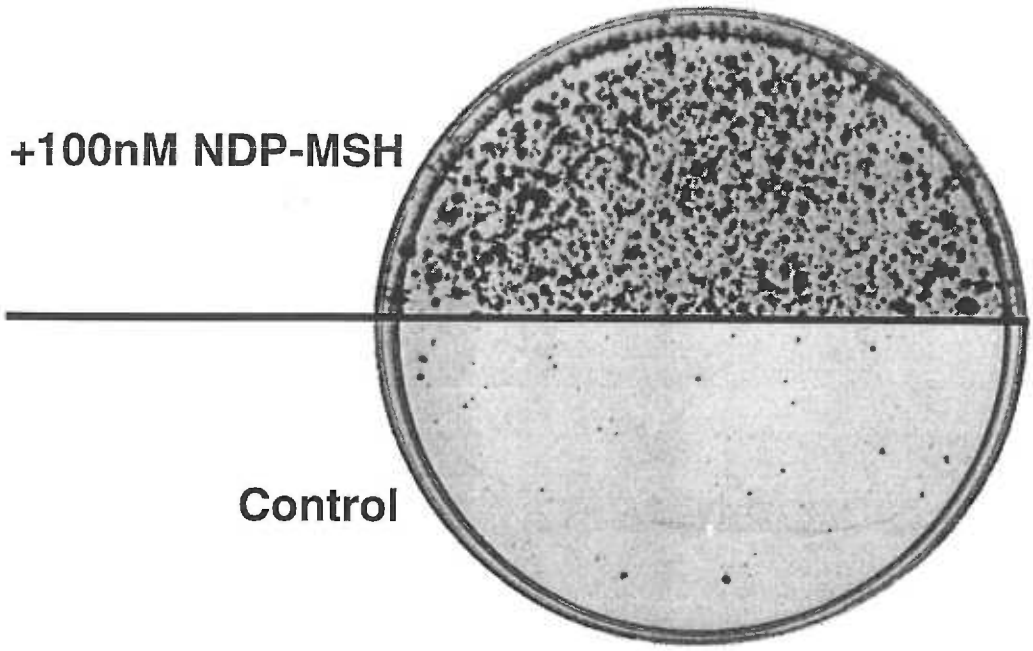
Figure 3. Activation of Gs or Gq signaling pathway confer hygromycin resistance in C5HyTK cells.

Cells were transfected with plasmids indicated as above by lipofectin reagents (Gibco BRL), and selected with 150 μg/ml of hygromycin B for a week. They are subsequently maintained in medium containing 400 μg/ml of G418 for 2 weeks before being stained with crystal violet.

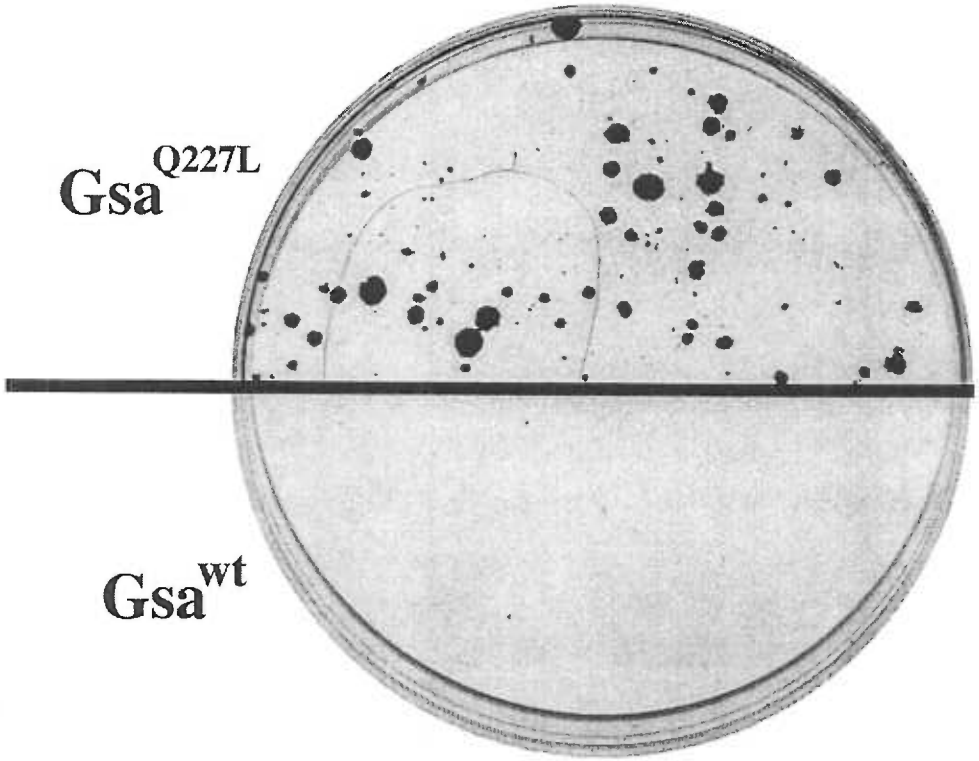
3A. Expression of a constitutively active MC1-R (MC1-R^{som}) resulted in hygromycin resistance.


