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Development of an in vitro cell culture gene expression technique to examine coat colour genes in cattle

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This writing business. Pencil and what-not.
Overrated if you ask me.

The old grey donkey Eeyore in
"Winnie-the-Pooh" by A.A. Milne

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Table of contents

SUMMARY I

ZUSAMMENFASSUNG III

LIST OF FIGURES V

LIST OF TABLES VII

LIST OF ABBREVIATIONS IX

1 INTRODUCTION 1

2 LITERATURE REVIEW 5

2.1 Pigmentation in mammals 5

2.1.1 Eumelanin and Pheomelanin biosynthesis 5

2.1.2 Melanocytes 8

2.2 Regulation of pigmentation 8

2.2.1 Hormones controlling the pigmentation 13

2.3 Melanocortin 1 receptor 16

2.3.1 Cloning and characterisation of MC1R and other MCRs 16

2.3.2 Allelic variants of MC1R 19

2.3.3 Structure and function of MC1R 26

2.4 Agouti 30

2.4.1 Isolation and characterisation of agouti in mice 31

2.4.2 Agouti expression 33

2.4.3 Agouti mutations 33

2.4.4 Mechanisms of agouti signalling 35

2.4.5 Agouti in cattle 40

2.4.6 Agouti in other species 41

2.5 Attractin (Mahogany) 41

2.5.1 Characterisation of the mahogany gene 42
2.5.2 Function of attractin 44
2.5.3 Attractin in cattle 46

3 MATERIALS AND METHODS 47

3.1 Animals 47

3.2 DNA methods 47
3.2.1 Genomic DNA extraction 47
3.2.2 DNA quantification using spectrophotometer 48
3.2.3 Polymerase Chain Reaction (PCR) 49
3.2.4 Agarose gel electrophoresis 50
3.2.5 DNA extraction from the agarose gel 50
3.2.6 Screening of the BAC library 50
3.2.7 BAC DNA extraction 50

3.3 RNA Methods 52
3.3.1 General precautions working with RNA 52
3.3.2 Total RNA extraction from tissue samples 52
3.3.3 Total RNA extraction from cultured animal cells 53
3.3.4 RNA quantification using spectrophotometer 54
3.3.5 Electrophoresis of RNA 54
3.3.6 Reverse transcription 54

3.4 Cloning of DNA 55
3.4.1 Cloning of PCR-products into the pGem®-T Easy vector 55
3.4.2 Subcloning of the inserts from the pGem®-T Easy vector into the pcDNA3.1(+). 59

3.5 Sequencing 60
3.5.1 Sequencing of PCR-products and plasmid clones 60
3.5.2 Sequencing of BAC clones 61
3.5.3 Sequencing analysis 61

3.6 Polymorphism detection in cattle MC1R DNA sequence 62
3.6.1 PCR-RFLP assays 62
3.6.2 AFLP assay 63

3.7 Rapid amplification of the 5' cDNA ends (5' RACE) 63
3.7.1 First strand cDNA synthesis 63
3.7.2 Purification of cDNA 64
3.7.3 Tailing reaction of cDNA 64
3.7.4 PCR amplification of dA-tailed cDNA 64
3.7.5 Second PCR amplification (nested PCR) 65

3.8 Real time PCR 65
3.8.1 Primers and probe design 67
3.8.2 Thermal cycling parameters 68
3.8.3 Optimisation of primer and probe concentration 68
3.8.4 Real time PCR reaction mix 69

3.9 Creation of stable cell lines expressing specific genes 69
3.9.1 Recovery of cryopreserved 293 cells 70
3.9.2 Maintenance of 293 cells in monolayer 71
3.9.3 Viable cell counts using trypan blue 71
3.9.4 Cell cryopreservation 72
3.9.5 Transfection of 293 cells with pcDNA3.1(+) clones 72

3.10 Cyclic AMP measurement 73
3.10.1 Stimulation the cells with α-MSH 73
3.10.2 Cyclic AMP Assay 74

4 RESULTS 77

4.1 MC1R 77
4.1.1 Cloning and sequencing of the bovine MC1R alleles 77
4.1.2 Creation of the stable lines expressing MC1R alleles 87
4.1.3 Pharmacological characterisation of the bovine MC1R alleles 89

4.2 Agouti 92
4.2.1 Sequencing of the bovine agouti coding sequence 92
4.2.2 Pharmacological characterisation of the bovine ASIP 94

4.3 Attractin 96
4.3.1 Amplification of the bovine attractin coding sequence 96

4.3.2 5' end of the bovine attractin 96
4.3.3 Sequencing of the attractin coding sequence from different cattle breeds 97
4.3.4 Sequence analysis of the bovine attractin 98

5 DISCUSSION 105

5.1 MC1R 105
5.1.1 Dominant black MC1R allele (E^D) 105
5.1.2 Recessive red MC1R alleles (e and e^f) 108
5.1.3 MC1R alleles similar to the wild type (E^{d1} and E^{d2}) 110

5.2 Agouti 111
5.2.1 Agouti coding sequence and expression 111
5.2.2 Agouti function 111

5.3 Attractin 114

5.4 In vitro cell culture gene expression technique in livestock species 115

6 REFERENCES 117

7 APPENDIX 133

7.1 Primers 133

7.2 Solutions and Media 136

7.3 Reagents 139

7.4 Sequences 143

CURRICULUM VITAE 157

Summary

The coat colour in mammals is determined by the relative amounts of the two pigments eumelanin (black/brown) and pheomelanin (red/yellow) produced in melanocytes. In the mouse, the eumelanin/pheomelanin ratio was shown to be controlled by three loci, extension (MC1R), agouti (ASIP) and mahogan*y* (attractin). Melanocortin 1 receptor (MC1R) is a receptor expressed on the cell surface of melanocytes and activated by α-MSH stimulation. Stimulated MC1R causes activation of adenylyl cyclase and subsequent increase of intracellular cAMP, which activates tyrosinase, a rate-limiting enzyme of melanin synthesis. High activity of tyrosinase leads to eumelanin production, whereas under basal activity of this enzyme pheomelanin synthesis prevails. Agouti signalling protein (ASIP) acts as an antagonist to α–MSH by binding to the MC1R. Attractin is an accessory receptor for ASIP, facilitating ASIP-MC1R interactions.

These three bovine genes were used to create an *in vitro* cell culture gene expression system for the investigation of gene function and mutations which affect gene function. MC1R was previously sequenced in cattle, and three alleles have been characterised: **E+** (wild type), $\mathbf{E^D}$ (dominant black) and **e** (recessive red). Three additional variants are described in this study: the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ in Brown Swiss breed (grey/brown coat colour), and the $\mathbf{e^f}$ allele found in the heterozygous form with the **e** allele in the Simmental breed (red coat colour). $\mathbf{E^D}$, **e**, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$ and $\mathbf{e^f}$ MC1R variants were cloned into a mammalian expression vector and transfected into HEK 293 cells. cAMP was measured in the cell lines expressing MC1R variants as a response to α-MSH stimulation. The $\mathbf{E^D}$ and **e** alleles were unresponsive to the wide range of α-MSH concentrations, and their pharmacological profile was indistinguishable. The $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$ and $\mathbf{e^f}$ responded in a dose-dependent manner, however, the stimulation curve of the $\mathbf{e^f}$ allele was shifted to the right, when compared to the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ stimulation curves. Cells transfected with the $\mathbf{e^f}$ allele reached the same cAMP concentrations as the cells transfected with the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ variants at more then 10 times a higher concentration of α-MSH. In conjunction with the mode of inheritance of coat colour these results indicate that the **e** allele encodes for a non-functional allele of MC1R, the $\mathbf{E^D}$ allele is a constitutively active receptor, the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ MC1R variants are similar to the wild type receptor, and the $\mathbf{e^f}$ is a partial loss-of-function allele.

No differences were found in the coding sequence of ASIP between the Holstein (black), Brown Swiss (grey/brown), Red Holstein and Simmental (red) cattle breeds. Incubation of the cells expressing bovine ASIP together with the cells expressing the $\mathbf{E^{d1}}$ or $\mathbf{E^{d2}}$ MC1R variants caused a significant decrease of cAMP production, indicating the presence of functional ASIP in cattle.

The coding sequence of bovine attractin was amplified using cross-species PCR and 5' RACE, and subsequently sequenced. For sequencing of the 5' end of the gene, a BAC clone was used. However, it was not possible to amplify the complete coding sequence of the bovine attractin.

Investigation of the influence of mutations on gene function can be performed by expressing a gene (alleles of a gene) in the *in vitro* cultured cells, followed by measuring biochemical processes which are affected by an expressed gene. Interaction of the genes can be studied by expressing several genes simultaneously. Using this approach is of general interest in livestock genetics, as it has lower costs and is less time consuming when compared with breeding experiments or production of transgenic animals.

Zusammenfassung

In Säugetieren wird die Fellfarbe durch das Verhältnis der Pigmente Eumelanin (schwarz/braun) und Pheomelanin (rot/gelb) bestimmt. Diese Pigmente werden in den Melanozyten synthetisiert. In der Maus ist bekannt, dass das Verhältnis von Eumelanin/Pheomelanin durch drei verschiedene Loci bestimmt wird: Extension (MC1R), Agouti (ASIP) und Mahogany (attractin). MC1R ist ein Rezeptor, der auf der Zelloberfläche der Melanozyten ausgebildet wird. Die Stimulation des Rezeptor geschieht durch α-MSH und löst eine ganze Kaskade aus. Adenylatzyklase wird aktiviert, was zur Folge hat, dass das intrazelluläre cAMP ansteigt. cAMP aktiviert die Tyrosinase, welche das limitierende Enzym der Melaninsynthese ist. Der Grad der Aktivität der Tyrosinase bestimmt welcher Farbstoff synthetisiert wird. Hohe Tyrosinaseaktivität führt zur Eumelaninproduktion und niedere Aktivität zur Pheomelaninsynthese. ASIP wirkt als ein Antagonist zu MC1R. ASIP bindet an den Rezeptor und verhindert so die Interaktion von MC1R und α-MSH. Attractin ist ein zusätzlicher Rezeptor für ASIP, welcher die Bindung zwischen ASIP und MC1R ermöglicht. Es wurde ein *in vitro* System mit diesen drei Genen des Rindes erstellt, um mehr über die Genexpression und die Auswirkung von Mutationen auf die Funktion herauszufinden. Das Gen MC1R des Rindes ist bereits bekannt und die drei folgenden Allele wurden beschrieben: **E**+ (Wildtyp), $\mathbf{E^D}$ (dominant schwarz) und **e** (rezessiv rot). Drei zusätzliche Allele wurden in dieser Arbeit beschrieben: die Allele $\mathbf{E^{d1}}$ und $\mathbf{E^{d2}}$, welche in der Rasse Braunvieh (grau/braune Fellfarbe) und das Allel $\mathbf{e^f}$, welches bis jetzt nur in der heterozygoten Form mit dem e Allel in der Rasse Simmental (rote Fellfarbe) gefunden wurde. Die Allele von MC1R : $\mathbf{E^D}$, **e**, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$ und $\mathbf{e^f}$ wurden in einen Säugetierexpressionsvektor kloniert und in HEK 293 Zellen transfektiert. Die Zelllinien wurden mit α-MSH stimuliert und anschliessend cAMP gemessen. Die $\mathbf{E^D}$ und **e** Allele reagierten nicht auf die unterschiedliche α-MSH Konzentrationen. Die Allele $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$ und $\mathbf{e^f}$ reagierten dosisabhängig. Der $\mathbf{e^f}$ Rezeptor reagierte erst mit höhere α-MSH Konzentration im Vergleichung zu der $\mathbf{E^{d1}}$ und $\mathbf{E^{d2}}$ Rezeptoren. Durch der Art der Vererbung der Fellfarbe und den Resultaten dieser Arbeit wird klar, dass das **e** Allel für einen nicht funktionellen Rezeptor kodiert, das $\mathbf{E^D}$ Allel kodiert für eine konstitutiv aktiven Rezeptor, und die Allele $\mathbf{E^{d1}}$ und $\mathbf{E^{d2}}$ des Gens MC1R sind sehr ähnlich wie der Wildtyprezeptor und das Allel $\mathbf{e^f}$ steht für einen schwach funktionierenden Rezeptor.

Es wurde kein Unterschied gefunden in der kodierenden Sequenz von ASIP zwischen den Rassen Holstein (schwarz), Braunvieh (grau/braun), Red Holstein und Simmental (rot). Zellen, welche ASIP exprimieren wurden zusammen mit Zellen die entweder $\mathbf{E^{d1}}$ oder $\mathbf{E^{d2}}$ Rezeptor expremieren, inkubiert. Das hat bewirkt, dass die cAMP Konzentration stark reduziert war. Dies gilt als Beweis, dass ASIP auch beim Rind als Antagonist zu MC1R wirkt.

Die kodierende Sequenz von Attractin im Rind wurde amplifiziert und sequenziert anhand der cross-species PCR und 5' RACE. Für das Sequenzieren des 5' Ende des Gens wurde ein BAC–Klon verwendet. Es war jedoch nicht möglich die ganze kodierende Sequenz von Attractin zu amplifizieren.

In *in vitro* Zellsystemen kann man durch Messen der veränderten biochemischen Aktivitäten die Auswirkung von Mutationen erforschen. Die Interaktion von Genen können durch gleichzeitige Expression von verschieden Genen in einem *in vitro* System beobachtet werden. Genexpression in *in vitro* Systemen ist für die Nutztierzucht eine interessante Methode, weil sie ökonomischer und weniger zeitaufwendig ist als Zuchtexperimente oder die Herstellung von transgenen Tieren.

List of figures

Figure 1: Typical coat colour variants of Swiss cattle **2**

Figure 2: The scheme of melanogenesis (according to Prota, 1992) **7**

Figure 3: The chemical structure of cAMP **10**

Figure 4: Schematic representation of the melanocyte intracellular signalling **12**

Figure 5: a) Structure of the proopiomelanocortin precursor and b) amino acid sequence of melanocortin peptides **14**

Figure 6: Pseudo structural plot of the mouse MC1R **27**

Figure 7: Black and agouti hair **30**

Figure 8: Genomic organisation and multiple forms of wild type $\mathbf{A^w}$ transcripts **32**

Figure 9: Amino acid sequences of the mouse and human ASIP **37**

Figure 10: Schematic representation of ASIP and MC1R interactions (according to Virador et al., 2000) **38**

Figure 11: Domain structure of mouse attractin, human soluble attractin and hypothetical *C.elegans* protein F33C8.1 (according to Gunn et al., 1999) **43**

Figure 12: The primers and TaqMan probe position for real time PCR amplification **66**

Figure 13: Definition of C_T (according to TaqMan® Universal PCR Master mix manual) **67**

Figure 14: Scheme of competitive binding of labelled and unlabelled cAMP to the specific protein **74**

Figure 15: An example of an RFLP assay for the 310/311G deletion **78**

Figure 16: An example of the T296C RFLP **79**

Figure 17: An example of C667T RFLP **79**

Figure 18: Part of the sequencing image of the PCR fragment of Fleurons MC1R gene **81**

Figure 19: Alignment of the coding sequences of the five MC1R variants characterised in this study $\mathbf{E^D}$, **e**, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$ and $\mathbf{e^f}$ as well as **E**+ allele reported by Klungland et al. (1995, accession number Y13957) **84**

Figure 20: Comparison of the deduced amino acid sequences of six bovine MC1R variants ($\mathbf{E^D}$, **E+**, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$, **e** and $\mathbf{e^f}$) **85**

Figure 21: Comparison of the bovine MC1R amino acid sequence with that of the other mammalian species human (X65634), mouse (X65635), pig (AF326520), sheep (Y13965), horse (AF288357), dog (AF064455) and fox (X90844) **87**

Figure 22: Amplification plot for the $\mathbf{E^D}$ and **e** cell lines **88**

Figure 23: Accumulation of intracellular cAMP in 293 cells transfected with **e** (■) and $\mathbf{E^D}$ (◆) MC1R variants in response to increasing concentrations of α-MSH **90**

Figure 24: Accumulation of intracellular cAMP in 293 cells transfected with $\mathbf{E^{d1}}$(■), $\mathbf{E^{d2}}$(◆) and $\mathbf{e^f}$ (▲) MC1R variantss in response to increasing concentrations of α-MSH **91**

Figure 25: Accumulation of intracellular cAMP in the two cell lines of Fleuron(◆) and Fichte(■) transfected with the $\mathbf{e^f}$ MC1R variant in response to increasing concentrations of α-MSH **91**

Figure 26: Comparison of the ASIP deduced amino acid sequences of cattle, sheep (Parsons et al., 1999), pig (AJ251837), fox (Y09877), horse (AF288358), mouse (L06941) and human (NM 001672) **93**

Figure 27: Comparison of the amino acid sequences of bovine ASIP and AGRP (AJ002025) **94**

Figure 28: Accumulation of intracellular cAMP in the cells transfected with $\mathbf{E^{d1}}$ MC1R variant co-incubated with the cells transfected with ASIP (grey columns) and the cells transfected with $\mathbf{E^{d1}}$ MC1R variant alone (white columns) **95**

Figure 29: Accumulation of intracellular cAMP in the cells transfected with $\mathbf{E^{d2}}$ MC1R variant co-incubated with the cells transfected with ASIP (grey columns) and the cells transfected with $\mathbf{E^{d2}}$ MC1R variant alone (white columns) **95**

Figure 30: Scheme of the amplification and sequencing of the bovine attractin **98**

Figure 31: Comparison of the attractin deduced amino acid sequences of cattle, mouse (AF116897), two secreted forms of human attractin "man1" (AF034957) and "man2" (AF106861), membrane form of human attractin "man3" (AF218915) and rat (AB049248) **102**

Figure 32: Comparison of the bovine attractin DNA sequence from the Genbank (AF3010089) and of the bovine attractin sequenced in this study **103**

Figure 33: In vitro cell system created to investigate MC1R and ASIP functioning **112**

Figure 34: Location of the primers, restriction enzyme sites and polymorphic sites on bovine MC1R sequence **145**

Figure 35: Coding cDNA and deduced amino acid sequence of bovine attractin gene **155**

List of tables

Table 1: Distribution and function of melanocortin receptors (according to Lu et al., 1998) **17**

Table 2: Reagents for real time PCR **69**

Table 3: cAMP protocol **75**

Table 4: A combination of the four polymorphic sites and predicted MC1R genotypes in Swiss cattle **80**

Table 5: C_T values for real time PCR amplification of RT-products and RNA of cell lines expressing MC1R variants **88**

Table 6: Dominant black MC1R alleles in different mammalian species **106**

Table 7: Universal and kit primers **133**

Table 8: Bovine MC1R specific primers **134**

Table 9: Bovine agouti specific primers **135**

Table 10: Bovine attractin specific primers **135**

List of abbreviations

6-BH4	L-erythro 5,6,7 tetrahydrobiopterin
α–MSH	α-melanocyte stimulating hormone
A	absorption
A	alanine
ABAM	antibiotic-antimicotic
ACTH	adrenocorticotrophic hormone
AFLP	amplification fragment length polymorphism
AGRP	agouti related protein
Ala	alanine
AP-2	activator protein-2
APS	ammonium peroxodisulfate
Arg	arginine
ASIP	agouti signalling protein
Asp	asparagine
ATP	adenosine triphosphate
Atrn	attractin
BAC	bacterial artificial chromosome
C	cysteine
cDNA	complementary DNA
CIP	calf intestinal alkaline phosphatase
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CUB	first found in C1r, Cis, uEGF and bone morphogenetic protein
Cys	cysteine
DEPC	diethylpyrocarbonate
df	dilution factor
DHI	5,6-dihydroxyndole
DHICA	5,6-dihydroxyndole-2-carboxylic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
Dopa	dihydroxyphenylalanine
dpm	desintegrations per minute
E	glutamic acid
Ec	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EL	extracellular loop
F	phenylalanine
FCS	fetal calf serum
G	glycine
GITC	guanidine isothiocynate
Gln	glutamine
Glu	glutamic acid
Gly	glycine

GPCR	G-protein coupled receptor
H	histidine
HEK	human embryonic kidney
His	histidine
I	isoleucine
IAP	intracisternal A-particle
IBMX	3-isobutyl-1-methylxanthine
IL	intracellular loop
Ile	isoleucine
IPTG	isopropylthio-β-D-galactoside
K	lysine
L	leucine
Leu	leucine
LINE	long interspersed element
LTR	long terminal repeat
Lys	lysine
M	methionine
MC1R	melanocortin 1 receptor
MCS	multiple cloning site
Met	methionine
Mitf	micrphtalamia-associated transcription factor
MOPS	morpholino propane sulfonic acid
MSHR	melanocyte stimulating hormone receptor
NDP-MSH	Nle^4, D-Phe^7-α-MSH
N	asparagine
OD	optical density
ORF	open reading frame
P	proline
PLG	phase lock gel
PCR	polymerase chain reaction
Phe	phenylalanine
POMC	proopiomelanocortin
Pro	proline
Q	glutamine
R	arginine
Raly	ribonucleic protein associated with lethal yellow
RFLP	restriction fragment length polymorphism
rpm	rotations per minute
S	serine
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
Ser	serine
SP-1	specificity protein 1
T	threonine
T_a	annealing temperature
TEMED	N,N,N',N'-tetramethylethylenediamin
Ter	termination (stop) codon
Thr	threonine
TM	transmembrane domain
T_m	melting temperature
Trp	tryptophan

Tyr	tyrosine
Tyr	tyrosinase
TYRP	tyrosinase related protein
UTR	untranslated region
UV	ultra violet
V	valine
Val	valine
W	tryptophan
Y	tyrosine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1 Introduction

Genome research in mice and man has expanded rapidly, particularly during the last decade. Recently, sequencing has triggered an explosive growth in the identification of genes. Further genome research will lead to more information about gene function and will allow better insights into the development of biological systems. Characterisation of a gene in livestock is possible using information on physiological homology, comparative mapping and homology of amino acid and DNA sequences from different species. Comparative genetics approaches will lead to the identification of genetic markers, which can be candidate genes, causing specific phenotypes. Linked markers need to be informative in the population and a control for recombination has to be established. Recognition of a causative mutation is advantageous for widespread use of genetic selection in breeding programs. However, the breeding selection for alleles carrying a mutation can be time consuming and expensive. Transgenic techniques are commonly used to investigate if a mutation affects the phenotype, but producing transgenic farm animals for research purposes is hardly applicable. The gene variants of livestock breeds can, nevertheless, be introduced into mice, but the mouse phenotype can not always be compared to the phenotype in the donor species. An alternative approach which can be used is expressing the gene of interest in *in vitro* cultured cells. The cell lines expressing different alleles of a gene (e.g. a wild type allele and a mutated allele) can be established. The cell line can be stimulated by the external factor and the cell response can be estimated quantitatively or qualitatively, and compared between allelic variants of the gene. *In vitro* cell culture systems are relatively free of factors affecting *in vivo* system such as environment and interaction with other genes. The *in vitro* cell system can be build up and two or more genes can be introduced simultaneously into the cell culture, and thus the interaction of allelic variants of several genes can be studied. However, for creating an *in vitro* cell culture system a certain amount of knowledge about gene function is required, i.e. for which type of protein (an enzyme, a receptor, secreted protein etc.) the gene encodes, if any external stimulation is required for the protein to exhibit functional response and which biochemical pathways are affected and can be measured.