mMCIR^Som
mMCIR^WT

3B. Cells expressing wild-type MC1-R survived in hygromycin (150 μg/ml) in a NDP-α-MSH dose dependent manner.


+100nM NDP-MSH
Control

3C. A constitutively active Gs (Gs^{Q227L}) enhanced hygromycin tolerance.


Gsa Q227L
Gsa wt

3D. Introduction of a constitutively active Ca^{++}/calmodulin kinase IV also induced colony formation in the presence of hygromycin.

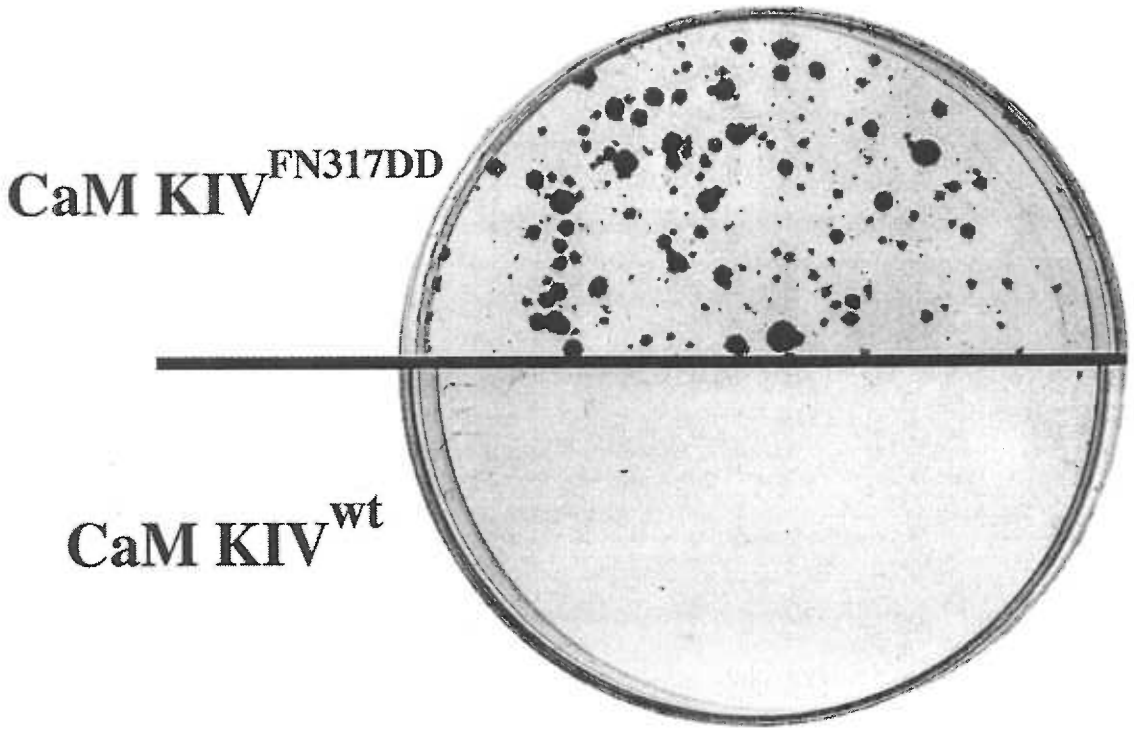

CaM KIV^FN317DD
CaM KIV^wt

Figure 4. Rescue of diluted MC1-R^{som}.

C5HyTK cells were transfected with 10 ug of pcDNA3/MC1-R^{wt} plus 10ng pcDNA3/ MC1-R^{som}. After selection in 150 μg/ml hygromycin for a week, cells were then allowed to grow in normal medium containing 400 μg/ml G418 for 10 days. Three clones were recovered in the plate. The clones were transferred to thin-walled tubes. The identity of the transgene was determined by PCR and Bbs I digestion as depicted.

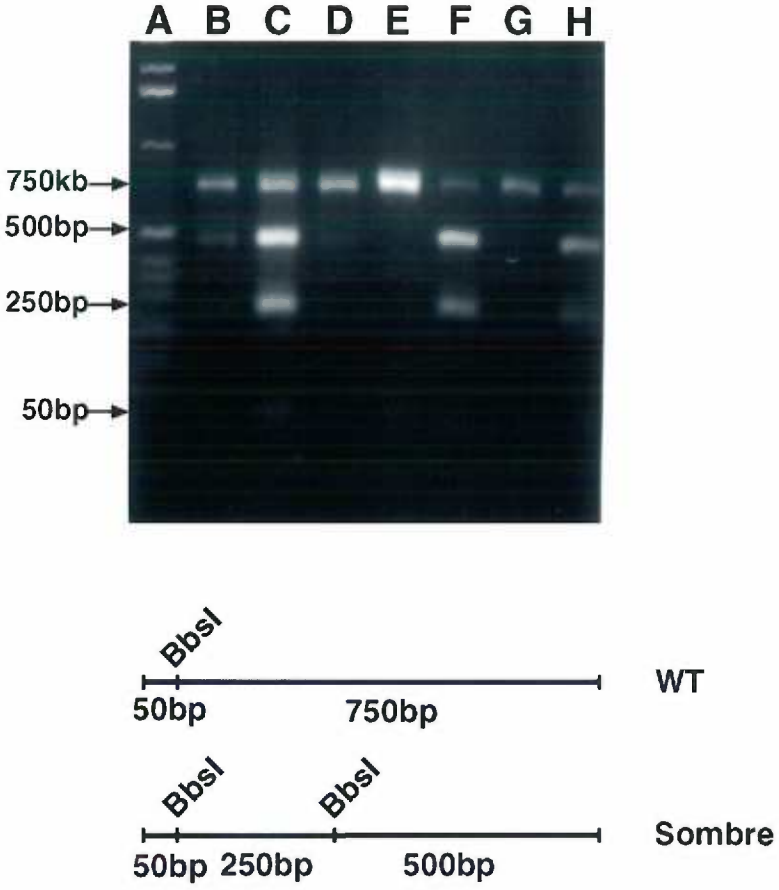
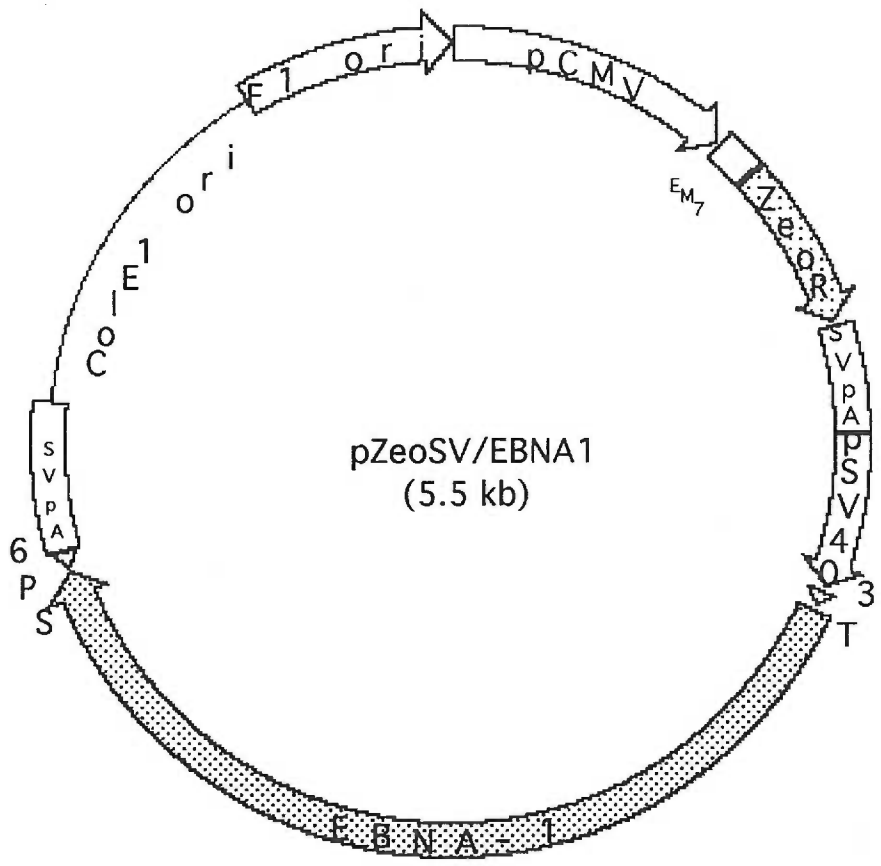
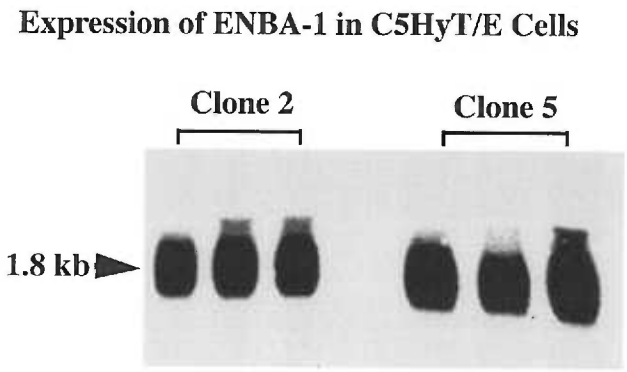