The coat colour genes of cattle were chosen to create such an *in vitro* gene expression system, due to the certain knowledge about the inheritance of the coat colour and also biochemical pathways in which these genes are involved. Thus comparison of the results obtained by using *in vitro* cell culture gene expression system with the results of breeding can be made.

Although specific coat colours or patterns have rarely been shown to influence production traits, they have been used for artificial selection of farm animals including cattle. Nowadays, many cattle breeds are expressing specific coat colours and/or patterns. The most typical coat colour variants present in Switzerland were taken for this study: Holstein including black and red Holstein, which is either red or black with white spots, Simmental, which is red, unicoloured or white-spotted and has white face and Brown Swiss, which possesses uniform coat colours varying in range from brown to grey (Figure 1).

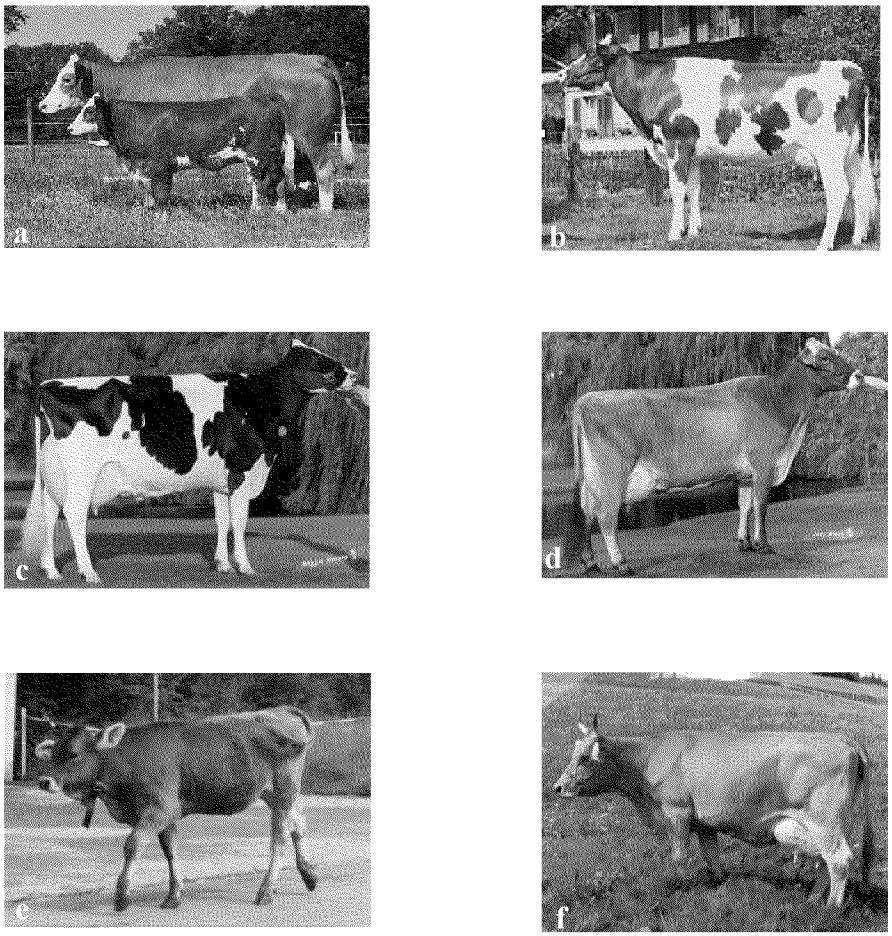


Figure 1: Typical coat colour variants of Swiss cattle

a) Simmental; b) Red Holstein; c) Black Holstein; d), e), f) Brown Swiss

The coat colour in mammals is determined by relative amounts of two pigments pheomelanin (red) and eumelanin (black) (Prota, 1992). The relative amounts of these pigments are known to be controlled by three genetic loci: extension (MC1R), agouti (ASIP) and *mahogany* (*attractin*) (Barsh et al., 2000). MC1R is a receptor, expressed on the melanocytes, which is activated by α-MSH, and subsequently couples to adenylyl cyclase, in turn activating

tyrosinase a rate limiting enzyme, which controls eumelanin and pheomelanin synthesis (Cone et al., 1996). High activity of tyrosinase leads to eumelanin synthesis, whereas the basal activity is sufficient for pheomelanin synthesis (Ito et al., 2000). Agouti is an antagonist of MC1R, which down-regulates the receptor activity (Lu et al., 1994). Attractin is an accessory receptor for agouti, facilitating its binding to MC1R (He et al., 2001). The coding sequence of cattle MC1R was sequenced and mutations associated with recessive red coat colour cattle and dominant black coat colour were found (Klungland et al., 1995; Joerg et al., 1996). The white coat colour in mammals is due to the absence of pigment synthesis and can be caused by a mutation in tyrosinase, or in the genes involved in the development, proliferation and migration of melanocytes (Shibahara et al., 1998). The colour patterns (e.g. roan, spotted, white face, belted) are also thought to be caused by mutations in the genes other than extension, agouti and attractin (http://sask.usask.ca/~schmutz/colors.html).

The aim of the present study was to clone and sequence the coding sequences of MC1R, agouti and attractin, to search for polymorphic sites, and to investigate the function of the genes and the influence of mutations on the gene function, using *in vitro* cell culture systems. MC1R is activated by α-MSH stimulation (Cone et al., 1996), therefore α–MSH was taken as an external stimulation factor for MC1R. Generally, response for α–MSH stimulation can be quantified using different assay systems (Eberle, 1988):

1. melanin assay
2. tyrosinase activity assay
3. cAMP production assay
4. adenylyl cyclase activity assay
5. protein posphorylation assay
6. hormone-receptor binding assay

Measuring of melanogenesis and tyrosinase activation is possible only in melanocytes, as tyrosinase expression is restricted to the pigment producing cells (Ganss et al., 1994), which are difficult to isolate and cultivate, except when they originate from melanoma, cases which are rarely found in cattle. Therefore, transfection of MC1R cloned into a mammalian expression vector and into cells other then melanocytes should be undertaken. cAMP production assay and hormone-receptor binding assay are commonly used to investigate the differences between MC1R alleles in the mouse and human. The receptor-binding assay quantifies the affinity of the receptor to the ligand, but can not always show whether the receptor has a loss, gain or reduction of function, as the mutated receptor can bind to the ligand with the same affinity as the wild type receptor, but is unable or less able to couple to

G-proteins and stimulate cAMP production. Alternatively the receptor can be constitutively active, but not able to bind to the hormone. Thus, the cAMP production assay can show loss-of-function, reduction, increase or constitutive activation of the receptor, but it does not distinguish, whether reduction in cAMP signalling occurs due to reduced binding or reduced signal transduction.

Expression of bovine agouti gene can be established in heterelogous cells, not expressing agouti. Cell lines expressing MC1R and agouti can be cultured together, mimicking hair bulb cell composition, and can be stimulated with α-MSH. In the case of the functional agouti protein a reduction of cAMP production is expected when compared with MC1R cells cultured without agouti cells.

Due to the widespread expression pattern of attractin (Gunn et al., 1999), it will be difficult to find cell lines, not expressing this gene.

In vitro cell culture gene expression can be applied to investigate different genetic models. The colour inheritance has been chosen as a model to study the expression of the mutated alleles as it is phenotypically visible and have been studied intensively in the biochemical pathways.

2 Literature review

2.1 Pigmentation in mammals

Melanins are the major biological polymers responsible for the diversity of colouration found in mammals. These pigments are also widespread in other organisms, for example in the mushrooms, the mould *Neurospora*, flatworms, many insects and practically all vertebrates (Searle, 1968; Prota, 1992).

The colour of the animals is usually not uniform all over the body. Spots, stripes, mottled appearance are found in many animals, as well as numerous shades of yellow, red, black and brown are seen in mammalian hair. These colour variations can arise due to different amounts, size and distribution of the pigment granules (melanosomes). Optical effects such as diffraction, scattering, and interference also contribute significantly to the palette of colours seen in animal skin and hair. However, at the chemical level, two distinct groups of pigments are found: the black and brown insoluble eumelanins and the alkali-soluble pheomelanins, ranging from yellow to reddish-brown (reviewed by Prota, 1992).

2.1.1 Eumelanin and Pheomelanin biosynthesis

Eumelanin is highly heteregeneous polymer consisting of the 5,6-dihydroxyindole (DHI) and the 5,6-dihydroxylindole-2-carboxylic acid (DHICA) units in the reduced and oxidised form and the pyrole units derived from their peroxidative cleavage. Pheomelanin consists mainly of sulfur-containing benzothiazine derivatives (Prota, 1992).

Both eumelanin and pheomelanin derive from the same precursor, L-tyrosine, and initially have a common metabolic pathway (reviewed by Prota, 1992): tyrosine is hydroxylated at the 3' position to dihydroxyphenylanine (dopa) which is further oxidised to dopaquinone. Both steps are catalysed by tyrosinase (monophenol dihydroxyphenyl-alanine: oxygen oxidoreductase, EC 1.14.18.1), a key enzyme in the melanin biosynthesis. Dopa formation from tyrosine is slow and irreversible, while oxidation to dopaquinone is fast and reversible. In the absence of the thiol compounds dopaquinone undergoes a spontaneous irreversible intramolecular cyclisation reaction to leucodopachrome which further undergoes several conversion steps and polymerisation into eumelanin (Prota, 1992; Figure 2). Apart from

tyrosinase two tyrosinase related proteins 1 and 2 (TYRP1 and TYRP2) are also involved in the eumelanin synthesis (Aroca et al., 1990; Kobayashi et al., 1994; Olivaries et al., 2001).

The *albino* (*c*) locus encodes a tyrosinase, which is a rate-limiting melanogenic enzyme. Mutations in the *albino* locus elicit dramatic effects on the quantity of melanin produced and usually little or no melanin is produced in mutant animals (Shibahara et al., 1998). The loss-of-function mutations in the human tyrosinase gene are associated with oculocutaneous albinism type I (Tomita et al., 1989). The *slaty* locus encodes for DOPAchrome tautomerase (TYRP2) an enzyme catalysing the conversion of DOPAchrome to DHICA. Mutations in the *slaty* gene result in the synthesis of eumelanin consisting mainly of DHI monomer units and thereby changing the colour of skin and hair to a dark grey (Jackson et al., 1992). Although the function of TYRP1 is not entirely clear, it is known that mutations in this gene (*brown* locus) result in the production of a lighter brown eumelanin in mice (Kobayashi et al., 1994). The function of this enzyme is apparently not the same in all mammalian species, in the mouse it was shown to catalyse the oxidation of DHICA, and in the human this conversion is catalysed by tyrosinase (Olivaries et al., 2001).

Intervention of thiols, such as cysteine, gives rise exclusively to thiol adducts of dopa, termed cysteinyldopas. Further oxidation of the thiol adducts leads to pheomelanin production via benzothiazine intermediates (reviewed by Prota, 1992) (Figure 2). No enzymes, except tyrosinase have been found to be involved in pheomelanin biosynthesis.

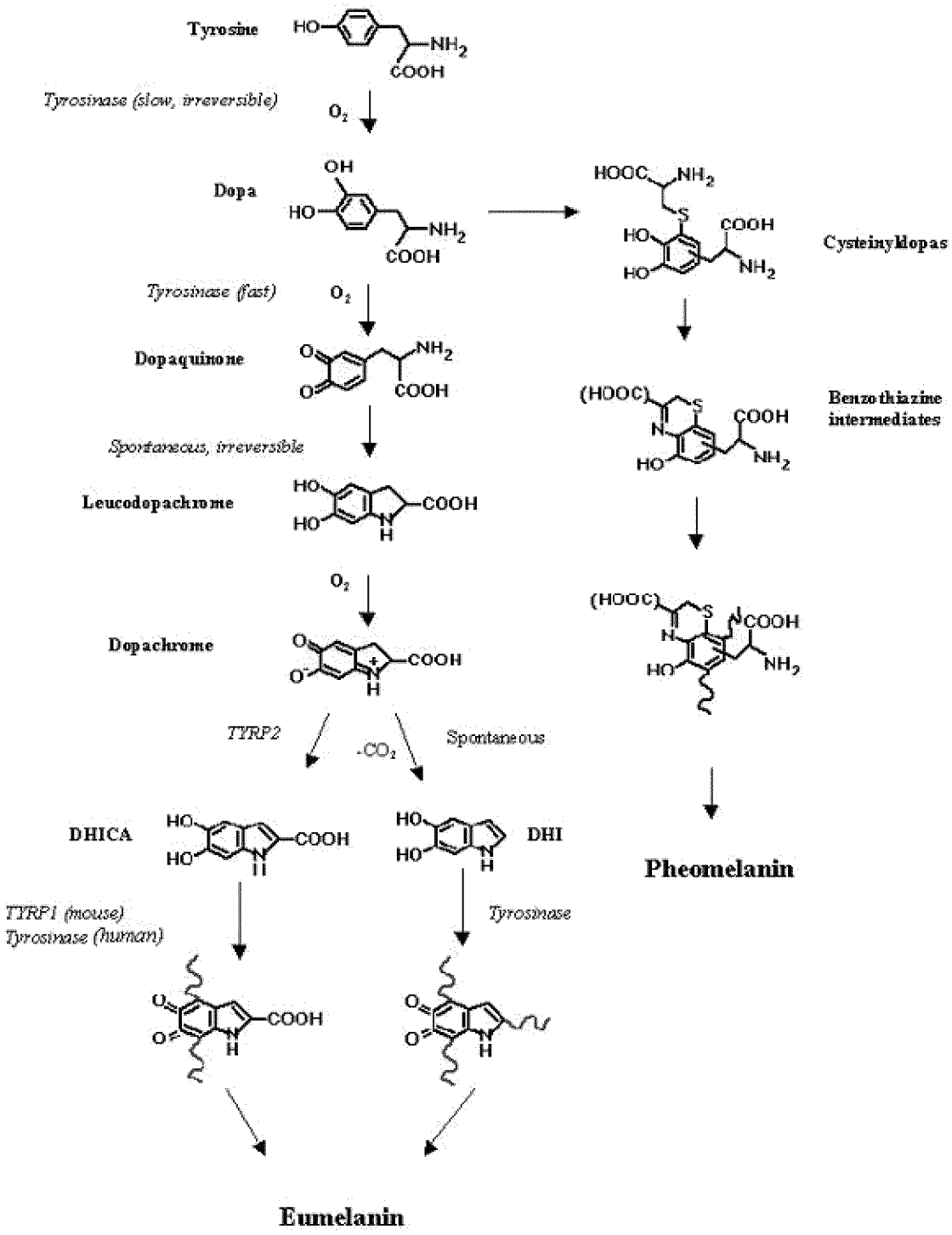


Figure 2: The scheme of melanogenesis (according to Prota, 1992)

2.1.2 Melanocytes

Melanin granules are produced in melanocytes in homotherm vertebrates (mammals and avians) and melanophores in cold-blooded vertebrates (reviewed by Prota, 1992). Both melanocytes and melanophores are derived embryologically from a stem population of melanoblasts which originate from the neural crest (Duncker, 1985). During embryogenesis of mammals undifferentiated melanoblasts migrate from the neural crest into the dermis and later invade in epidermis, differentiate into melanocytes and partly incorporate into the developing hair follicle. Melanocytes vary in size and shape, dependent on their population density. In low density areas melanocytes have very long dendrites and in high density areas melanocytes are more round and have shorter dendrites.

Melanin production takes place within a melanocyte in a special cytoplasmic organelle known as a melanosome (Prota, 1992). A melanosome consists of structural proteins, tyrosinase and possibly other functional proteins. Mature melanosomes are transfered to the surrounding cells: keratinocytes in the epidermis and Malphigian cells in the hair bulb. Two types of melanosomes have been distinguished dependent on the type of pigment produced: black or brown eumelanosome and yellow or reddish pheomelanosome. The eumelanosome is a large ellipsoidal organelle with a highly ordered protein matrix, and the pheomelanosome is spherical and composed of loosely aggregated small granules.

The melanocytes of the hair bulb only synthesise melanin during the growing stage of the hair cycle. In this stage the melanin granules are actively produced and transferred to the Malphigian cells of the upper bulb which then become the cells of the medulla and the cortex.

The melanin producing activity of the hair bulb can often differ qualitatively and quantitatively from that of the epidermal melanin unit. For example, in most mammals dark hair grows from colourless or from red skin, or alternatively the melanocytes of the hair follicle can lose their function, while the epidermis retains the ability to synthesise melanin, like in the polar bear. In many mammals glabrous skin, i.e. the paws, genitalia and snout are pigmented due to the actively melanising epidermal melanocytes (Eberle, 1988).

2.2 Regulation of pigmentation

Pigmentation in mammals can be divided into *constitutive* (intrinsic), which designates the genetically determined levels of melanin in the absence of any direct or indirect influences, and *facultative* (inducible), which characterises the increase in melanin pigmentation above the constitutive level and is induced by environmental factors and hormones (Eberle, 1988).

Constitutive pigmentation is determined by the rate of melanin synthesis in melanocytes, the rate of transfer of the melanin-containing melanosomes to keratinocytes and Malphigian cells, and the relative amounts of eumelanin and pheomelanin synthesised by melanocytes.

There are numerous biochemical factors which can affect melanogenesis, including concentrations of the substrate, i.e. tyrosine and cysteine, enzyme activities, ion concentrations as well as hormonal regulation.

The amino acids tyrosine and cysteine are two crucial substrates required for activation of melanogenesis. L-tyrosine is produced in melanocytes from L-phenylalanine. This conversion is regulated by the enzyme phenylalanine hydroxylase and the co-factor L-erythro-5,6,7,8-tetrahydrobiopterin (6BH4). Under certain pathological conditions phenylalanine hydroxylase activity can be inhibited significantly by the non-enzymatically produced 7-isomer of 6BH4. One of the consequences of this defect is a shortage on L-tyrosine supply for melanogenesis as only micromolar levels (4×10^{-5} M) are required to drive this process. The cell can partially substitute this defect with the direct L-tyrosine uptake, but it is significantly slower than uptake of L-phenylalanine (Schallreuter et al., 1998).

The presence of tyrosine is required for both eumelanin and pheomelanin synthesis, while cysteine is crucial to switch to pheomelanogenesis. The addition of cysteine to dopaquinone (6×10^{6} M^{-1} s^{-1}) proceeds much faster than its intramolecular cyclisation of dopaquinone (0.9 s^{-1}) (Thompson et al., 1985). These kinetic data indicate that even at concentrations of cysteine as low as 1 μM, this addition reaction proceeds faster, and thus pheomelanogenesis is preferred as long as cysteine is present (Ito et al., 2000).

Ito et al. (2000) investigated how melanogenesis is affected by tyrosinase activity, and proposed a model of switch from pheomelanin to eumelanin synthesis. When tyrosinase activity is low, the production of cysteinyldopas takes place with no pigment formation. When tyrosinase activity becomes higher, cysteindopas are oxidised to produce pheomelanin and, finally, when tyrosinase activity becomes much higher, eumelanin production begins to take place. Thus pheomelanin is always formed first and then eumelanin is deposited on the pre-formed pheomelanin. This model is also confirmed by the finding that black, brown, light brown and blond hair contain the same amount of pheomelanin and differ only in their eumelanin content (Ito et al., 2000). Thus, tyrosinase is a rate-limiting enzyme in the cascade of melanin synthesis.