A B C D E F G H
750kb
500bp
250bp
50bp
BbsI
WT
50bp
750bp
BbsI
BbsI
Sombre
50bp 250bp
500bp

Figure 5. Functional expression of EBNA-1 in C_5HyTKE cells.

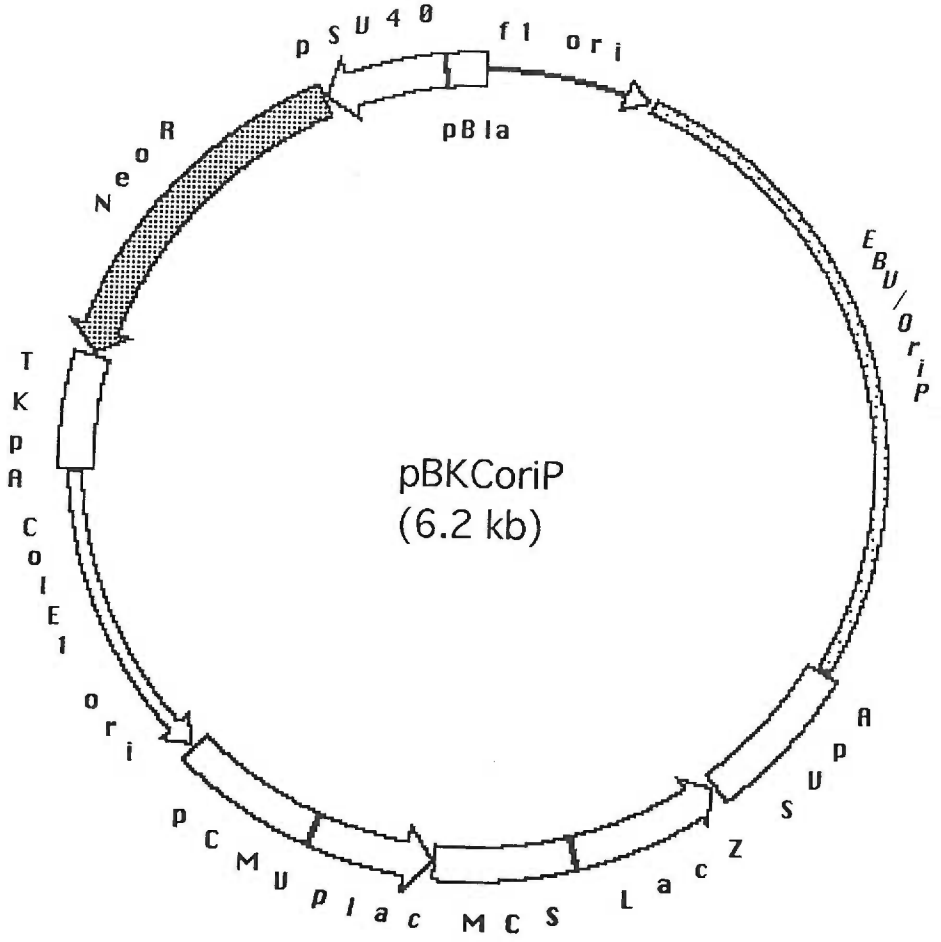
5A. Map of pZeoSV/EBNA-1.



5B. EBNA-1 is high expressed in C5HyT/E cells. Ten mg of total RNA isolated from confluent plates of C5HyTH/E cells was included in each lane.