Activity of tyrosinase is regulated by the melanocortin peptides, mainly by the α-melanocyte stimulating hormone (α–MSH) and adrenocorticotropic hormone (ACTH) (see chapter 2.2.1.1). Melanocortin peptides exert their effects through melanocortin receptors (MCRs),

and in the case of the melanocytes through MC1R. MCRs belong to the family of G-protein coupled receptors.

G-protein coupled receptors (GPCRs) are the large family of the seven transmembrane domains receptors, which mediate the cellular responses to an enormous diversity of signalling molecules, including hormones, neurotransmitters, and local mediators, which can be proteins, small peptides, amino acids and fatty acid derivatives. The trimeric GTP-binding proteins (G-proteins) functionally couple the receptors with their target enzymes or ion channels. When an extracellular ligand binds to a GPCR, the receptor changes its conformation and switches on a G-protein, thus causing a G-protein to eject guanosine diphosphate (GDP) and replace it with guanosine triphosphate (GTP). This switch of the G-protein allows the protein to dissociate and deliver its message to a downstream target. The switch back to the inactive form of a G-protein occurs by its hydrolysis of bound GTP into GDP (reviewed by Birnbaumer, 1990.)

Stimulation of MC1R with the melanocortin peptides causes activation of adenylyl cyclase by coupling through stimulatory G-protein (Gs). Thus stimulation of MC1R causes increase of intracellular cAMP. cAMP (Figure 3) is synthesised from adenosine triphosphate (ATP) by a plasma membrane-bound enzyme adenylyl cyclase, and is rapidly continuously destroyed by phosphodiesterases (PDE), which hydrolyse cAMP to adenosine 5'-monophosphate (5'-AMP) (Fimia and Sassone-Corsi, 2001).

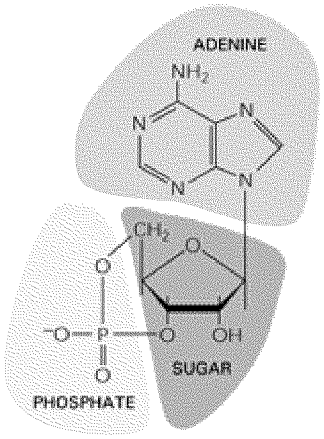


Figure 3: The chemical structure of cAMP

Increase of cAMP activates protein kinase A (PKA), which catalyses phosphorylation of specific serines and threonines of selected proteins, including tyrosinase, thereby up-regulating its activity (Tsatmali et al., 2002; Figure 4).

cAMP can also increase the transcription of the tyrosinase gene via *Mitf* (Microphtalmia-associated transcription factor). Up-regulation of cAMP results in the phosphorylation and subsequent activation of the CREB family of transcription factors. The melanocyte specific promoter of *Mitf* contains a consensus cAMP responsive element (CRE), which is activated by the cAMP responsive element binding protein CREB (Fisher, 2000). The consequence of the elevation of the *Mitf* protein is up-regulation of the tyrosinase promoter. However, the stimulation of cells with melanocortins resulting in the increased cAMP levels leads only to a short-pulse of *Mitf* expression, even at long term activation of MC1R. Thus, any sustained increase in pigmentation should arise either from activation of the other transcriptional factors or posttranscriptional activation of tyrosinase (Goding, 2000). Interestingly, the MC1R promoter can also be regulated by *Mitf*, at least in mast cells (Adachi et al., 2000), raising the possibility that *Mitf* and MC1R participate in a positive feedback loop.

Hence, melanin pigment formation in melanocytes is under the control of MC1R, whereby activation of the receptor results in the formation of increased amounts of black/brown eumelanin and down-regulation of the receptor activity results in the formation of a greater proportion of red/yellow pheomelanin. Agouti signalling protein (ASIP) is an antagonist of MC1R, which prevents melanocortin binding to the receptor and down-regulates receptor signalling (Barsh et al., 2000). Attractin is a low affinity receptor for ASIP, which facilitates it's binding to MC1R (He et al., 2001; Figure 4).

In addition to its receptor-mediated effects, there is evidence suggesting that α-MSH is able to regulate melanogenesis independently from MC1R. α-MSH can bind to the 6BH4, which has been shown to up-regulate the tyrosine supply and down-regulate the activity of tyrosinase in melanocytes. If α-MSH levels exceed that of free 6BH4, the hormone itself can function as a poor substrate for tyrosinase due to a tyrosine residue in position 2 (Schallreuter et al., 1998). Furthermore, α-MSH can also stimulate melanocyte dendricity. While dendrites are important for the formation of the epidermal-melanin unit and the transfer of melanin, the regulation of dendricity by α-MSH may be vital for the pigmentary response. The mechanisms of α-MSH stimulation are not completely elucidated, but it is likely that it depends on cAMP, which increases actin disorganisation and promotes melanocyte dendricity (Tsatmali et al., 2002).

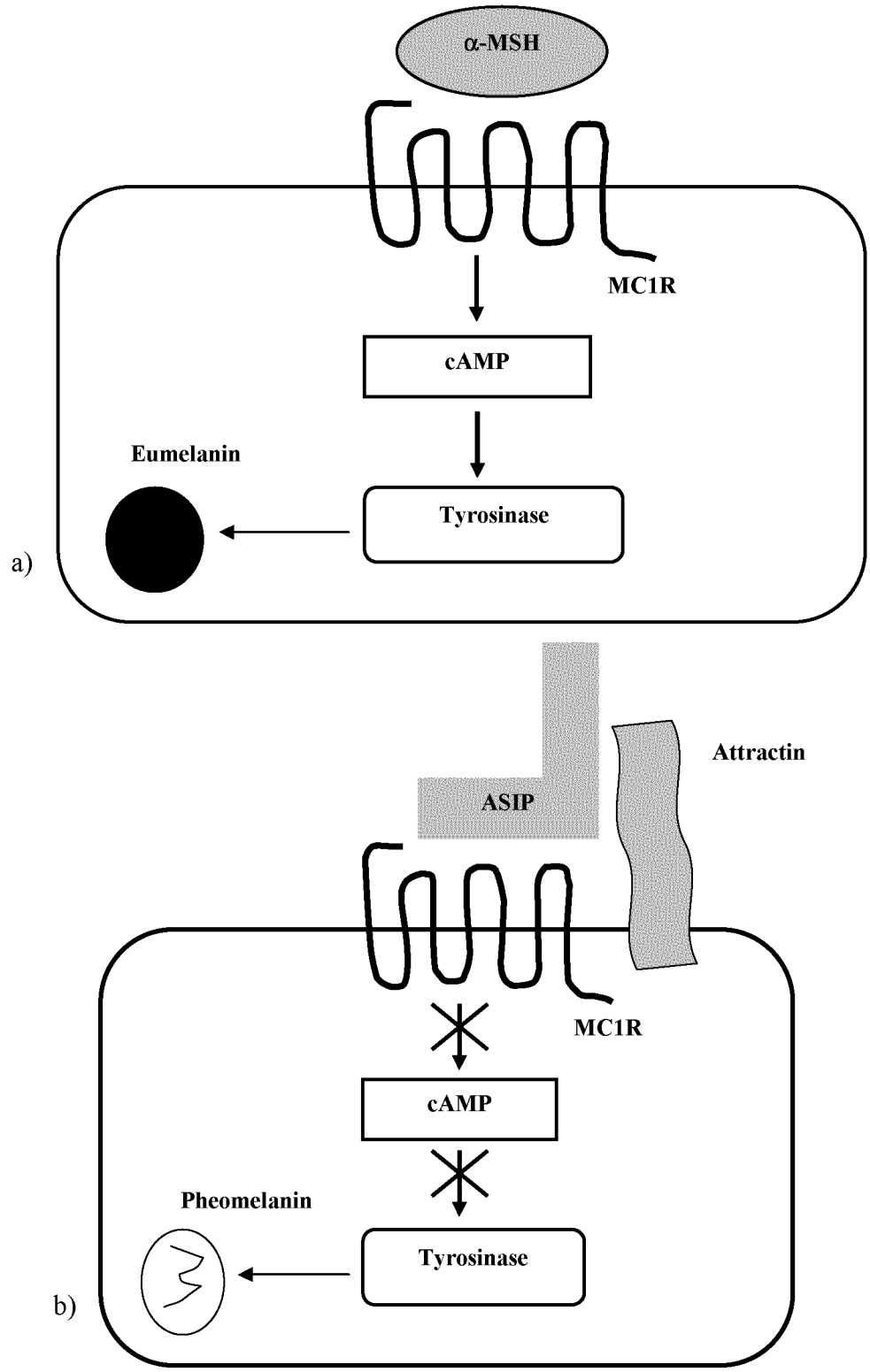


Figure 4: Schematic representation of the melanocyte intracellular signalling

a) α-MSH binds to MC1R, thus activating adenylyl cyclase via G-protein which results in an increase of intracellular cAMP. cAMP activates tyrosinase, thereby elevating eumelanin synthesis. b) ASIP prevents α-MSH binding to the MC1R, thus blocking the whole pathway activating tyrosinase and therefore only pheomelanin is synthesised. Attractin is a receptor for ASIP, which helps ASIP to bind to MC1R.

2.2.1 Hormones controlling the pigmentation

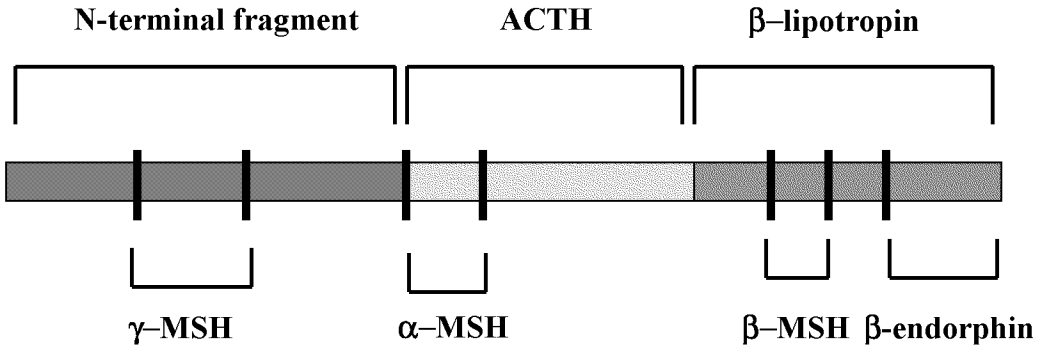
2.2.1.1 Melanocortins

The functions of melanocytes are controlled by a family of hormones, known as the melanocortins. The term "melanocortin" refers to peptides produced by the proteolytic cleavage from the larger proopiomelanocortin (POMC) polypeptide precursor and possess melanotropic and/or adrenocorticotropic activity (Figure 5). Initially POMC was identified in the anterior and intermediate lobe of the pituitary gland, and to date POMC is recognised to be expressed in various brain regions, including the hypothalamus, as well as in some peripheral tissues and organs, such as the gastrointestinal tract, the gonads and the skin (O'Donohue and Dorsa, 1982; Thody et al., 1983). The melanocortins include the α, β and γ–melanocyte stimulating hormones (MSH) and the adrenocorticotropic hormone (ACTH). Although the melanocortins derive from different regions of the POMC, all of them share the common sequence His-Phe-Arg-Trp (H-F-R-W), which is crucial for melanogenic activity (Eberle, 1988). In the skin POMC peptides have been shown to be produced in keratinocytes, melanocytes and to a lesser extent in Langerhans cells. Thus, the melanocortins in the skin function as endocrine, paracrine and autocrine hormones (Wakamatsu et al., 1997). α-MSH and ACTH also interact with different cytokines and have immunomodulatory and anti-inflammatory actions (Catania and Lipton, 1993; Bhardwaj and Luger, 1994). Nonetheless, the most prominent role of the melanocortins in the skin of vertebrates is the regulation of pigmentation: melanin synthesis and melanocyte proliferation.

It is generally thought, that α-MSH is the most potent pigmentary peptide (Eberle, 1988). This however reflects the situation in the lower vertebrates and in the mouse, but it might be different in humans. A comparison of the affinity of the melanocortins to the MC1R and their ability to stimulate cAMP formation, tyrosinase activity and proliferation of melanocytes in the human follows the order α-MSH~ACTH>β-MSH>γ-MSH (Suzuki et al., 1996; Wakamatsu et al., 1997), while in mice the affinity of MC1R to ACTH is lower than to α–MSH (Mountjoy, 1992; Cone et al., 1996). Moreover, a truncated form of the adrenocorticotrophic hormone ACTH1-17 was found to be the most potent stimulant to increase the intracellular cAMP in the human (Wakamatsu et al., 1997) and it binds to the MC1R on the human melanoma cells with an affinity that is 22-fold higher than that of α–MSH (Siegrist et al., 1989). The complete form of ACTH (1-39) has been shown to be equipotent with α-MSH in stimulating melanogenesis in human melanocytes (Hunt et al.,

1994), and since it is present in the circulation at greater concentrations than α-MSH, it may also be an important natural ligand for MC1R. Hence, in human the role of ACTH peptides in the regulation of pigmentation is likely to be more prominent than that of α-MSH. γ-MSH peptides have little or no effect on melanogenesis on their own, but can amplify (γ2-MSH) or inhibit (γ3-MSH) the melanogenic effect of β-MSH (Slominski et al., 1992).

a)



b)

ACTH	H_2N-SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF-OH
α-MSH	Ac-SYSMEHFRWGKPV-NH_2
β-MSH	H_2N-AEKKDEGPYRMEHFRWGSPPKD-OH
γ-MSH	H_2N-YVMGHFRWDRFG-OH
NDP-MSH	Ac-SYSXEHZRWGKPV-NH_2

Figure 5: a) Structure of the proopiomelanocortin precursor and b) amino acid sequence of melanocortin peptides

All peptides are derived from POMC with the exception of the Nle^4, D-Phe^7-α-MSH (NDP-MSH), a potent synthetic analogue. The conserved H-F-R-W core sequence is underlined. In the sequence of NDP-MSH, X represents norleucine and Z represents D-phenylalanine (according to Cone et al., 1996).

POMC is encoded by a gene consisting of three exons and producing a mRNA of approximately 1100 nucleotides which encodes a protein of 267 amino acids. POMC is processed by cleavage with two prohormone convertases (PC1 and PC2). POMC peptides are evolutionary conserved, for example no differences has been found between the α-MSH sequences in mammalian species (Eberle, 1988). The processed melanocortins undergo a number of posttranscriptional modifications, such as C-terminal amidation, N-terminal

acetylation and glycosylation. C-terminal amidation serves as a protection from digestion by carboxypeptidases. N-terminal acetylation has a profound influence on the biological activity of melanocortins, while acetylated forms are more potent in the activation of MC1R (Wakamatsu et al, 1997; Lu et al., 1998).

2.2.1.2 Melanin concentrating hormone

Melanin concentrating hormone (MCH) was originally isolated from the chum salmon pituitary gland (Kawauchi et al., 1983). Salmon MCH induces melanosome aggregation within melanophores, which causes whitening of the colour. In addition to melanin concentrating activity, salmon MCH has a potent inhibitory effect on corticotropin-releasing factor-induced α-MSH and ACTH secretion in vitro by the teleost pituitary gland (Baker et al., 1986). MCH has been found in high concentrations in human and rat brains, however no MCH has been found in the peripheral blood of these species. Treatment of human melanocytes with MCH has no effect on the expression of tyrosinase and melanin synthesis. The physiological role of MCH in mammals seems to be different from those in fishes (Shibahara et al., 1998).

2.2.1.3 Melatonin

Synthesis and release of melatonin from the pineal gland is stimulated by darkness and inhibited by light, acting via the eyes and the nervous system (Reiter, 1986). Melatonin is responsible for lightening the colour in amphibia (Bagnara and Hadley, 1973) and was shown to be involved in regulation of the seasonal control of pigmentation in the weasel, a moulting mammal, by suppressing the release of pituitary melanocortins (Rust, 1969). However, it was also shown that mammalian melanocytes express specific surface receptors, that can bind melatonin (Garratt et al., 1995).

2.2.1.4 Sex hormones

The female sex hormones, especially oestrogen, have been shown to be potent melanogenic stimulants in epidermal melanocytes, but not to hair follicular melanocytes (Snell, 1967). Melanocytes express receptors for these hormones. However, it is not clear whether oestrogen acts directly on melanocytes, or whether it stimulates the melanocortins release from the pituitary gland. Increase of the pigmentation observed during pregnancy may be due to higher oestrogen levels (Hearing, 1998).

2.3 Melanocortin 1 receptor

2.3.1 Cloning and characterisation of MC1R and other MCRs

Prior to cloning, two melanocortin receptors, melanocyte stimulating hormone receptor (MSHR) and adrenocorticotrophic hormone receptor (ACTHR) were known from classical physiological and pharmacological studies as G protein-coupled receptors (GPCRs). The MSHR was specifically found in melanocytes and melanoma cells and ACTHR had been shown to be expressed in adrenocortical cells and adipocytes (reviewed by Cone et al., 1996).

MSHR cDNA was first isolated from a human melanoma sample known to express a high number of MSH binding sites. Degenerate primers were designed to recognise all GPCR sequences and used for PCR with the cDNA from the human melanoma tissue. A large number of resulting fragments were cloned and sequenced, and one fragment was identified as the putative MSH receptor due to its ability to recognise mRNA specifically expressed by melanocytes. This fragment was used to screen a mouse cDNA library constructed from Cloudman S91 melanoma cells and a human genomic DNA library (Mountjoy et al., 1992). Using a similar method, the human MSHR was isolated from a cDNA library by Chhajlani and Wikberg (1992).

Amplification from human melanoma tissue also produced a sequence fragment that was clearly a unique GPCR closely related to MSHR. Northern blot hybridisation analysis clearly demonstrated, that this fragment recognised a mRNA specifically expressed in the adrenal tissue and was thus a candidate for the ACTHR gene. This probe was used to isolate ACTHR genomic sequences from human (Mountjoy et al., 1992) and bovine (Cone and Mountjoy, 1993) genomic DNA libraries.

PCR using degenerate primers and low-stringency hybridisation with the existing MSHR and ACTHR sequences rapidly lead to the discovery of three additional members of the melanocortin receptor gene family, which were called MC3R, MC4R and MC5R, named in the chronological order of their discovery. To avoid confusion, the MSHR and ACTHR were renamed to MC1R and MC2R, respectively. However, both nomenclatures are used in the literature (reviewed by Cone et al., 1996).

MC3R has been found to be expressed in the brain (hypothalamus and limbic system), placenta, stomach, duodenum and pancreas (Gantz et al., 1993a) and MC4R mRNA has been found to be expressed only in the brain (Gantz et al., 1993b). However, detailed neuroanatomical mapping by *in situ* hybridisation demonstrated that MC4R mRNA is much

more widely expressed in the rat brain, also being expressed in the cortex, thalamus, hypothalamus and limbic system, brain stem and spinal cord, than MC3R (Mountjoy et al., 1994). The MC5R mRNA has a remarkably wide distribution of expression, being found in the skin, muscle, thymus, spleen, ovary, testis, adrenal cortex, lung, brain and pars tuberalis (reviewed by Lu et al., 1998). The expression sites and function of all five MCRs is summarised in the Table 1.

Table 1: Distribution and function of melanocortin receptors (according to Lu et al., 1998)

Receptor	**Sites of expression**	**Functions**
MC1R (MSHR)	Melanocytes	Pigmentation
MC2R (ACTHR)	Adrenal cortex, adipocytes	Steroidogenesis
MC3R	Hypothalamus, limbic system, placenta, gut	Unknown
MC4R	Hypothalamus, limbic system, cortex, brain stem	Regulation of feeding and metabolism, thermoregulation
MC5R	Exocrine glands, muscle, brain, adipocytes	Regulation of exocrine gland function

The five melanocortin receptors are 39-61% identical to one another on the amino acid level. Compared with the other members of the G protein-coupled receptor superfamily, the melanocortin receptors are smaller (298-372 amino acids), resulting from a short amino-terminal extracellular domain, a short carboxyl-terminal intracellular domain, a small second extracellular domain, and a small fifth transmembrane domain (TM). Furthermore, some residues, conserved among the most G protein-coupled receptors, are absent in the melanocortin receptors. These include the proline residues in the TM4 and TM5, which are thought to introduce a bend in the α-helical structure of the transmembrane domains. They are thought to participate in the formation of a binding pocket, and one or both of the two cysteine residues are thought to form a disulfide bond between the first and second extracellular loops of most GPCRs (Mountjoy et al., 1992).

Each of the melanocortin receptors couples to activation of adenylyl cyclase, but displays a unique pharmacological profile for activation by different melanocortin peptides. MC1R and MC2R are the most specialised in terms of ligand recognition. The MC2R exhibits absolute

specificity for ACTH, requiring two peptides for recognition and activation, the core H-F-R-W sequence, present in all melanocortin peptides and a highly basic motive found only in ACTH. The H-F-R-W core sequence is a full agonist, albeit at low affinity of the other four melanocortin receptors. MC1Rs of the most vertebrates demonstrate a preference for α-MSH over ACTH. α–MSH is five times more potent than ACTH at the murine receptor, but may be as high as 1000-fold for the MC1R in lizards and amphibians (Eberle, 1988). The human MC1R is apparently unique in being equally activated by ACTH and α-MSH (see chapter 2.2.1.1). MC3R, MC4R and MC5R are less selective, being equipotently activated by α-MSH and ACTH (reviewed by Cone et al., 1996).

2.3.1.1 Characterisation of MC1R

Both mouse and human MC1R genes contain no introns like many other G-protein coupled receptors, and encode a protein of 315 and 317 amino acids, respectively (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992).

The extension locus, characterised in a wide variety of mammalian species was named after the extension of black pigmentation that results from the expression of dominant extension alleles. The MC1R have been mapped near to the extension locus in the mouse at the distal end of chromosome 8 by fluorescent *in situ* hybridisation (Gantz et al., 1994) and using an intersubspecific mapping panel (Magenis et al., 1994). Sequencing and pharmacological characterisation of the mouse MC1R alleles **E+**, **e**, $\mathbf{E^{tob}}$, $\mathbf{E^{so}}$, $\mathbf{E^{so\text{-}3J}}$ have demonstrated that these receptor variants have genotypic and phenotypic properties in agreement with their corresponding extension locus alleles. In conjunction with the mapping of MC1R at the same location as extension, these results indicate that the extension locus encodes MC1R (Robbins et al., 1993).

The transcription unit and promoter region of MC1R have been characterised in the human. The size of the human MC1R transcript was estimated to be approximately 2.3 kb (Smith et al., 2001). 5' RACE analysis of the transcriptional initiation sites of MC1R gene in human melanoma cells revealed multiple initiation sites within a region of approximately 600 bp upstream from the start codon (Moro et al., 1999). The most probable cap site was designated as the A residue at the position -388 which aligns with the consensus initiator sequence YYCARR, ccc<u>A</u>GC (Smith et al., 2001). Sequence analysis of the 3' UTR of the MC1R revealed a polyadenylation signal, AATAAA, 18 bp upstream from the beginning of the poly-A tail of the transcript and 736 bp downstream from the stop codon (Smith et al., 2001). A promoter assay revealed that the minimum region exhibiting promoter activity is located

between nucleotides -517 and -282. Analysis of the nucleotide sequence has shown that the MC1R promoter is GC rich, and no TATA and CAAT sequences were found near the site of initiation of transcription (Moro et al., 1999). However, a potential TATA-like element, TTTAAA, was found at the position -420 (Smith et al., 2001). The expression of MC1R has been reported to be induced by its ligand α-MSH (Chakraborty et al., 1995), indicating that cAMP production may be responsible for up-regulation of MC1R expression. This suggestion is supported by the fact, that CRE, SP-1 and AP-2 elements which are known to be activated by cAMP have been found in the promoter of the human MC1R. An atypical TATA-box consensus has been identified in a number of cAMP-responsive genes and this is also in agreement with the suggestion, that the MC1R gene expression may be regulated through a cAMP signalling pathway (Smith et al., 2001). The mouse MC1R promoter has not been as well characterised as the human promoter. Even so two E-box elements (from nucleotides -477 to -472 and from -466 to -461) have been found to be essential for transactivation of the murine MC1R by *Mitf* (Adachi et al., 2000). The human promoter region also contains an E-box, however promoter deletion analysis and transactivation studies have failed to show activation through this element by *Mitf* (Smith et al., 2001).

Although expressed by melanocytes and melanoma cells, and considered to be a control point of pigmentation, MC1R is also found to be expressed at lower levels in other cell types, such as human monocytes (Bhardwaj et al., 1997), endothelial cells (Hartymeyer et al., 1997), keratinocytes (Chakraborty et al., 1999), in the periaquaductal grey of the CNS (Xia et al., 1995) and in neutrophiles (Catania et al., 1996).

2.3.2 Allelic variants of MC1R

The various alleles of the extension locus either extend or diminish the amount of eumelanin in the coat, with an opposite effect on the amount of yellow pigment. The extension of the black pigment is dominant ($\mathbf{E^D}$), and yellow or red coat colour is caused by the recessive allele of extension (**e**) (Searle, 1968). The dominant alleles of extension are frequently associated with the constitutive activation of MC1R, while recessive alleles normally result from loss-of-function mutations of the receptor. The term constitutive activation has been used to refer to those G-protein coupled receptors which are active even in the absence of a ligand (Lu et al., 1998).

2.3.2.1 MC1R in mouse

Four alleles of MC1R have been characterised in the mouse: wild type (**E+**), recessive yellow (**e**), sombre (including two independent sombre alleles, $\mathbf{E^{so}}$ and $\mathbf{E^{so\text{-}3J}}$) and tobacco ($\mathbf{E^{tob}}$).

The recessive yellow mutation arose spontaneously in the C57BL inbred strain; **e/e** animals are almost entirely yellow, with a small number of dark hairs found dorsally and black eyes. The **e** allele has a deletion of one nucleotide at position 549 of the coding sequence and therefore a frameshift of open reading frame (ORF) at position 183 of amino acid sequence in the second extracellular loop. This results in a prematurely terminated MC1R. The $\mathbf{E^{so}}$ allele arose spontaneously in the C3H strain, and $\mathbf{E^{so}}$ homozygotes are with the exception of a few yellow hairs are entirely black and have darkened skin as well. $\mathbf{E^{so}}$**/E+** animals have yellow hairs at the flanks and have grey bellies. The $\mathbf{E^{so\text{-}3J}}$ allele arose spontaneously in the CBA/J strain and is phenotipically similar to $\mathbf{E^{so}}$. The $\mathbf{E^{so}}$ allele has a base pair substitution, which results in conversion of Leu into Pro at amino acid position 98. The allele $\mathbf{E^{so\text{-}3J}}$ has also a point mutation converting a Glu into Lys at amino acid position 92. Both of these mutations occur in the second transmembrane domain (TM2). $\mathbf{E^{tob}}$ is a naturally occurring extension allele present in tobacco mouse *Mus poschiavinus*. The $\mathbf{E^{tob}}$ allele produces a darkening of the dorsum, which is visible after 8 weeks, when the flanks become agouti. The point mutation found in this allele converts the Ser at amino acid position 69 in the first intracellular loop (IL) into a Leu. The $\mathbf{E^{tob}}$, $\mathbf{E^{so}}$ and $\mathbf{E^{so\text{-}3J}}$ alleles are epistatic over agouti (Robbins et al., 1993).

To understand the mechanism by which the described alleles cause their pigmentation phenotypes, the **E+, e**, $\mathbf{E^{tob}}$, and $\mathbf{E^{so\text{-}3J}}$ MC1R alleles were cloned into mammalian expression vectors and transfected into human embryonic 293 kidney cells. The stable cell lines expressing MC1R alleles were analysed for their α–MSH responsive intracellular cAMP accumulation or adenylyl cyclase activity. The **e** allele of receptor was shown to be non-functional, while it was unable to couple to adenylyl cyclase even at 10^{-4} M concentration of α–MSH. The cells transfected with the **E+** allele of MC1R responded to α–MSH stimulation in a dose-dependent manner with a 2- to 5-fold increase in intracellular cAMP production and a 7-fold increase in adenylyl cyclase activity. The $\mathbf{E^{so\text{-}3J}}$ allele was constitutively active and unresponsive to α-MSH concentrations from 10^{-9} M to 10^{-4} M. The average amount of adenylyl cyclase activity was approximately 60% of the maximal achievable adenylyl cyclase activity in the **E+** allele. Pharmacological data of the $\mathbf{E^{so}}$ allele was obtained by Cone et al. (1996) which was very similar to the $\mathbf{E^{so\text{-}3J}}$ allele. It is notable, that NDP-MSH (a synthetic analogue of α-MSH) was capable of activating $\mathbf{E^{so\text{-}3J}}$ allele in a dose

dependent manner, indicating different mechanisms of activation by α-MSH and NDP-MSH (Cone et al., 1996).

In contrast with the $\mathbf{E^{so}}$ and $\mathbf{E^{so\text{-}3J}}$ alleles, the dominant phenotype of the $\mathbf{E^{tob}}$ MC1R allele does not result from a constitutive activation of the receptor. The $\mathbf{E^{tob}}$ MC1R allele encodes a hormone-responsive receptor with slightly higher basal activity then **E+** receptor and a median effective concentration (~5 x 10^{-9} M) also slightly higher than that of the **E+** receptor (~2 x 10^{-9} M). However, the $\mathbf{E^{tob}}$ allele appeared to have a much greater ability to elevate intracellular cAMP than the **E+** MC1R allele, particularly at α-MSH concentrations above 3 x 10^{-9} M. Thus, the mechanism by which the $\mathbf{E^{tob}}$ acquires a dominant genotype relative to **E+** is clearly different from those of the $\mathbf{E^{so}}$ and $\mathbf{E^{so\text{-}3J}}$ alleles. Greater activation of adenylyl cyclase can be accomplished by a greater off-rate for G-protein coupling, which is consistent with the mutation being present in the intracellular loop (Robbins et al., 1993).

2.3.2.2 MC1R in human

Population studies have revealed that the coding region of the human MC1R is highly polymorphic with over 30 allelic variants identified to date (Sturm et al., 2001).

Extensive polymorphism of the MC1R coding sequence has been observed in the Northern European population; in population-based studies the variant MC1R alleles were identified in approximately 50% to 70% of these individuals (Smith et al., 1998; Flangan et al., 2000; Palmer et al., 2000). MC1R variants associated with red hair in Europe are rarely found in other populations. Conversely, **Arg163Gln**, a variant rarely found in Europeans, is present in over 70% East/Southeast Asians and Native Americans (reviewed by Schaffer and Bolognia, 2001). In contrast, the African population completely lacks non-synonymous MC1R variants. Two hypotheses of two different groups of authors explain the described situation. Rana et al. (1999) argue that the diversifying selection took place in the North European population against dark pigmentation in areas of low ambient UV radiation due to the resultant impaired cutaneous production of vitamin D and predisposition to rickets. Harding et al. (2000) suggest that a strong functional constraint to a wild type of MC1R exists in Africa, where eumelanin is required to protect against UV radiation induced burning as well as skin cancer. In this case the European MC1R diversity does not result from enhanced selection, but rather reflects neutral evolution.

All variants of human MC1R found to date are either associated with the red hair and/or fair skin and/or poor tanning ability, or have no significant association with any pigmentation phenotype. Furthermore, no dominant black alleles similar to the murine $\mathbf{E^{tob}}$, $\mathbf{E^{so}}$ and $\mathbf{E^{so\text{-}3J}}$

MC1R alleles have been reported so far. Mutations found in the human MC1R, which were investigated from the point of view of affecting the receptor function, are summarised below.

2.3.2.2.1 Loss-of-function mutations

Three particular variants **Arg151Cys** (IL2), **Arg160Trp** (IL2) and **Asp294His** (TM7) have in several studies been shown to have highly significant association with red hair, fair skin and poor tanning ability and are thereafter referred to as "red-hair-colour alleles". The odds of having red hair increase 9- to 16-fold with the presence one of these variant alleles, and individuals carrying two of these alleles almost always have red hair (Box et al., 1997; Smith et al., 1999). The **Arg142His** (IL2) variant was only detected in red hair individuals, but the sample size was insufficient to statistically test the association of this variant with hair or skin type. The **Val60Leu** (TM1) was most frequent in fair/blonde and light brown hair (Box et al., 1997). The binding assay for all of these MC1R variants showed very similar affinities for α–MSH and NDP-MSH as for the wild type receptor. However, each of the mutant receptors was unable to increase intracellular cAMP in response to α-MSH, with the exception of a slight increase being noticed for the **Val60Leu** variant (Frändberg et al., 1998; Schiöth et al., 1999). Thus, the mutations make the MC1R unable to couple with G-proteins and activate adenylyl cyclase. It is also known that the IL2 contains the consensus sequence for cAMP-dependent protein kinase recognition between amino acids 142-145 and 151-154 (Eberle et al., 1993). Therefore, changes at positions 142 and 151 may block phosphorylation and hence receptor signalling.

Two single nucleotide insertion mutations **86insA** and **537insC** have also been found in individuals with red hair and fair skin (Flanagan et al., 2000). No functional analysis has been done for these alleles of the receptor, but because single nucleotide insertions produce a frameshift and result in a prematurely terminated receptor, they can be considered loss-of-function mutations.

2.3.2.2.2 Partial loss-of-function mutations

The **Ile40Thr** and **Val122Met** variants are relatively frequent in the Spanish population and are possibly associated with fair skin. The **Ile40Thr** mutation is located on the extracellular side of the TM1, **Ile40** is conserved in mammalian MC1Rs and also present in the mouse and human MC4R and MC5R. The **Val122Met** is located in the TM3, next to **Asp121**, a residue found to be critical for agonist binding and effective coupling to the cAMP cascade (Yang et al., 1997). The valine at position 122 is also present in the most mammalian MC1Rs. Both

variants showed a right shift to the dose-response curves of cAMP accumulation when stimulated with NDP-MSH with a significant increase in the median effective concentration (EC_{50}). The **Ile40THr** variant of the receptor showed a four-fold decreased affinity for NDP-MSH and the median effective concentration (EC_{50}) of α-MSH for adenylyl cyclase coupling was increased by approximately 100-fold, compared to the wild type of the MC1R, indicating that this mutation impairs both hormone binding and G-protein coupling. Conversely, the affinity of the **Val122Met** for NDP-MSH and the EC_{50} for coupling to cAMP production is affected to approximately the same extent. This suggests that the rightward shift in the cAMP curve is caused by decreased binding affinity. The basal and maximal hormone induced cAMP levels were similar for wild-type and **Ile40Thr** receptors, but significantly lower for the **Val122Met** mutant (Jimenez-Servantes, 2001).

2.3.2.2.3 Mutations with undefined influence on function

Val92Met (TM2) was reported to be frequent in individuals with red hair/fair skin (Valverde et al., 1995), however it was also found in the samples of fair skin and light brown hair of Chinese and British individuals, and valine is not conserved between species (Rana et al., 1999). α-MSH binding assays on the **Met92** receptor expressed in COS-1 cells showed this variant to have an approximately five times lower potency in displacing the radio-labelled analogue of α–MSH by endogenous α-MSH when compared to the wild type receptor (Xu et al., 1996). However, no loss of binding potency has been detected by testing the ability of the receptor to activate adenylyl cyclase, predicting its functional equivalence to the wild type receptor (Koppula et al., 1997).

Arg163Gln (TM4) variant may also be associated with pheomelanin-rich skin because it has only been identified in such individuals from East and Southeast Asian populations and American Indians (Rana et al., 1999). The arginine at position 163 is conserved in the MC1R of higher primates, cow, fox, horse and mouse.

Recent studies have provided evidence for a dosage effect of MC1R variants in both hair colour and skin type. Most individuals with red hair are homozygous or compound heterozygous (i.e. have two mutant alleles for a particular locus) for MC1R gene mutations. Flanagan et al. (2000) reported that in North European individuals with red hair 85% are homozygous or compound heterozygous for MC1R mutations and 13% have a single variant allele. However, the majority of the latter group have auburn or strawberry blonde hair, rather then pure red. A significant heterozygote effect on sun sensitivity has also been reported for

loss-of-function MC1R mutations. North European population individuals with one variant allele are intermediate with regard to both skin type and tanning ability compared with those having two variants and those with none (Healy et al., 2000). A significant dosage effect of MC1R variants on the number of cutaneous freckling sites has also been described, but no relationship between MC1R variants and eye colour has been reported (Flanagan et al., 2000). Rare individuals with red hair have homozygous wild-type MC1R alleles, indicating presence of unidentified changes outside the coding sequence of MC1R or in the other loci (Schaffer and Bolognia, 2001).

2.3.2.3 MC1R in cattle

The complete coding sequence of bovine MC1R was amplified using cross-species primers designed based on murine and human sequences and subsequently sequenced (Klungland et al., 1995). The coding region of cattle MC1R is 954 bp and encodes a protein of 317 amino acids. Three alleles of MC1R were identified through sequencing of animals which had different coat colour phenotypes in Norwegian and Icelandic cattle populations. The $\mathbf{E^D}$ allele results from a substitution of Leu to Pro at position 99 (at the very end of TM2) and is inherited as dominant black. The **e** allele results from the deletion of a G at position 310/311 of coding DNA serquence and **e/e** animals are always red. The animals homozygous or heterozygous for wild type allele **E+/E+** and **E+/e** can be black, brown and red (Klungland et al., 1995). Simultaneously Joerg et al. (1996) found that red coat colour in Holstein cattle is associated with the same deletion 310/311G. French red breeds such as Blonde d'Aquitaine, Charolais, Limousine and Salers were shown to be exclusively composed of homozygous **e/e** animals (Rouzaud et al., 2000).

The **E** locus was genetically mapped to the bovine chromosome 18 (Klungland et al., 1995), which is in agreement with comparative genome mapping (O'Brien et al., 1993). The dominant $\mathbf{E^D}$ bovine allele is similar to the dominant murine $\mathbf{E^{so}}$ allele, which results from a conversion of Leu at position 98 into Pro, which indicates the same mechanism of creating a constitutively active receptor.

Genotyping of different coat colour phenotypes in Norwegian and Icelandic populations have revealed several black animals processing **E+/E+** and **E+/e** genotypes. This is in agreement with the model of two loci interaction proposed by Adalsteinsson et al. (1995). According to this model the black phenotype of the **E+/E+** and **E+/e** animals is caused by homozygosity of the recessive agouti allele (**a**), which is unable to block the wild type receptor **E+**. This model is also supported by the existence of red animals genotyped as **E+/E+** and **E+/e**. In this case

the wild type receptor **E+** is inhibited by agouti and thus eumelanin production is suppressed (Klungland et al., 1995).

2.3.2.4 MC1R in other species

The MC1R coding sequence has been determined in some mammalian species, in such as the pig, horse, sheep, dog and fox and several variants, associated with red and black coat colour were found:

Recessive red/yellow alleles (e):

The **Ser83Pro** (TM2) variant was found to be associated with a chestnut (uniform red) coat colour in horses (Marklund et al., 1996). No dominant black alleles of MC1R were found in the horse, indicating that the black coat colour results from recessive agouti alleles (Rieder et al., 2001). The **Ala240Thr** (TM6) variant is associated with red coat colour in pigs (Kijas et al., 1998). The **Arg306Ter** mutation, when the CGA codon encoding arginine is mutated into a stop codon TGA was found to be associated with red/yellow pigmentation in several dog breeds such as Yellow Labradors, Golden Retrievers and Irish Setters. The mutated receptor lacks 12 residues at carboxyl terminal, thus abolishing **Cys315** which is palmitoylated and participates in receptor localisation or trafficking (Newton et al., 2000).

Dominant black alleles (E^D):

Two variants of pig MC1R are associated with black coat colour: **Leu99Pro** (at the very end of TM2), which is identical to the bovine E^D allele and **Asp121Asn** (TM3) (Kijas et al., 1998). The **Ser90Gly** (TM2) variant found in the black dog breeds Newfoundland and Black Labrador was proposed to be a constitutively activated receptor. The same mutation was also present in Yellow Labradors together with the **Arg306Ter** substitution, therefore any role that **Ser90Gly** might play in receptor activation is inhibited by the **Arg306Ter** mutation (Newton et al., 2000).

The **Cys125Arg** (TM3) variant was found specifically in dark-pigmented foxes carrying the Alaska Silver allele (E^A). This change when introduced in the murine MC1R by site directed mutagenesis was shown to activate adenylyl cyclase in the absence of any hormone stimulation at levels between 25-90% of maximum levels of the wild type MC1R stimulated with α–MSH. This indicates that the E^A variant is a constitutively active receptor (Vage et al., 1997). Interestingly, both the **Cys123Arg** mutated variant of the mouse MC1R and the fox E^A variant expressed at a very low copy number, compared with the wild type receptor. This may be due to the potent down-regulation of the constitutively active receptor, also reported for the **Lys296Glu** variant of rhodopsin receptor (Li et al., 1995). The **Cys123Arg** MC1R in the

mouse retained a high affinity to the NDP-MSH. The constitutive activity of the fox $\mathbf{E^A}$ variant of the MC1R was not reliably reproducible, probably due to the low level of expression of the receptor in the cells. It is remarkable, that in striking contrast to the mouse the heterozygosity for $\mathbf{E^A}$ is not sufficient to override inhibition of eumelanin production by agouti. The **E^AE+, Aa** foxes produce significant amount of red pigment around the flanks, midsection and neck. One possible explanation of this phenomena is that while mouse agouti behaves as a classical competitive antagonist, fox agouti is a negative agonist and can inhibit the constitutively active $\mathbf{E^A}$ allele of MC1R. A second hypothesis would invoke another target of agouti action on the melanocyte in addition to MC1R (Vage et al., 1997).

A combination of the two mutations **Met73Lys** (IL1) and **Asp121Asn** (TM3) have shown complete co-segregation with the dominant black colour in sheep (Vage et al., 1999). To investigate their role in the receptor function these changes were introduced into the mouse MC1R separately and together. Pharmacological analysis revealed that the **Asp119Asn** mutation alone in context of the murine MC1R did not constitutively activate the receptor. Furthermore, replacement of the Asp at this position with Lys, Asn, Val or Ala significantly decreases ligand binding (Yang et al., 1997; Lu et al., 1998). The **Met71Lys** mutation alone potently activated the mouse MC1R to approximately 40% of maximum stimulation levels in the absence of the ligand. However, the receptor could be further activated to maximum levels by both α-MSH and NDP-MSH. The **Met71Lys** variant had an increased affinity to the ligand, compared with the wild type receptor. When **Met71Lys** and **Asp119Asn** were introduced together (double mutant), the extent of constitutive activation of the receptor was lowered and the potency of α-MSH was reduced to undetectable levels. This negative effect on the receptor activation could indicate, that the **Asp119Asn** is not critical for the black coat colour observed in sheep, and that **Met71Lys** alone can produce a similar phenotype (Vage et al., 1999). However, the reduced potency of the double mutant receptor is still sufficient for constitutive activation of the receptor and subsequent eumelanin production. It is notable that the **Asp121Asn** substitution in the MC1R in the pig leads to the arising of a dominant black allele, most probably a constitutively active receptor (Kijas et al., 1998).