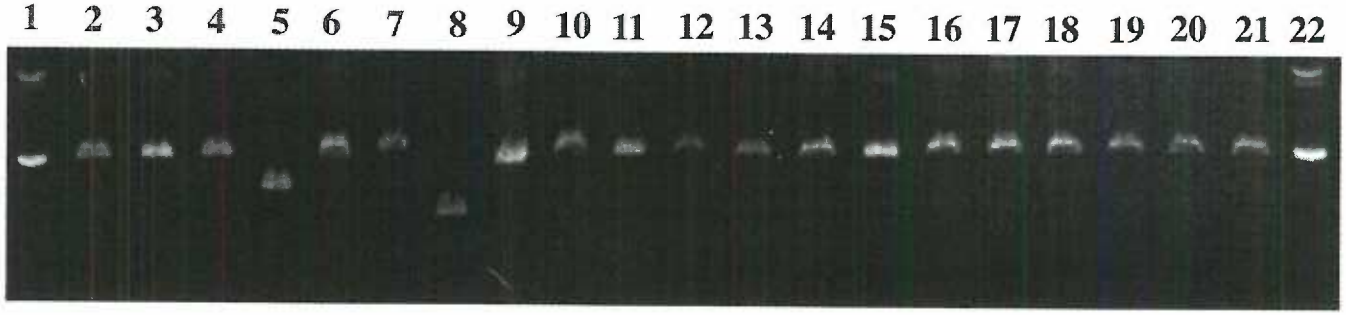
5C. Map of pBKCP.



Order of MCS: BssH II, Pst I, Sal I, BamH I, EcoR I, Hind III, Xho I, Sca I, Xba I, Not I, Cla I, Kpn I

5D. OriP containing plasmid maintain episomally in C_5HyTKE cells.

The cells were transfected with pBKCP or pcDNA3, and selected with 400 μg/ml of G418 for Four weeks before Hirt treatment. Plasmids from 20 random kanamycin resistant clones were analysed. Lanes 1 and 22, control plasmids. Lanes 2 to 21, rescued plasmids from individual clones. Only 2 of them showed gross deletion.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

CONCLUSIONS

The physiological function of MC5-R has been defined by gene targeting in the mouse. MC5-R is involved in the melanocortin stimulated release and/or synthesis of lipids, proteins, and porphyrins in sebaceous, lacrimal and Harderian glands, respectively. As a result, lack of MC5-R function in these glands results in diminished production of sterol esters by sebaceous gland; marked reduction of porphyrin synthesis in Harderian gland; and loss of melanocortin stimulated protein secretion by lacrimal gland. Consequently, decreased hair lipids leads to impaired water repulsion and heat insulation in the MC5-R deficient mice. Given the previously demonstrated importance of the pituitary gland for the activity of sebaceous and Harderian glands, it is conceivable that the MC5-R mediated function is influenced by circulating pituitary derived melanocortin peptides. However, the possible involvement of autocrine/paracrine derived melanocortins can not be excluded.

The function of MC5-R in multiple exocrine glands suggests the existence of a hypothalamic-pituitary-exocrine gland axis. This axis differs from the well characterized neuroendocrine axes in that it releases compounds to external environment. Therefore, direct feedback regulation may not be possible. In this scenario, the MC5-R may provide the molecular basis for the coordinated hormonal regulation of secretion of products from a number of exocrine sources, including Harderian, lacrimal, preputial, and sebaceous glands. Although the biological significance of this pituitary-exocrine gland axis is not fully understood, it could play a role in the control of pheromone secretion.

The expression of MC5-R, a receptor for two stress-regulated hormones, α-MSH and ACTH, in pheromone producing exocrine glands suggests that MC5-R may mediate stress-induced pheromone release. Pheromones are chemicals excreted to the external

environment by exocrine glands for intraspecific communication. Pheromonal interaction has been observed in a number of social organisms from earthworms to primates. In rodents, pheromones have been shown to serve as sex attractants, alarm substances, territorial markers, and individual odors (Gleason and Reynierse, 1969). Most interestingly, stress has a profound influence on pheromone release as indicated by the alteration of behaviors in conspecific animals. For example, both rats and mice dislike odors of stressed conspecifics, odors of stressed rats elicit opioid analgesia and alarm responses, and stressed mice invoke aggression and produce odors that alter immune activities (see chapter one). Both physical and psychological stressors stimulate the secretion of ACTH and α-MSH by the pituitary. The stress-induced increase of serum ACTH and α-MSH may stimulate the MC5-R expressing exocrine glands to release pheromonal substances along with proteins or porphyrins in the lacrimal gland and Harderian gland, respectively. It is also possible that activation of MC5-R in preputial gland may have the same effect.

The most important questions to address at this time are: 1). What is the source of melanocortin ligand for the MC5-R? and 2). How does the availability of this ligand regulate exocrine gland function? Future research should be on the influence of stress on the activities of exocrine glands using porphyrin release and synthesis as a model, and the characterization of the role of MC5-R in stress-induced behavioral changes.

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