2.3.3 Structure and function of MC1R

The two dimensional structure (location of transmembrane domains and extracellular and intracellular loops) of the mouse and human MC1R was predicted by hydrophobicity plot analysis and comparison of the MC1R amino acid sequence with the sequences of other

G-protein coupled receptors (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992; Cone et al., 1996) (Figure 6).

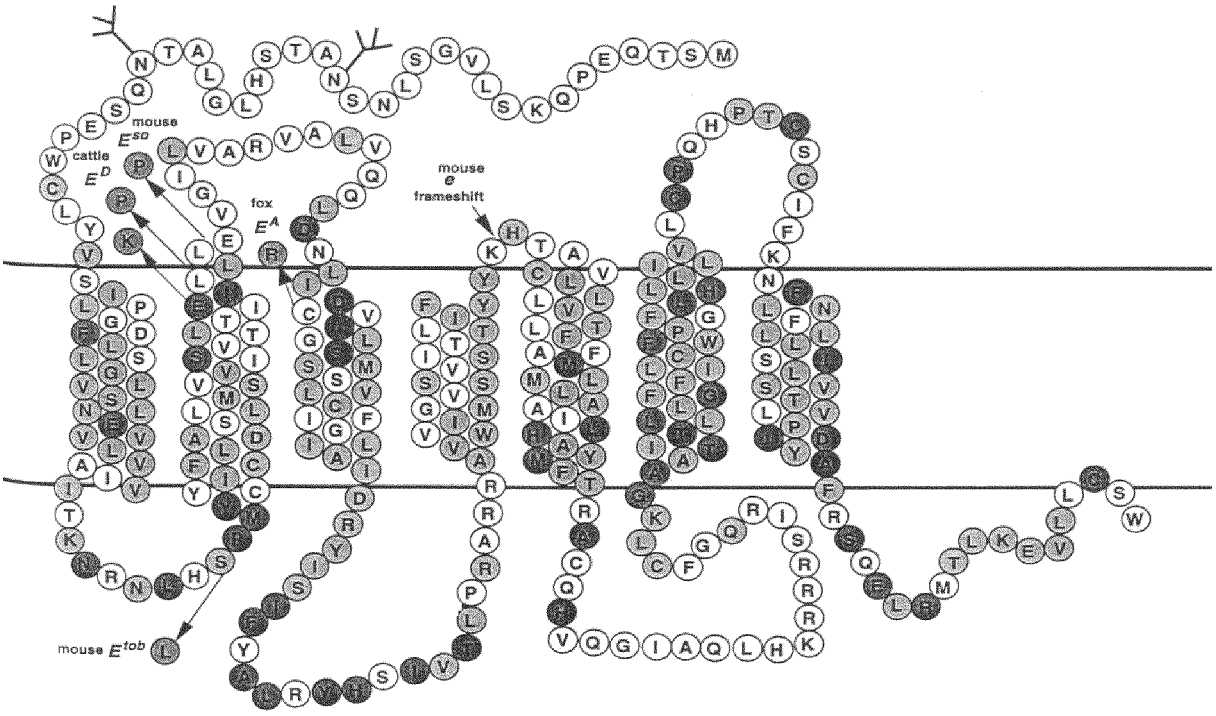


Figure 6: Pseudo structural plot of the mouse MC1R

Variant alleles in some species are indicated. Light and dark shading indicate residues conserved or identical in all of the melanocortin receptors (according to Cone et al., 1996).

Comparison of the melanocortin receptor amino acid sequences revealed the highest homology between TM2, TM3 and TM7, and the lowest homology between TM4, TM5, IL1, IL3 and carboxyterminal loop (Prusis et al., 1995; Schiöth et al., 1998). The regions of the highest homology can be considered important for the function of the receptors, and the regions of the lowest homology may be the cause of the different specifities of the receptors to their ligands. However, it is also possible, that they were less preserved during evolution due to the unimportant role in receptor function.

The involvement of the particular amino acids in the function receptor can be investigated by site-directed mutagenesis of these specific amino acids. Subsequently, binding assays of the mutated and wild type receptor expressed in the cells can be performed to find differences in the binding affinities of the receptors. Measurement of intracellular cAMP (or adenylyl cyclase activity) as a response to increased α-MSH stimulation can be performed to determine the differences in potency of the wild type and the mutated receptors. Comparison of the

binding affinity and potency of the mutated receptor with the wild type receptor, can clarify if it affects ligand binding and/or G-protein coupling.

The molecular basis for the interaction of the melanocortins and their synthetic analogues with the MC1R is not yet fully clear. Several three dimensional molecular models were proposed by different groups of authors (Prusis et al., 1995; Cone et al., 1996; Haskell-Luevano et al., 1996). All models implicate the involvement of an ionic binding pocket composed mainly of acidic amino acids in TM2, TM3 and/or TM6 and/or TM7. Prusis et al. (1996) docked a cyclic analogue of the α-MSH into a binding pocket of the human MC1R constituted of **Asp117** (TM3), **Asp121** (TM3), **His260** (TM6) and probably **Glu55** (TM1). Specific amino acids proposed by Haskell-Luevano et al. (1996) to constitute this binding pocket of the human MC1R binding to NDP-MSH include acidic residues **Glu94** (TM2), **Asp117** (TM3) and **Asp121** (TM3) and the non-acidic residues **Phe280** and **Asn281** in TM7. The computer model by Cone et al. (1996) implicates that five amino acids [**Phe43** (TM1), **Glu92** (TM2), **Asp115**, **Asp119** (TM3) and **Phe278** (TM7)] of mouse MC1R interact with the His-Phe-Arg-Trp core sequence of melanocortins. The partial discrepancies of the models can be explained by differences of the ligand specificities used in these studies. However, the proposed models of receptor ligand interactions are at least partially confirmed by site-directed mutagenesis studies. The results of Yang et al. (1997) have shown, that individual and complex mutagenesis of the acidic acids **Glu94**, **Asp117** and **Asp121** into non-polar alanine have significantly decreased binding and potency of NDP-MSH and α-MSH in the human MC1R. Introducing the **Asp119Asn** mutation into the **Met73Lys** variant of murine MC1R significantly decreased both affinity and potency of the receptor (Vage et al., 1999). However, it is notable that the **Asp121Asn** variant of the MC1R is associated with the black dominant colour in pigs, probably producing a constitutively active receptor (Kijas et al., 1998). Frändberg et al. (1994) showed, that mutagenesis of **Asp117** into alanine, as well the substitution of **His260** by alanine leads to a drop in affinity for the linear α–MSH, but not to NDP-MSH. Haskell-Luevano et al. (1996) also propose the presence of a second hydrophobic binding pocket, consisting of a series of aromatic residues (phenylalanine and tyrosine) spanning TM4, TM5 and TM6 involved in hydrophobic (aromatic-aromatic) interactions with **D-Phe7** and **Trp9** of NDP-MSH. Substitution of the individual phenylalanines or tyrosines by alanine caused either very small or no loss of the NDP-MSH binding affinity and potency. However paired aromatic mutations caused a significant decrease in NDP-MSH binding affinity and potency.

Studies of many G-protein coupled receptors demonstrated, that conserved cysteines play a critical role in the receptor function due to their ability to form disulfide bonds. Site-directed mutagenesis revealed that cysteines are also involved in the functioning of MC1R. Single point mutations of four cysteines in extracellular loops of the human MC1R (**Cys35, 267, 273** and **275**) into glycine resulted in a complete loss of binding to NDP-MSH. Moreover, mutants with normal ligand binding for NDP-MSH at positions **Cys191**(TM5), **Cys215** (3IL) and **Cys315** (C-terminal) failed to generate a cAMP signal in response to both α–MSH and NDP-MSH (Frändberg et al., 2001). The site-directed mutagenesis of the amino acids in the third intracellular loop of human MC1R revealed, that amino acid residues present in C-terminal part of the IL 3 are involved in G-protein coupling and that the region of four amino acids **Lys226-Arg227-Gln228-Arg229** is essential for coupling of MC1R to G-proteins (Frandberg et al., 1998).

The mouse constitutively active MC1R $\mathbf{E^{so\text{-}3J}}$ variant results from **Glu92Lys** substitution, which is analogous to the **Glu94** of human MC1R and which was proposed to be involved in ligand binding (Haskell-Luevano, 1996). Substitution of the **Glu92** could constitutively activate the receptor, however only when it is substituted with a basic residue. Alteration of the glutamic acid to an isoleucine or glutamine disrupts the affinity of the receptor for the ligand, but produces no constitutive activation (Cone et al., 1996). Lu et al. (1998) showed that conversion of the transmembrane residues **Glu92**, **Asp119** and **Cys125** in the mouse MC1R into basic residues is required for constitutive activation. However these substitutions significantly reduce agonist affinity and potency of the receptor. Cone et al. (1996) suggest, that the **Glu92Lys** change introduces a positive charge capable of electrostatic interaction with one of the aspartic acid residues, and constitutively activates the receptor by indirectly disrupting the constraining bonds that these residues form. This model, however, was not confirmed, because only a substitution with a basic amino acid at this position results in constitutive activation of the receptor and no conserved basic residue was found that could serve as a potential counterpart for **Lys92** (Lu et al., 1998). Lu et al. (1998) proposed a model of constitutive activity of MC1R caused by mutations at **Glu92**, **Leu98**, **Asp119**, **Cys123** occurring by ligand mimicry. According to this model, the arginine residue at position 8 of α–MSH, known to be essential for high affinity ligand binding normally binds in the pocket formed by these residues, interacting electrostatically with **Asp119**. Replacement of **Glu92**, **Asp119** or **Cys123** with a basic residue thus mimics the effects of the ligand arginine on receptor confirmation. Interestingly, the **Asp121Asn** (analogous to the mouse **Asp119Asn**) change in the pig results in the dominant black allele of MC1R, although **Asn** belongs to the

class of uncharged polar side chain amino acids. The same change in the sheep is not sufficient for constitutive activity of the receptor without the **Met71Lys** mutation.

2.4 Agouti

The agouti locus was identified due its effect on the type and temporal deposition of eumelanin and pheomelanin. It was named after the South American rodent (*Dasyprocta aguti*), which shows the wild type pattern at this locus very clearly (Searle, 1968). Hairs in the wild type agouti coat are characterised by a sub-terminal or terminal yellow band, the rest of the hair shows black or brown eumelanin pigment (Figure 7). Thus, the agouti locus regulates a switch in the pigmentation system of the hair bulb between eumelanin to pheomelanin.

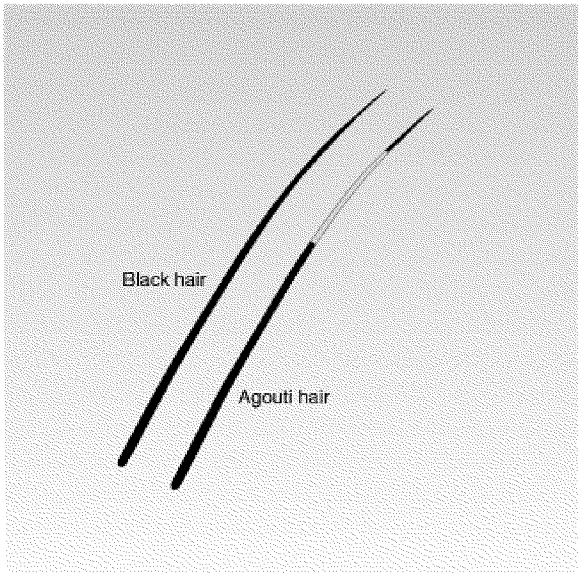


Figure 7: Black and agouti hair

Transplantation experiments in which melanocytes from one mouse were allowed to migrate into developing hair follicles of another mouse demonstrated that the banded hair pattern is not determined by the agouti genotype of the melanocytes, but instead by the genotype of surrounding cells (Silvers and Russell, 1955). The effects of agouti are limited to the hair bulb melanocytes, and melanocytes elsewhere in the body are not affected (Siracusa, 1994). Genetic analysis of numerous agouti locus mutants in mice led to the identification of at least 18 dominant and recessive alleles of the agouti gene (Silvers, 1979; Green, 1989). A complex dominance hierarchy exists in which alleles associated with pheomelanin production are generally dominant over alleles associated with eumelanin production (Silvers, 1979). This relationship can be exemplified by several agouti alleles:

A^y (lethal yellow) produces a yellow phenotype, obesity, diabetes and development of tumours in various tissues in the heterozygous form and is responsible for the embryonic death in the homozygous form

A^{vy} (viable yellow) produce a yellow phenotype, obesity, diabetes and development of tumours in various tissues

A^w (light-bellied agouti) gives rise to an agouti dorsum and cream-colour ventrum

a^t (black and tan) gives rise to an all black dorsum and an all yellow ventrum

a (non-agouti) produces a predominantly black phenotype, except for small amounts of pheomelanin around the pinnae, nipples and perineum

a^e (extreme non-agouti) produces a completely black phenotype

The extension locus is epistatic over agouti, which means, that allelic substitutions at the agouti locus do not affect the coat colour of animals that carry either loss-of-function or gain-of-function MC1R alleles (Wolff et al., 1978), which suggests that agouti may act by preventing α-MSH binding to MC1R (Jackson, 1993).

2.4.1 Isolation and characterisation of agouti in mice

Genetic mapping of the agouti locus on the murine chromosome 2 and characterisation of the chromosome rearrangements within this region associated with different agouti phenotypes allowed to perform positional cloning of the agouti gene (Bultman et al., 1992; Miller et al., 1993). Sequencing analysis of cosmid clones containing the agouti gene has revealed, that it is composed of four non-coding and three coding exons, with alternatively spliced 5' untranslated exons present in transcripts found in the dorsum and ventrum (Bultman et al., 1994; Siracusa 1994). Both types of transcripts result in mRNA fragments approximately 0.8 kb in size, the transcript I is expressed both in the dorsal and ventral skin and is hair cycle growth specific, and the transcript II is expressed only in the ventral skin (Figure 8).

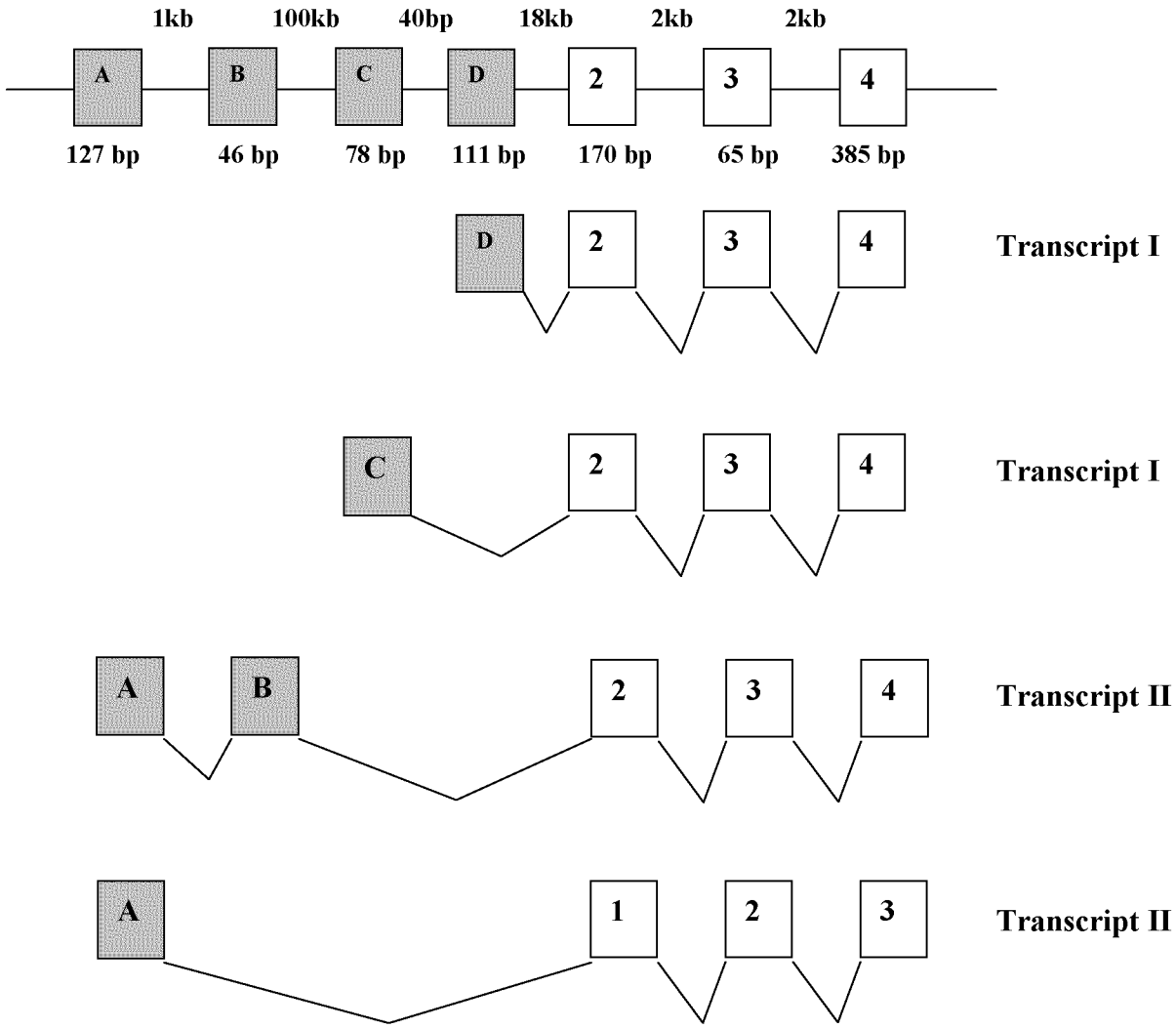


Figure 8: Genomic organisation and multiple forms of wild type $\mathbf{A^w}$ transcripts

Exons are shown in boxes and the numbers below the boxes indicate the length of the exons. Exons A, B, C and D are non-coding, whereas exons 2, 3 and 4 contain the protein-coding sequence. Numbers between the exons indicate the sizes of the introns. Two different forms are expressed in both ventrum and dorsum regions of neonatal skin during days 2-7 of embryonic development and are hair cycle specific (transcript I); and two other alternatively spliced forms are expressed continuously in the ventrum (transcript II) (according to Siracusa, 1994).

Exons 2, 3 and 4 encode for a 131 amino acid agouti signalling protein (ASIP). ASIP has an N-terminal domain with the characteristics of an eukaryotic signal sequence and a hydrophobic domain. The middle part has a large proportion of basic amino acids, and the C-terminal part is rich in cysteine residues (Miller et al., 1993).

2.4.2 Agouti expression

The normal pattern of agouti expression correlates with its function. The production and deposition of pheomelanin in the growing agouti hair occurs during days 3-7 of the neonatal development, which is in agreement with the maximal levels of ASIP expression during days 2-7 of the neonatal development. In $\mathbf{a^t}$ and $\mathbf{A^w}$ mice ASIP is highly expressed in the yellow-coloured hair of the ventrum, and expression is absent in the black-coloured hair of the dorsum of $\mathbf{a^t}$ mice and as well as in the dorsum and the ventrum of $\mathbf{a^e}$ mice; ASIP is expressed at very low level in the skin of **a** mice (Bultman et al., 1992; Miller et al., 1993). ASIP was also found to be expressed in the neonatal skin of $\mathbf{W/W^v}$ mice (which lack hair bulb melanocytes) (Bultman et al., 1992). This is consistent with the experimenrs of Silvers and Russell (1955) indicating that agouti is expressed within a follicular environment, and not within melanocytes. Expression of ASIP in the tissues of wild type adult animals was limited to testes, which were showing multiple transcripts ranging from 1.0 to 7.5 kb in size. A common feature of the dominant agouti mutations, which result in obesity and the diabetic condition, is an ectopic overexpression of size-altered ASIP RNA in most tissues, although the protein sequence is unaltered (Bultman et al., 1992; Siracusa, 1994).

Interestingly, RT-PCR with various tissues from humans has shown that ASIP is expressed in adipose tissue (Kwon et al., 1994) and the heart, ovary and testes, and at lower levels in liver, kidney and foreskin (Wilson et al., 1995).

2.4.3 Agouti mutations

Sequencing of the agouti locus has revealed that many agouti phenotypes, resulting in a reduced or ubiquitous ASIP expression are associated with rearrangements in the 5' region of the locus. The $\mathbf{A^y}$ mutation is the most dominant of all agouti alleles, and apart from yellow coat colour, obesity, diabetes and high susceptibility to development of tumours, it is also responsible for embryonic lethality in the homozygous form. This mutation occurs due to a deletion of a 5' upstream sequence, which removes the neighbouring Raly (ribonucleic protein associated with lethal yellow) gene coding sequence, resulting in ubiquitous expression of 1.1 kb agouti transcript (Miller et al., 1994; Siracusa et al., 1994). The altered transcript is spliced from 5'-UTR of the Raly gene to exon 2 (first coding exon) of the agouti gene, thus $\mathbf{A^y}$ mice express the same protein as wild type agouti mice, but as Raly encodes for ubiquitously expressed RNA-binding protein, the agouti gene with the promoter of Raly is also expressed

ubiquitously. The embryonic lethality in the homozygous $\mathbf{A^y}$ condition is due to the absence of the Raly expression (Duhl et al., 1994; Michaud et al., 1994). Like $\mathbf{A^y}$ the other agouti dominant alleles $\mathbf{A^{iy}}$ (intermediate yellow), $\mathbf{A^{iapy}}$ (intercisternal A-particle yellow), $\mathbf{A^{sy}}$ (sienna yellow) and $\mathbf{A^{vy}}$ (viable yellow) are ubiquitously expressed, but they have insertions of variable sizes in the 5' region (Michaud et al., 1994).

It was noticed, that the original non-agouti allele **a** reverts to two more dominant agouti alleles $\mathbf{a^t}$ (black and tan) and $\mathbf{A^w}$ (white-bellied agouti) with an exceptionally high frequency. Sequencing of genomic clones from 5' region of the agouti locus has shown, that the **a** allele contains an insertion of 11 kb in the promoter region, which consists of an entire 5.5 kb VL30 element (a retrovirus like structure, dispersed at ~100-200 copies in rodent genomes), plus an internal 5.5 kb of additional sequence flanked by direct terminal repeats of 526 bp. The $\mathbf{a^t}$ reverted allele has a 6 kb insertion, including the entire VL30 element and only one 526 repeat internally. The $\mathbf{A^w}$ reverted allele has a 0.6 kb insertion corresponding to a single intact VL30 long terminal repeat (LTR). It has been proposed that reversion from **a** to $\mathbf{a^t}$ occurs by excision of 5 kb of DNA (the sequence integrated into VL30), brought by a homologous recombination between 526 bp terminal repeats, and reversion from a to $\mathbf{A^w}$ occurs by excision of the entire insertion, except for one LTR, brought by homologous recombination between VL30 LTRs. Retrovirus-like elements, particularly intracisternal A-particles have been shown to affect the expression of numerous genes, even when integrated in the opposite transcriptional orientation (Kuff and Lueders, 1988). Therefore, it has been speculated, that insertion of the VL30 element along with the 5.5 kb additional sequence shuts off ASIP expression in the **a** mutant by negatively regulating both transcripts I and II. Additionally, the inserted segment in the $\mathbf{a^t}$ mutants (dark dorsum, yellow ventrum) down-regulates the production of transcript I, but does not significantly affect the production of transcript II, which is specifically produced in the ventrum. The remaining LTR repeat in the reverted $\mathbf{A^w}$ allele does not alter the expression pattern of both transcripts, and thus reverts to the wild type (agouti-banded dorsum and light yellow ventrum) (Bultman et al., 1994).

Isolation and characterisation of the human homologue of the mouse agouti gene revealed, that the sequences of these two proteins are moderately well conserved and that the biological properties of the human and mouse ASIP are similar (Wilson et al., 1995). It seems unlikely that variation at the agouti locus may account for any variation in red hair colour seen in human populations as there is no known analogous phenotype to the agouti banded hair pattern in humans. Therefore, there has been little interest in searching for polymorphisms at this locus that can be associated with the human pigmentary phenotypes. Nevertheless, some

polymorphism screens have been performed for the coding sequence of the human ASIP, but no functional variability has been identified to date. However the non-coding parts of the human agouti gene have not been investigated, leaving a possibility that a functional polymorphism exists in this area (Voisey et al., 2001).

2.4.4 Mechanisms of agouti signalling

2.4.4.1 Agouti and agouti related protein

Non-pigmentary effects of ectopic ASIP expression reflect the normal function of agouti-related protein (AGRP), which is normally expressed in the hypothalamus and the adrenal glands, and its size, sequence and biochemical activity is similar to agouti protein (Ollmann et al., 1997; Dinulescu and Cone, 2000). ASIP and AGRP share a signal sequence, a relatively conserved internal sequence (D46-S59) and a highly conserved cysteine-rich region from C105 to the C terminus (Virador et al., 2000). The cysteine-rich carboxyl domain of ASIP and AGRP contains a canonical pattern similar to the inhibitor cysteine knot (ICK) pattern, found in a family of secreted invertebrate toxins. The ICK is thought to serve as a scaffold that allows different toxin family members to antagonise a wide variety of molecular targets, usually calcium channels.

2.4.4.2 Agouti antagonism of melanocortin receptors

As there is no primary sequence similarity between ASIP or AGRP and melanocortin peptides, it was initially suggested that ASIP might antagonise the effects of melanocortin receptor signalling indirectly, possibly through the calcium channels (Conklin and Bourne, 1993; Zemel et al., 1995). However, recent studies show, that ASIP and AGRP bind directly to melanocortin receptors (Lu et al., 1994; Ollman et al., 1998; Yang et al., 1999). Genetic studies on mice based on interactions between *albino* (tyrosinase), agouti and extension loci mutations have also shown, that MC1R not only contributes, but is absolutely required for the pigmentary effects of ASIP (Barsh et al., 2000). Responsiveness to ASIP in melanocytes from mice expressing a functional or mutant MC1R gene also showed, that a functional MC1R is required for ASIP to exert its effects, as measured by decreased levels of tyrosinase, TYRP1 and TYRP2 expression (Abdel-Malek et al., 2001).

Although it is clear that ASIP inhibits MC1R function, there is no full agreement on the mechanism of ASIP and MC1R interaction. Initially it was proposed, that ASIP protein is a

competitive antagonist of MC1R, inhibiting α-MSH binding to MC1R (Lu et al., 1994; Willard et al., 1995). However, recent studies with mouse melanocytes showed, that ASIP-MC1R interactions can not be solely explained by inhibition of α-MSH binding to MC1R, as ASIP suppresses melanogenesis in absence of α-MSH (Graham 1997; Sakai et al., 1997). These observations reflect the dual role of ASIP as a competitive antagonist (Lu et al., 1994) and inverse agonist of MC1R (Siergist et al., 1997; Virador et al., 2000; Eberle et al., 2001). This is also in agreement with the recent determination of the 3-D structure of carboxyl terminal AGRP in solution, which reveals the three large loops of which two are structurally well characterised (Bolin et al., 1999). One loop of AGRP or ASIP, contains the Arg-Phe-Phe (116-118) sequence, which has chemical properties, resembling the core sequence (His-Phe-Arg-Trp) of melanocortin peptides. These may compete directly with the melanocortin peptides, while another loop reinforces or refines interaction of ASIP and AGRP with melanocortin receptors (Barsh et al., 2000).

Mouse ASIP have been shown to antagonise MC1R and MC4R, and to a lesser extent MC2R and MC3R. It does however not have any effect on MC5R (Voisey and van Daal, 2002). Yang et al. (1997) showed, that human ASIP antagonise all known melanocortin receptors, suggesting a wider function of human ASIP.

2.4.4.3 Structure - function relationship of agouti signalling protein

The mouse ASIP contains an N-terminal 22-amino acid signal peptide, which is processed on secretion, yielding agouti protein 23-131 (Willard et al., 1995). The 109-residue peptide can be subdivided into three regions (Figure 9):

1. An amino terminal, containing a hydrophobic sequence (40-50) and two glycosylation sites **His**^{23} and **Asn**^{39}
2. The middle basic region, containing 12 Lys/Arg
3. A cysteine-rich C-terminal region

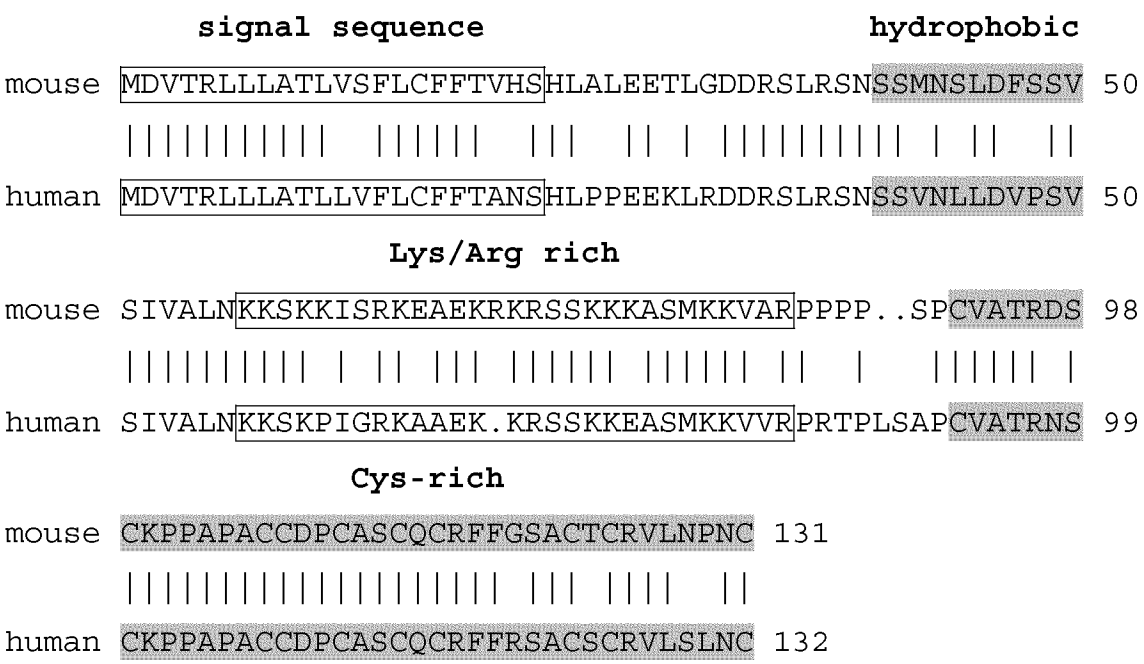


Figure 9: Amino acid sequences of the mouse and human ASIP

The C-terminal region (83-131) of ASIP has been shown to be as potent in antagonising melanocortin receptors as the full length protein (Willard et al., 1995). Alanine scanning mutagenesis indicates, that amino acids Arg-Phe-Phe (116-118) are critical to the affinity of ASIP to MC1R, MC3R and MC4R (Kiefer et al., 1998). Not surprisingly these three residues have chemical properties that resemble the core sequence (His-Phe-Arg-Trp) of melanocortin peptides (Kiefer et al., 1998).

Virador et al. (2000) have investigated the bio-activity of the small parts of the mouse agouti peptide and found that the KVARP (82-86) sequence in the Lys/Arg rich region is the minimal functional domain in this region that can elicit significant down-regulation of melanogenic enzyme expression and function. Helical wheel analysis of ASIP residues 80-88 suggest that residues **Lys81**, **Lys82** and **Arg85** would align on one side of the helical wheel and might thus be responsible for binding to an extracellular loop of MC1R through electrostatic interactions. Furthermore, residue **Arg85** is conserved between the mouse and human ASIP and AGRP, and was therefore suggested to be possibly involved directly in MC1R binding (Virador et al., 2000). However, Eberle et al. (2001) showed, that this part of ASIP is not required for the agouti effects on MC1R, and a part of ASIP from 91 to 131 residue is sufficient for the down-regulation of adenylyl cyclase activity and melanin production. In agreement with this, the 15-mer peptides from the Cys-rich region, all containing Arg-Phe-Phe motive were also able to down-regulate melanogenic enzyme expression and function (Virador et al., 2000). The other parts of the ASIP did not have any significant effect on melanogenesis. Interestingly, the

hydrophobic region between residues 40 and 50 is relatively conserved between ASIP and AGRP. However, this part of the protein has no significant effect on tyrosinase expression and activity. Thus, the hydrophobic sequence, which includes a predicted β-strand, may be relevant to the functioning of ASIP and AGRP, different from MC receptors signalling, for example binding to lipids or to membrane phospholipids (Virador et al., 2000).

Based on these results, Virador et al. (2000) proposed a model of ASIP and MC1R interaction (Figure 10). According to this model the agouti protein binds to two sites on MC1R, one site is extracellular loops 1 and 2, where it can replace α-MSH binding, and which is in agreement with AGRP binding to the extracellular loops of MC4R (Yang et al., 1999). The other site is different from that binding to α-MSH, it may be an N-terminal loop, which is consistent with the binding of larger peptides to G-protein coupled receptors (Ji et al., 1998). This model does not contradict the data of Eberle et al. (2001), showing that 91-131 is sufficient for down-regulating MC1R activity.

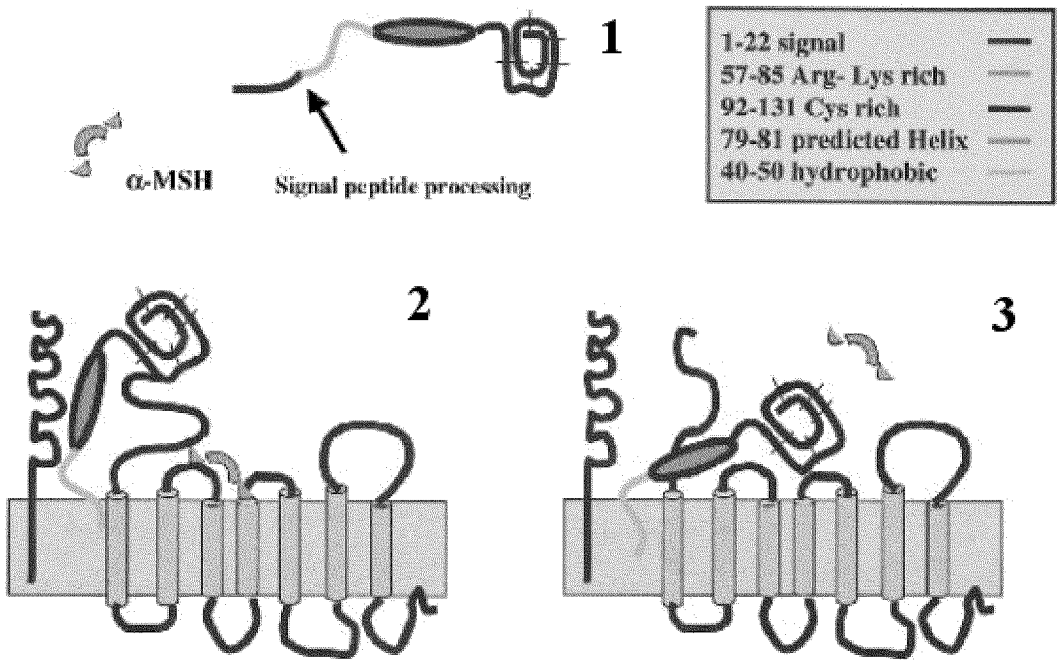


Figure 10: Schematic representation of ASIP and MC1R interactions (according to Virador et al., 2000)

(1) ASIP is generated *de novo* and then secreted, leaving behind its signal sequence and moving to the vicinity of melanocytes. (2) ASIP may interact at the melanocyte membrane with phospholipids and extracellular matrix components and/or the attractin receptor through hydrophobic and basic domains which would provide local concentration and

stability of ASIP. The ASIP epitope located in the predicted helix interact with the N-terminus of MC1R. (3) The C-terminus of ASIP may alternatively interact with extracellular loops 1 and 2 of the MC1R.

2.4.4.4 Agouti and obesity

One of the pleiotropic effects of the mouse agouti mutations is the obesity with the degree of weight increase being proportional to the intensity of the yellow coat colour (Argeson et al., 1996).

MC4R is expressed in the hypothalamus and plays an important role in the regulation of feeding and metabolism. MC4R knockout mice are obese but do not have a yellow coat colour (Voisey and van Daal, 2002). It seems likely that the ubiquitous expression of the agouti protein results in chronic antagonism of MC4R by disrupting its function. Thus the ubiquitous expression mimics the function of AGRP, a natural ligand of MC4R. Transgenic mice over-expressing AGRP develop obesity but not yellow coat colour, as AGRP does not antagonise MC1R (Ollmann et al., 1997). However, agouti antagonism of MC4R is unlikely to be solely responsible for the obesity phenotype as administration of a melanocortin agonist to mice over-expressing agouti does not reverse the obesity phenotype (Zemel et al., 1998), but only inhibits food intake (Fan et al., 1997). MC3R is also implicated in body weight regulation, as MC3R knockout mice have an increased fat mass, reduced lean mass and a greater feeding efficieny (Chen et al., 2000). In addition, knockout mice for both MC3R and MC4R gain significantly more weight compared with MC4R knockout mice (Voisey and van Daal, 2002).

Claycombe et al. (2000) demonstrated, that ASIP expression in the adipose tissue of transgenic mice up-regulates synthesis and secretion of leptin, a product of the obesity gene (ob), which regulates food intake and energy balance primarily by binding to hypothalamic leptin receptors (Halaas et al., 1995). In addition it exerts direct metabolic effects in adipose tissue itself (Seeley et al., 1997). Unlike the mouse agouti, human ASIP is normally expressed in adipose tissue, even in non-obese individuals (Kwon et al., 1994). This is in agreement with studies on transgenic mice which showed, that agouti expression in adipose tissue is not sufficient for the development of obesity and diabetes. However, treatment of transgenic mice expressing ASIP in adipose tissue with insulin, leads to a significant gain of weight (Mynatt et al., 1997). Thus, it can be speculated, that ASIP, when expressed ubiquitously in addition to elevating the leptin secretion from adipose tissue also causes the increased secretion of insulin from the pancreas. In this way ASIP and insulin have a synergistic effect on the body weight.

It was shown, that mouse adipocytes express high levels of MC2R (Boston and Cone, 1996). Hence, it is likely, that ASIP can act as an antagonist of MC2R, down-regulating ACTH-mediated lipolysis, in the same way as it does for MC1R in melanocytes and MC4R in hypothalamus.

Thus, there are at least two ways, how agouti signalling protein can cause obesity, one way is affecting feeding behaviour through MC4R and MC3R and another way is regulating the metabolism of adipocytes, including lipogenesis and lypolysis.

2.4.5 Agouti in cattle

Although various authors have discussed possible alleles of the agouti locus in cattle, that can be homologous to those found at this locus in other species (Lauvergene, 1966; Searle, 1968; Olson and Willham, 1982; Adalsteinsson et al., 1995), the agouti locus mutants in cattle are not completely understood. The most convincing results are from Adalsteinsson et al. (1995), who investigated the coat colour inheritance in black, brown and red Icelandic cattle and subsequently proposed the existence of at least two agouti alleles **a** and **A+.**

The **a** (non agouti) allele is associated with recessive black coat colour and modifies the **E+** allele of extension in a such way, that α-MSH stimulation is not antagonised by ASIP, and thus only eumelanin is produced. The existence of the **a** allele for recessive black is in agreement with genotyping black animals as **E+/E+** and **E+/e** (Klungland et al., 1995).

The **A+** is a wild type allele of agouti, which encodes for a functional agouti protein, which is capable of antagonising the MC1 receptor. This results in the production of both pigments eumelanin and pheomelanin, which produces a brown coat colour.

Interaction of extension and agouti can explain the changing coat colour from red to brown/black during the ageing from calf to adult cattle. There are cases, when the calves which are born red, change the colour to brown or black at the first hair coat change, either all over the body as in Holstein cattle (http://sask.usask.ca/~schmutz/colors.html) or on the extremities and lower parts of the body as in Icelandic cattle (Adalsteinsson et al., 1995). According to the model proposed by Adalsteinsson et al. (1995) for Icelandic cattle, the α–MSH binding to MC1R is antagonised completely by ASIP during fetal development and only pheomelanin is produced, and thus the calf is born red. During the first change of hair coat, the expression of ASIP is concentrated in a pattern, specific to restricted areas on the animal body.

The cattle agouti coding region was sequenced and it was reported to be expressed in the kidney (accession numbers X99691 and X99692), indicating an expression pattern similar to that in humans. No polymorphism at this locus in cattle has been reported so far.

2.4.6 Agouti in other species

Phenotype of some dog breeds, such as Rottweilers, Airedales, Gordon setters and German Shepherd, very much resembles the black and tan agouti allele of mise, however the agouti gene have not yet been cloned in the dog. The complete genomic structure of the agouti locus was established for the pig, but no functional polymorphism was found (Leeb et al., 2000). The white-woolled phenotype of modern sheep breeds is considered to have a pheomelanin background from the dominant white/tan ($\mathbf{A^{wt}}$) agouti allele (Adalsteinsson et al., 1987), but no causative mutations were found in the coding sequence of the agouti gene (Parsons et al., 1999), indicating that they might be located in non-coding regions as in mice. A deletion of 11 bp in the second coding exon of the agouti gene resulting in a frameshift of ORF was found to be associated with recessive black coat colour in the horse (Rieder et al., 2001). The Standard Silver fox has dark pigmented fur and is phenotypically identical to the Alaska Silver Fox, which carries a constitutively active allele of MC1R. The dark coat colour in the Standard Silver fox is due to the recessive allele of agouti (**a**), missing the first coding exon, which is likely to result in the absence of agouti expression (Vage et al., 1997). Interestingly, the heterozygosity for the dominant $\mathbf{E^A}$ allele in fox is not sufficient to override inhibition of eumelanin production by the wild type allele of agouti (**A**). Thus foxes genotyped as $\mathbf{E^AE+}$, **Aa** produce significant red pigment around the flanks, midsection and neck. These results indicate that interaction between MC1R and agouti in the fox is distinct from the epistatic interaction seen in mice. Two hypothesis are proposed to explain this finding, the first hypothesis suggests that the ASIP of the fox is capable of down-regulating activity of a constitutive active MC1R, and the second hypothesis invokes another target for agouti action on the melanocytes in addition to MC1R (Vage et al., 1997).

2.5 Attractin (Mahogany)

The mahogany mutation was recognised approximately 40 years ago as a modifier of pigment type switching whose effects were similar to that caused by loss-of-function agouti mutations or gain-of-function MC1R mutations (Lane and Green, 1960). Three phenotypically distinct mahogany alleles *mg*, mg^{3j} and mg^{L} have been characterised so far. The mg^{3j} allele has the

darkest pigmentation phenotype with no visible yellow pigment in coat hairs of homozygous animals. The *mg* is the next strongest mutation, which produces homozygous animals with dark backs, but some yellow pigment in their flank and ventral hairs. Mice homozygous for the mg^{L} allele have the weakest phenotype: they appear similar to *mg* homozygous mice but with a narrower dorsal stripe of black and more visible yellow pigment. As might be expected for an additive interaction, the mg^{3j}/mg compound heterozygotes are darker than mg^{3j}/mg^{L}, which are in turn darker, than mg/mg^{L} mice (Gunn et al., 1999). The observation, that mice homozygous for the *mahogany* mutated allele as well as for the recessive yellow allele of MC1R (**e**) are yellow like homozygotes for the **e** allele of MC1R in conjunction with the ability of mutants for *mahogany* to suppress the yellow pigmentation and obesity, caused by dominant alleles of agouti indicates that the *mahogany* gene acts upstream of MC1R and downstream of agouti (Nagle et al., 1999).

2.5.1 Characterisation of the mahogany gene

Gunn et al. (1999) and Nagle et al. (1999) independently and simultaneously cloned the mouse mahogany gene, using a positional cloning strategy. The mouse mahogany locus was found to span over 160 kb and 30 exons were identified (Nagle et al., 1999). The mRNA of the mahogany gene is approximately 9 kb and encodes a predicted 1.428 amino acid type I transmembrane protein (Gunn et al., 1999). The extracellular domain of the predicted protein is 93% identical to attractin, a human serum protein glycoprotein secreted from activated T cells that has been implicated in monocyte spreading and T-cell clustering, and is encoded by a 4 kb cDNA (Duke-Cohan et al., 1998). Northern hybridisation with RNA from different human tissues using a probe corresponding to the cDNA encoding for the N-terminal part of the mahogany gene revealed two main RNA isoforms of 8.5 and 4 kb. The 8.5 kb form is likely to encode the transmembrane form of human attractin because it was also detected with a probe from the C-terminal part of mahogan*y* gene (Gunn et al., 1999). Subsequently it was shown that human attractin contains an insertion of a LINE-1 (long interspersed nuclear element-1) in the exon upstream the sequence coding for the transmembrane domain, which contains a stop codon and a polyadenylation signal. Thus the mRNA for soluble attractin does not contain exons encoding for transmembrane and cytoplasmic domains, and the mRNA isoform for the membrane attractin splices over the LINE-1 exon and includes five exons encoding for transmembrane and cytoplasmic domains identical to those in mouse gene (Tang et al., 2000). Due to this homology between mouse mahogany and human attractin, the mouse

gene was renamed to attractin (*Atrn*), and mahogany mutations were subsequently renamed to $Atrn^{mg\text{-}3j}$, $Atrn^{mg}$ and $Atrn^{mg\text{-}L}$ (Barsh et al., 2000).

The extracellular part of the predicted mouse attractin protein as well as the human attractin contain two epidermal growth factor (EGF) domains flanking a CUB domain near the N-terminal, a C-type lectin domain in the central part and two laminin-type EGF domains close to the predicted transmembrane domain of mouse attractin (Figure 11). Mouse and human attractin also share EGF, CUB and laminin-type EGF domains homology with the hypothetical *Caenorhabditis elegans* perlecan-like protein F333C8.1 (Gunn et al., 1999).

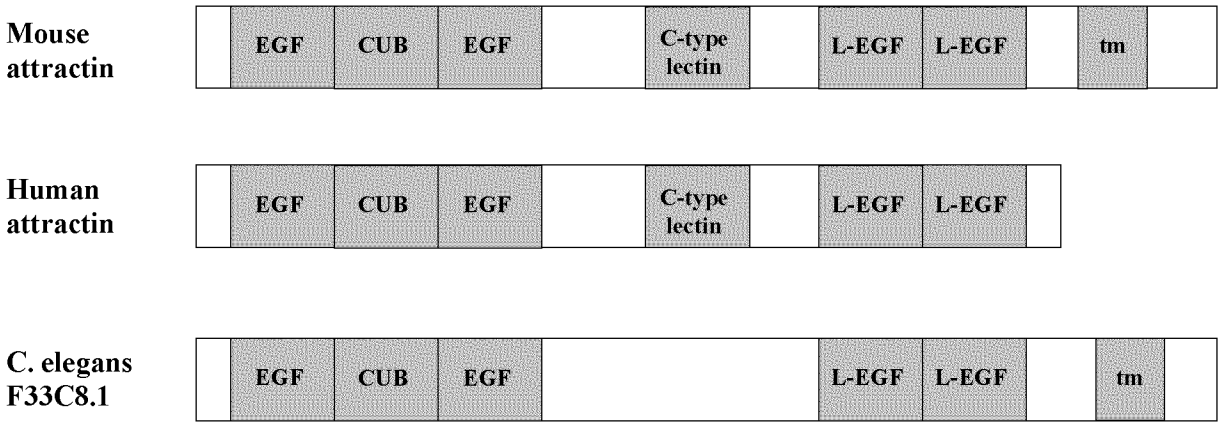


Figure 11: Domain structure of mouse attractin, human soluble attractin and hypothetical *C.elegans* protein F33C8.1 (according to Gunn et al., 1999)

Multiple EGF domains are commonly found in type I transmembrane proteins (membrane proteins with their N-terminal in the cytosol) that are involved in cell adhesion and receptor-ligand interactions. Laminin-type EGF modules are found in several proteoglycans such as perlecan and heparin sulphate proteoglycan. As CUB domains are frequently found in glycosylated proteins and C-type lectins bind carbohydrate, attractin protein is predicted to be heavily glycosylated and carbohydrate interactions are believed to be essential for the functioning of attractin (Nagle et al., 1999). The cytoplasmic tail of attractin is short (126 amino acids) and has no previously defined signalling domain. However, it contains an 8 amino acid stretch, which is conserved in proteins from mouse and human paralogue sequences and the *C.elegans* homologue sequence, indicating a functional significance of this 8 amino acid stretch (Nagle et al., 1999).

2.5.1.1 Expression of attractin

Attracin RNA is widely, but not ubiquitously expressed and is found at high levels in melanocytes and other components of the skin, as well as in brain, heart, kidney, liver and

lung, but not in the uterus, muscle or spleen (Duke-Cohan et al., 1988; Gunn et al., 1999; Nagle et al., 1999).

2.5.1.2 Attractin mutations

The severity of pigmentation defects in mice carrying different *Atrn* alleles is consistent with the changes observed at expression levels of mutated alleles. According to the Northern blot hybridisation $Atrn^{mg\text{-}3j}$, mutant animals produce no detectable transcript, while $Atrn^{mg}$ and $Atrn^{mg\text{-}L}$ mutants make a small amount of normal-sized transcript as well as more abundant aberrant isoforms (Gunn et al., 1999). Nagle et al. (1999) found that the mutation in $Atrn^{mg\text{-}3j}$ mice has a 5 bp deletion near the end of exon 16 that results in frameshift of the ORF. Therefoe the first 914 codons are unchanged but codon 915 is changed from serine to a leucine and a premature stop codon is introduced at position 916. This mutation presumably results in an instability of the transcript due to the nonsense-mediated decay of RNA (Gunn et al., 2001). Both $Atrn^{mg}$ and $Atrn^{mg\text{-}L}$ mutations contained insertions of an IAP element approximately 5 kb in size, which lie in adjacent introns and downstream exons encoding for the transmembrane domain (Gunn et al., 1999). In the $Atrn^{mg\text{-}L}$ animals the IAP element is inserted into intron 27 in a 5' to 3' orientation, while the IAP element in the $Atrn^{mg}$ animals is inserted in intron 26 in reverse orientation (Gunn et al., 2001).

Western blot analysis using a polyclonal antisera against a part of mouse *Atrn* showed that a normal ~210 kD protein in extracts of the brain of non-mutant mice and no protein expression in brain of $Atrn^{mg\text{-}3j}$ mutant animals. Extracts from the brain of $Atrn^{mg\text{-}L}$ animals show slightly reduced levels of the normal-sized protein, and brain extracts from $Atrn^{mg}$ mice show an aberrant protein reduced by approximately 20 kD in size (Gunn et al., 2001).

2.5.2 Function of attractin

Expression of the transgene attractin under driving of the promoters specific to different cell types in the mice homozygous for $Atrn^{mg\text{-}3j}$ mutation have shown, that expression of *Atrn* in melanocytes is sufficient to restore the ability of those melanocytes to respond to ASIP (He et al., 2000). Thus it can be anticipated, that *Atrn* acts as a receptor for ASIP, which facilitates ASIP binding to MC1R. Subsequently He et al. (2000) have confirmed this hypothesis by showing ASIP and *Atrn* binding using the surface plasmon resonance method to measure real time interaction of the two proteins. Interestingly, the N-terminal fragment of ASIP (His23-Arg85) showed a binding pattern similar to the full length protein, whereas the

C-terminal part (Ser73-Cys131) did not bind to *Atrn*. The strength of the *Atrn*-ASIP interactions indicates that *Atrn* acts as an accessory or low-affinity receptor (He et al., 2000). However, *Atrn* was not able to either bind to AGRP, or suppress obesity induced by AGRP overexpression, which is in agreement with observation that mutations in *Atrn* gene suppresses A^y induced obesity, but not hyperphagy (He et al., 2000). This indicates that the mechanism of the *Atrn* mutations suppression of obesity is distinct from the MC4 signalling pathway.

To investigate the role of *Atrn* in the body weight regulation Gunn et al. (2001) have examined the effects of each *Atrn* allele on body weight, body fat content and locomotor activity. Mice homozygous for $Atrn^{mg\text{-}3j}$ and $Atrn^{mg}$ have a 10-15% body weight and 20-40% body fat content reduction when compared to their non mutant litter-mates at 3 months of age. The locomotor activity was elevated by 20-30% in $Atrn^{mg\text{-}3j}$ mutants and by 55-70% in $Atrn^{mg}$ mutant mice. $Atrn^{mg\text{-}L}$ has no detectable effect on body weight, fat content and locomotor activity.

He et al. (2001) have carried out a histopathological survey of $Atrn^{mg\text{-}3j}/Atrn^{mg\text{-}3j}$ mice to search for microscopic lesions, which could provide additional clues to the functioning of *Atrn*. Comparison of normal and mutant mice revealed that mutant mice have apart from a reduced fat storage, countless vacuoles, or so-called "spongy" degeneration in the brain and spinal cord. The most consistently and severely affected were the brainstem, cerebellar medulla, granular layer of the cerebellum, pons, thalamus, hippocampus, caudate and putamen, somatosensory cortex and spinal cord grey matter. Gunn et al. (2001) compared the severity and timing of spongy degeneration in *Atrn* mutant mice. The $Atrn^{mg\text{-}3j}$ mice were the most severely affected with vacuoles found at 1 month of age and increasing with age. In $Atrn^{mg}$ mutants the vacuoles appeared between 2 and 4 months of age, were quantitatively less severe. Vacuoles were not visible in the $Atrn^{mg\text{-}L}$ mutants by 4 months of age, but mild vacuolation was apparent in 8 month old animals. These data taken together with reduced body weight, reduced body fat content and increased locomotor activity in $Atrn^{mg\text{-}3j}$ and $Atrn^{mg}$ mice is consistent with the *Atrn* expression pattern in the brain, when $Atrn^{mg\text{-}3j}$ does not show any expression, $Atrn^{mg}$ expresses an aberrant protein and $Attrn^{mg\text{-}L}$ show reduced levels of normal sized protein.

Histopathological abnormalities similar to those seen in $Atrn^{mg\text{-}3j}$ mice were observed in *zitter* rats ($Atrn^{zi}$) and black tremor hamsters ($Atrn^{bt}$), which both carry loss-of-function mutations in the attractin gene (Kuramoto et al., 2001; Kuramoto et al., 2002). $Atrn^{mg\text{-}3j}$ in mice, $Atrn^{zi}$ in rats and $Atrn^{bt}$ in hamsters were also shown to cause body tremor, which is probably the consequence of vacuole formation in the central nervous system (Kuramoto, 2001; Kuramoto 2002).

A role of attractin as an accessory receptor specific for ASIP does not explain either the spongy degeneration, reduced body weight, increased locomotor activity observed in mahogany mice, or the widespread pattern of attractin expression outside the skin. These observations suggest, that *Atrn* carries out one or more function that are unrelated to agouti or melanocortin signalling (Gunn et al., 2001; He et al., 2001). A wider function of *Atrn* is also supported by its evolutionary conservation. Melanin polymers composed of aromatic acid derivatives are apparent throughout the animal kingdom, but the pheomelanin and agouti-melanocortin system are found only in vertebrates (Gunn et al., 2001), except for the cysteine spacing pattern characteristic for ASIP and AGRP which is also found in several invertebrate toxins (Barsh et al., 2001). However, *Atrn* homologues are found in *C.elegans* (Gunn et al., 1999) and *Drosophila* (He et al., 2001), indicating that attractin family proteins in flies, worms and mammals carry out a homologous function that is unrelated to their action as specific ASIP receptor. Thus it is proposed that the major function of *Atrn* is maintenance or stabilisation of certain classes of cell-cell interactions and the absence of this stabilising function causes the vacuolation in the central nervous system. This subsequently causes tremor, increased locomotor activity and reduced body fat content (Gunn et al., 2001). It is notable, that the soluble form of attractin is not necessarily required for normal development, as mouse and hamster have only membrane form of attractin (Gunn et al., 1999; Nagle et al., 1999; Kuramoto et al., 2002), whereas the human and rat have both membrane and soluble forms (Tang et al., 1999; Kuramoto et al., 2001).

Gunn et al. (2001) also speculate, that *Atrn* has mild effects on pigmentation that are independent of agouti-induced pigment type switching, as *Atrn* mutant mice acquire a dark reddish appearance when they age and $Atrn^{mg\text{-}3j}$ mutant animals have a 20-30% reduction of eumelanin content between 5 and 11 months of age.

2.5.3 Attractin in cattle

A part of bovine *Atrn* with a size of approximately 980 bp spanning from exon 5 to 3' UTR was amplified using cross-species primers, sequenced (accession number AF194962) and a MspI digestion polymorphism was detected within this fragment. Subsequently *Atrn* was genetically mapped to bovine chromosome 13 (Edeal et al., 2000).

3 Materials and methods

3.1 Animals

Typical colour variants were used in this study: Simmental (red, Figure 1a), Red Holstein (red, Figure 1b), Holstein (black, Figure 1c), Brown Swiss (brown/grey, Figure 1d, 1e, 1f) and Red Holstein x Holstein cattle (black). Genomic DNA was isolated from blood or semen samples of living animals. Samples for RNA extraction were taken immediately after slaughter of the animals at the slaughter-house (Zurich). RNA was extracted from liver and/or testes.

3.2 DNA methods

3.2.1 Genomic DNA extraction

3.2.1.1 Genomic DNA extraction from blood

Frozen blood was thawed at 37°C. 5-10 ml of blood was washed with NE buffer. The blood sample was filled up to 30-40 ml with NE buffer, gently mixed and centrifuged at 2,000 xg for 10 min at 4°C and the supernatant was discarded. This step was repeated 2-3 times. The pellet was resuspended in 5 ml TNE buffer. RNA was digested by incubating the sample with 50 µl of RNase (10 mg/ml) for 30 min at 37°C. Proteins were digested by incubating the sample with 250 µl of 10% SDS and 40 µl of proteinase K at 50°C overnight. Subsequently a Phase Lock Gel (PLG) cartridge was placed at the top of a 15 ml centrifuge tube and centrifuged at 1,500 xg for 5 min to move the PLG to the bottom of the tube. The tube was filled with 6 ml of phenol-chloroform-isoamyl alcohol (approximately pH 7.8) and the sample was shaken vigorously and centrifuged at 1,500 xg for 5 min. Centrifugation caused the PLG to move to the inter phase and isolated the upper, aqueous phase containing the DNA from the lower phase, containing the phenol-chloroform. The upper phase was carefully poured into a new tube and the DNA was precipitated with 2.5 volumes of absolute ethanol. The precipitated DNA formed a flock pellet and was transferred to a new tube with a sterile glass hook. After

washing twice with 70% ethanol, the DNA was dried at room temperature and resuspended in 0.5 ml TE pH 8.0 at 60°C for 30 min.

3.2.1.2 Genomic DNA extraction from semen

The sperm cells from the straws (containing approximately 15 million cells) were washed with 1 ml of PBS, vortexed and centrifuged for 5 min at 2,500 xg. The pellet was resuspended in 1 ml of PBS and centrifuged for 5 min at 2,500 xg. The supernatant was removed and the pellet was dried for 10-20 min at room temperature. The pellet was resuspended in a mixture of 475 µl Sperm cell lysis buffer, prewarmed at 60°C, 25 µl of 1 mM Dithiothretol and 10 µl of proteinase K (20 mg/ml). The sample was incubated for 12 hours in a water bath at 65°C. Afterwards 160 µl of saturated NaCl solution were added to the sample, vortexed for 2 min and centrifuged for 10 min at 15,000 xg. 400 ml of the supernatant were removed to a tube with 1 ml of ice cold absolute ethanol. The sample was left on ice for 10 min, and centrifuged for 10 min at 15,000 xg. After removing the supernatant the DNA pellet washed with 200 µl of 70% ethanol, and dried at room temperature for 30 min. 100 µl of TE pH 8.0 were added to the pellet, and the sample was then incubated at 65°C in a water bath to dissolve the DNA. 1 µl of DNA solution was used for PCR.

3.2.2 DNA quantification using spectrophotometer

The concentration of the nucleic acids was determined by measuring the light absorption of the DNA in aqueous solution against blank at the wavelength of 260 nm using a spectrophotometer (Lambda Bio UV/Vis Perkin Elmer). The absorption of 1 OD (A) is equivalent to approximately 50 µg/ml (extinction coefficient) double stranded DNA. Thus the DNA concentration in a sample was calculated according to the formula:

$$c\ (\mu g/\mu l) = (OD_{260} \times df \times ec)/1000$$

where *OD260* is the optical density at 260 nm, *df* is the dilution factor and *ec* is the extinction coefficient, equal to 50 µg/ml. The ratio A260/A280 is used to estimate the purity of the nucleic acids, since proteins are absorbed at 280 nm. Pure DNA has a ratio of approximately 1.8.

3.2.3 Polymerase Chain Reaction (PCR)

3.2.3.1 Thermostable DNA polymerases

DNA amplification was performed with two types of thermostable DNA synthesis systems: Taq DNA polymerase and the Expand™ High Fidelity PCR System, which is composed of an enzyme mix containing Taq DNA and Pwo DNA polymerase. Due to the 3'-5' exonuclease proof-reading activity of Pwo DNA polymerase, the Expand™ High Fidelity PCR System results in a 3-fold increased fidelity of DNA synthesis (8.5×10^{-6} error rate when compared to Taq DNA polymerase, which has an error rate of 2.6×10^{-5}). The Expand™ High Fidelity PCR System was used when the high fidelity of the amplification was desired for subsequent cloning and/or sequencing of the PCR products. Taq DNA polymerase generates the products with a single 3'- A base overhang, while the products of the Pwo DNA polymerase are blunt.

3.2.3.2 PCR conditions

PCR was performed in a final volume of 25 µl, in a mixture containing 50-500 ng of genomic DNA, 10 pmoles of each primer (forward and reverse), each dNTP at a concentration of 0.2 mM, 2.5 µl of 10x buffer, which was supplied together with the DNA polymerase, and 2.5 units of Taq DNA polymerase or 1.75 units of Expand™ High Fidelity PCR System enzyme mix.

The amplification profile consisted of a pre-denaturation step at 95°C for 5 min, 35-40 cycles with 30 sec denaturation at 95°C, 30 sec annealing at primer specific temperatures (50-64°C) and elongation at 72°C. The duration of elongation depended on the size of the expected PCR-fragment (30 sec for fragments smaller then 1000 bp, 1 min for 1000 bp, increasing the time for 1 min for each 1000 bp). The amplification was completed with a final elongation at 72°C for 7 min.

3.2.3.3 Primer design

Primers were designed 18-30 nucleotides in length, with GC content of 40-60% and if possible with a G or C at the 3' end of the primer. Care was taken, that the primers did not contain repeats and palindromes and were not complementary to the other primers in the PCR mix. Melting temperature (T_m) was estimated according to the formula:

$$T_m = 2°C \times (\text{number } A+T \text{ residues}) + 4°C \times (\text{number } G+C \text{ residues})$$

Primer pairs were designed with similar melting temperatures, differing no more than 4°C. The annealing temperature applied was usually 10°C less than T_m. The annealing temperature was adjusted experimentally, if it was not optimal. When the whole coding sequence of a gene was amplified for subsequent subcloning into mammalian expression vectors, the forward primer contained a start codon and an A or a G in position -3, because it is important for proper initiation of translation (Kozak, 1987).

3.2.4 Agarose gel electrophoresis

DNA loading dye was added to a concentration 1/6 of the final loading volume of the DNA sample. The sample was loaded to a 1% agarose gel and run in a 1x TBE buffer at 70 V for 1.5 hours. A 100 base-pair ladder and KiloBase™ DNA marker were used as size references for the DNA fragments smaller and bigger then 1000 bp respectively. DNA was stained by adding ethidium bromide at a concentration of 0.1 µg/ml to the melted agarose and visualised in a UV-light trans-iluminator. The gels were photographed using a Polaroid camera.

3.2.5 DNA extraction from the agarose gel

Specific products were cut out of the gel and extracted with the QIAEX II Agarose Gel Extraction kit.

3.2.6 Screening of the BAC library

A bovine genomic DNA BAC library was screened by PCR using the gene (bovine Attractin) specific primers Mgex3F1 and Mgex3R1 (Table 10) by the Joint Research Unit for Radiobiology and Genomics (CEA, INRA).

3.2.7 BAC DNA extraction

A piece of agar containing a BAC colony was put into 5 ml of LB medium containing chloramphenicol at a concentration of 12.5 µg/ml and incubated overday (6-8 hours) in a shaker at 37°C at 250 rpm. 1 ml of the overday bacterial culture was transferred into 500 ml of

LB medium with 12.5 µg/ml chloramphenicol and grown overnight (12-16 hours) at 37°C at 300 rpm. The bacterial cells were harvested by centrifugation at 6,000 xg for 15 min at 4°C. A Qiagen Plasmid Maxi kit was used to extract the BAC DNA. The Qiagen plasmid purification protocol is based on a modified alkaline lysis procedure, followed by binding of BAC DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins and low-molecular weight impurities were removed by washing in medium-salt buffer. BAC DNA was eluted in a high-salt buffer and desalted by isopropanol precipitation. Qiagen anion-exchange resin is packed as Qiagen-tip (column) which is operated by gravity flow.

The bacterial pellet was gently resuspended in 10 ml of buffer P1, containing RNase A at a concentration of 100 µg/ml. 10 ml of buffer P2 were added to the cells, mixed gently by inverting 4-6 times and incubated at room temperature for 5 min to lyse the bacteria. 10 ml of ice cold buffer P3 were added to the lysed cells, immediately mixed and incubated on ice for 20 min to precipitate genomic DNA, proteins, cell debris and SDS. The cell lysate was centrifuged at 20,000 xg for 30 min at 4°C. The clear supernatant was transferred to a new centrifuge tube and again centrifuged at 20,000 xg for 15 min at 4°C to remove the traces of the precipitate. During the second centrifugation step the Qiagen-tip 500 was equilibrated by applying 10 ml of buffer QBT and allowing the column to empty by gravity flow. The obtained supernatant, containing BAC DNA was applied to the Qiagen-tip 500 and allowed to enter the resin by gravity flow. The Qiagen tip, containing BAC DNA was washed 2 times with 30 ml of buffer QC. The DNA was eluted with 15 ml of buffer QF prewarmed at 50°C. 0.7 volumes of room temperature isopropanol were added to the BAC DNA, mixed and immediately centrifuged at 15,000 xg for 30 min at 4°C. The supernatant was carefully decanted, the pellet washed with room temperature 70% ethanol and centrifuged at 15,000 xg for 10 min. The supernatant was removed and the pellet was air-dried for 10-15 min. The DNA was resuspended in 500 µl of 10 mM tris HCl pH 8.0. The concentration of the DNA was determined by UV spectrophotometry (chapter 3.2.2) and loading to an agarose gel. If required DNA was concentrated by precipitating with 1 volume of isopropanol and 0.1 volume of 3 M sodium acetate pH 5.0. BAC clones were cryopreserved as described in chapter 3.4.1.7.

3.3 RNA Methods

3.3.1 General precautions working with RNA

Certain precautions were taken when handling RNA, because of the ubiquity of ribonuclease (RNase) activity in the surrounding environment. RNases are a family of enzymes that degrade RNA molecules through both endonucleotic and exonucleotic activity. RNases have minimal cofactor requirements and are active over a wide range of pH. RNases of the pancreatic variety (RNases A) are particularly resistant, as these enzymes can renature quickly, following treatment with most denaturants, even after boiling. Therefore both equipment and reagents were purged of RNases before the onset of the experiment. All work with RNA was performed wearing gloves, as the oils from the finger prints are rich in RNase activity. All plastic-ware was for single use and sterile, glassware was autoclaved and the electrophoresis chamber was treated with 0.5% SDS (non-specific nuclease inhibitor) before use. All solutions were prepared using diethyl pyrocarbonate (DEPC, a non-specific RNase inhibitor) treated water. The RLT buffer used for cell lysis contained guanidine isothiocynate (GITC) which is a strong protein denaturant. RNasin was used in the reaction of reverse transcription. It is a protein purified from the human placenta, which inactivates RNases A, B and C by covalent binding and is compatible with the most biochemical reactions.

3.3.2 Total RNA extraction from tissue samples

RNA was extracted from testes and liver samples using the RNeasy kit. The tissue samples were taken from animals immediately after slaughtering, cut into small pieces and were immediately frozen in liquid nitrogen and later stored at -80°C. The tissues can be stored for several months at -80°C. Total RNA was isolated from 0.6 mg of the tissue. Frozen tissue was ground with a mortar and pestle in liquid nitrogen. The obtained powder was transferred into a 50 ml falcon tube and homogenised with a blender in 15 ml of buffer RLT, containing 0.143 M β-mercaptoethanol for at least 60 sec at maximum speed, until the sample was uniformly homogeneous. The blender was cleaned before starting homogenisation and between the samples by dipping and rotating once in bidest water, once in 1 N NaOH, three

times in bidest water and finally once in RLT. The homogenised tissue lysates in buffer RLT can be stored at -80°C for several months. Tissue lysate was centrifuged at 3,000 xg for 10 min at room temperature (all subsequent centrifuging steps were also performed at room temperature). The supernatant was carefully transferred to a new 50 ml centrifuge tube. 1 volume of 70% ethanol was added to the supernatant, the samples were mixed by vigorous shaking and put into an RNeasy maxi spin column placed in a 50 ml falcon tube. The column was centrifuged for 5 min at 3,000 xg and the flow-through was discarded. 15 ml of buffer RW1 were added to the RNeasy column. The column was centrifuged for 5 min at 3,000 xg to wash the column, and the flow-through was again discarded. 10 ml of buffer RPE were added to the column. The column was centrifuged for 2 min at 3,000 xg to wash the column and the flow-through was discarded. Another 10 ml of buffer RPE were added to the column and it was centrifuged for 10 min at 3,000 xg to dry spin the column membrane. The RNeasy column was transferred into a new 50 ml falcon tube and 0.8 ml of RNase-free water were pipeted onto the spin-column membrane. The column was allowed to stand for 1 min at room temperature and then centrifuged for 3 min at 3,000 xg. The flow-through, containing the RNA was stored at -80°C.

3.3.3 Total RNA extraction from cultured animal cells

Total RNA was extracted from confluent cells grown in five tissue culture dishes [100 x 20 mm style (Falcon ®), approximately 3-4 x 10^7 cells] using the RNeasy kit. The culture medium was removed and the cells washed with PBS. After placing the dishes on ice 1.5 ml of the buffer RLT, containing 0.143 M β-mercaptoethanol were added to each dish and the cells were detached by scraping. The cell lysate was collected in a 50 ml falcon tube and homogenised using a blender for at least 45 sec at maximum speed until the sample was uniformly homogeneous. The blender was cleaned as described in the previous chapter. 1 volume of 70% ethanol was added to the homogenised lysate and it was mixed by vigorous shaking. The sample was applied to an RNeasy maxi spin column placed in a 50 ml centrifuge tube and centrifuged at 3,000 xg for 5 min at room temperature (all subsequent centrifuging steps were also performed at room temperature), and the flow-through was discarded. 7.5 ml of buffer RW1 were pipetted into the spin column, and it was centrifuged for 5 min at 3,000 xg to wash the column. 30 µl of DNase I stock solution were mixed with 210 µl of buffer RDD. The DNase I incubation mix was pipetted directly onto the spin-column

membrane, and left at room temperature for 15 min. 7.5 ml of buffer RW1 were pipetted into the spin column, and the mixture was left at room temperature for 5 min and then centrifuged for 5 min at 3,000 xg and the flow-through discarded. 10 ml of the buffer RPE were added to the RNeasy column and it was centrifuged for 2 min at 3,000 xg to wash the column and the flow-through was discarded. Another 10 ml of buffer RPE were added to the RNeasy column, and it was centrifuged for 10 min at 3,000 xg to dry spin the column membrane. The RNeasy column was transferred to a new 50 ml collection tube. 0.8 ml of RNase-free water were pipetted directly onto the spin-column membrane, and left at room temperature for 1 min, and then centrifuged for 3 min at 3,000 xg. The flow-through, containing the RNA was collected and stored at -80°C.

3.3.4 RNA quantification using spectrophotometer

The RNA concentration was measured like the DNA concentration (see chapter 3.2.2). The extinction coefficient for RNA is 40 µg/ml for RNA and the A_{260}/A_{280} ratio is 2.

3.3.5 Electrophoresis of RNA

A mixture consisting of 1.5 µl of 10x MOPS, 2.5 µl of formaldehyde and 7.5 µl of formamide were added to 2.5 µg of RNA in final volume of 3.5 µl of DEPC H_2O. The RNA was heated at 55-60°C for 10-15 min to denature any secondary structures present in the RNA. 2 µl of RNA-loading dye were added and the mixture was loaded to a 1.25% agarose gel in 0.66 M formaldehyde, 1x MOPS. The gel was run in 1x MOPS at 40-60 V for 3-4 hours until the bromphenol blue had reached 2/3 of the way down. The gel was stained in methylen blue solution for 15-20 min, and then washed several times in tap water. Two bands of 18S and 28S RNA were visible after washing. In complete and intact RNA the 28S RNA band is twice as intense as the 18S band.

3.3.6 Reverse transcription

The reverse transcription reaction was performed in a final volume of 25 µl. 2.5 µg of total RNA and 100 pmoles of either the TVX (Table 7) or a specific reverse primer were made up

to 10 µl with DEPC H_2O and heated at 70°C for 5 min. The mixture was then cooled down to 25°C in 10 min to anneal the primer to the RNA, followed by 2 min at 25°C. During the cooling steps the following reagents were mixed: 5 µl of 5x RT-buffer, 2.5 µl of 2.5 mM dNTPs, 2.5 µl of DEPC H_2O, 40 units of RNasin (1 µl), 2.5 µl of 40 mM sodium pyrophosphate (preheated at 40°C) and 22.5 units of AMV reverse transcriptase (1.5 µl). The mixture was pipetted to the RNA and the primer during the 2 min at 25°C. The reaction was then incubated for 45 min at 42°C, 10 min at 50°C, 10 min at 55°C and stopped by heating at 72°C for 15 min. 5 µl of the obtained cDNA were taken for the subsequent PCR performed at 40 cycles.

3.4 Cloning of DNA

3.4.1 Cloning of PCR-products into the pGem®-T Easy vector

The pGem®-T Easy vector system was used for cloning of PCR products. The pGem®-T Easy vector contains single 3'-T overhangs at the insertion site, and this provides a compatible overhang for PCR products generated with Taq DNA polymerase. If PCR was performed using Expand™ High Fidelity PCR System, A-tailing of the PCR product was performed.

3.4.1.1 A-tailing of the blunt-ended PCR fragments

The A-tailing reaction was performed in a final volume of 20 µl, containing the PCR fragment after extraction from the gel in 10 mM tris HCl pH 8.0, dATP at final concentration of 0.2 mM, 2 µl of 10x PCR buffer, and 5 units of Taq DNA polymerase. The reaction was incubated at 70°C for 30 min. The A-tailed PCR fragment was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. Finally the DNA pellet was resuspended in 7 µl of 10 mM tris HCl pH 8.0 and up to 3.5 µl were used for subsequent ligation.

3.4.1.2 Ligation

Vector-to-insert molar ratios of 1:1 to 1:3 were normally used. To calculate the appropriate amount of insert the following formula was used:

$$ng\ of\ insert = \frac{(ng\ of\ vector \times kb\ size\ of\ insert) \times insert\ vector\ molar\ ratio}{kb\ size\ of\ the\ vector\ (3\ kb)}$$

The ligation reaction was carried out in a final volume of 10 µl containing 25 ng of pGem®-T Easy vector, the required amount of PCR product, 5 µl of 2x rapid ligation buffer and 3 units of T4 DNA ligase. The reaction was incubated overnight at 4°C. The following day the competent *E.coli* cells were transformed with half of the ligation reaction.

3.4.1.3 Preparation of the competent E.coli cells

Epicurian Coli® XL10-Gold™ Kan ultracompetent cells were used for the first transformation. The competent cells were stored at -80°C. Once they were defrosted, the cells loose their ability to be transformed with plasmid DNA. The rest of the Epicurian Coli® XL10-Gold™ ultracompetent cells were grown in LB medium and used to prepare 'home-made' competent cells. The transformation efficiency was 1 x 10^9 colonies/µg DNA for ultracompetent cells and 1-2 x 10^7 colonies/µg DNA for the 'home-made' competent cells. Although the transformation efficiency was almost 100 times higher for the ultracompetent cells, the 'home-made' competent cells were normally used, as the transformation efficiency of 10^7 colonies/µg DNA was enough.

The frozen competent cells were prepared according to Sambrock et al. (1989). An overnight culture was grown from frozen stock (-80°C) in LB medium containing 50 µg/ml kanamycin. 200 µl of the night culture was transferred to 100 ml of SOB medium supplemented with 20 mM $MgSO_4$. The culture was grown until OD_{600} = 0.4-0.5. The cells were transferred to ice cold 50 ml falcon tubes, kept on ice for 10 min and centrifuged at 3,300 xg for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 20 ml of ice cold FSB by gentle vortexing. The cells were kept on ice for a further 10 min and then centrifuged at the same conditions as before. After removing the supernatant the pellet was resuspended in 4 ml of ice cold FSB and 140 µl of DMSO (dimethylsulphoxide), mixed gently by swirling and kept on ice for 15 min. An additional 140 µl of DMSO were added to the cells. 400 µl aliquots were dispensed into ice cold eppendorf tubes. The tubes were immediately frozen in liquid nitrogen and stored at -80°C.

3.4.1.4 Transformation of the competent E.coli cells with ligation reaction

The XL10-Gold™ Kan competent cells were thawed on ice and 100 µl were put into 15 ml Falcon 2059 polypropylene tubes. Half of the ligation reaction was added to the competent

cells and swirled gently. As a positive transformation control 0.1 ng of pUC18 plasmid were used. The tubes containing the competent cells and ligated DNA were incubated on ice for 30 min and then heat pulsed in a 42°C water bath for 30 sec and put on ice for 2 min. 0.9 ml SOC medium preheated at 42°C were added to each tube, and the tubes were incubated in a shaker at 37°C and 100 rpm for 1 hour. For colour screening 50 µl of 2.5% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 50 µl of 2.5% isopropylthio-β-D-galactoside (IPTG) were plated on LB agar plates, containing 50 µg/ml ampicillin. The experimental transformation reaction was centrifuged at 2,000 xg for 5 min, approximately 900 µl of supernatant were removed and the pellet was resuspended in the residual supernatant. The experimental transformation reaction was plated onto the LB ampicillin plates containing X-Gal and IPTG. 100 µl of the transformation control reaction were plated onto a regular LB ampicillin agar plate. The plates were incubated at 37°C for 16-17 hours. Colonies containing plasmids without insert were blue and colonies with plasmids containing inserts were white. Plasmid DNA was extracted from 4-10 white colonies.

3.4.1.4.1 Antibiotic selection for the cells transformed with plasmid

The pGem®-T Easy vector contains an ampicillin resistance gene. This makes it possible to select for the transformed bacteria by adding ampicillin to the agar plates.

3.4.1.4.2 Colour selection for the recombinant plasmids

Many plasmid vectors (also pGem®-T Easy vector) carry a short fragment of *E.coli* DNA that contains the regulatory sequences and the beginning of the coding sequence of the β-galactosidase gene (*lacZ*). The multiple cloning site (MCS) is inserted into the coding region of the β-galactosidase gene without disruption of the open reading frame (ORF), but with the harmless interposition of a small number of amino acids into N terminal part of β–galactosidase. When such a vector is transfected into host cells which encode for the C terminal part of β-galactosidase, the peptides can associate and become an enzymatically active protein. The *lacZ*+ bacteria with the active β-galactosidase can be recognised by the blue colour of the colonies in the presence of the chromogenic substrate X-Gal and the lac operon inducer IPTG. Insertion of a fragment of a foreign DNA into the MCS of a plasmid, results in the disruption of the ORF of *lacZ* gene. Therefore bacteria colonies containing the recombinant plasmid remain white.

3.4.1.5 Recombinant plasmid DNA extraction

A single colony from a transformation plate was grown on an LB-ampicillin plate overnight for storage of the material. Simultaneously a part of the same colony was incubated in 5 ml of LB medium containing 50 µg/ml ampicillin overnight in a shaker at 37°C and 250 rpm. The grown cells were centrifuged at 2,000 xg and the pellet was resuspended in 250 µl of P1 (15 mM tris HCl pH 8.0, 10 mM EDTA, 20 mg/ml RNase A). 300 µl of P2 (200 mM NaOH, 1% SDS) were added, the cell suspension mixed gently and left at room temperature for 5 min to lyse the cells. 300 µl of P3 (3M potassium acetate pH 4.5) were added, the cell suspension gently mixed and incubated on ice for 5 min. A fluffy white material was formed. The sample was centrifuged at 15,000 xg for 15 min and the supernatant was transferred to a new eppendorf tube and centrifuged again under the same conditions. The clear supernatant was transferred to a new eppendorf tubes and plasmid DNA was precipitated with an equal volume of isopropanol. The DNA/isoprapanol mixture was kept at -20°C for 15-30 min and then centrifuged at 15,000 xg for 15 min. The DNA pellet was washed with 70% ethanol, dried at room temperature and resuspended in 50-100 µl of tris HCl pH 8.0.

3.4.1.6 Checking the plasmids for the presence of insert

The MCS of the pGem®-T Easy vector contains two EcoRI sites adjacent to the T3' overhangs, therefore the insert can be cut out of the vector using the EcoRI restriction enzyme. 200-500 µg of plasmid DNA were digested in the final reaction volume of 20 µl, containing 2 µl of 10x buffer H and 7.5 units (0.5 µl) of EcoRI at 37°C for 2 hours. The digested reaction was loaded to a 1% agarose gel. Digested plasmids containing an insert showed two bands: the linearised vector at 3 kb and an insert of the known expected size.

3.4.1.7 Cryopreservation of the plasmid clones

The plasmid clones could be frozen and stored at -80°C. A colony of the required clone was grown overnight in LB medium, containing 50 µg/ml of ampicillin, at 37°C and 250 rpm. 0.7 ml of liquid culture was mixed with 1 ml of cryopreservation medium (LB medium : glycerol 1:1) in Nunc Cryotube™ vials and frozen in liquid nitrogen. Frozen tubes were transferred to -80°C. The frozen bacteria can be partly melted and used for growing overnight cultures with subsequent plasmid DNA extraction. One frozen tube can be re-used for approximately eight times.

3.4.2 Subcloning of the inserts from the pGem®-T Easy vector into the pcDNA3.1(+)

pcDNA3.1(+) is a 5.4 kb vector designed for high-level stable and transient expression in mammalian host cells.

3.4.2.1 Preparation of the insert

1 µg of pGem®-T Easy clone DNA was digested with EcoRI at the conditions described in chapter 3.4.1.6. The digestion reaction was loaded to 1% agarose gel, the insert cut out and extracted from the gel using QIAEX II Agarose Gel Extraction kit.

3.4.2.2 Preparation of the vector

1 µg of pcDNA 3.1(+) was digested with EcoRI at the same conditions. The reaction was divided in two parts. One half (10 µl) was taken for the positive control and the other half was subjected to dephosphorylation with alkaline phosphotase. 9.5 µl of H_2O and 0.5 units (0.5 µl) of calf intestinal alkaline phosphotase (CIP) were added to the dephosphorylation reaction, which was incubated for 30 min at 37°C. The step of dephosphorylation was performed to remove 5'- phosphate residues and therefore suppress self-ligation and circularisation of the plasmid DNA. CIP was inactivated by heating the reaction at 70°C for 10 min. Both digested plasmid and digested/dephosporylated plasmid were purified using the QIAquick PCR Purification kit. The principle of the QIAquick purification is based on the binding of DNA to a specially adapted silica-gel membrane. DNA binds to the silica-gel membrane while contaminants pass through during the steps of microcentrifugation with special buffers. In the final step purified DNA is eluted in 10 mM tris HCl pH 8.0. The elution volume of 30 µl was used and 3 µl were loaded to a 1% agarose gel to verify that the digestion was complete. The completely digested plasmid should show up a single band at 5.4 kb.

3.4.2.3 Ligation

25 ng of the digested and dephosphorylated vector were ligated with insert added at the molar ratio from 1 to 3. The required amount of the insert was calculated using the same formula as described in chapter 3.4.1.2. The ligation reaction was performed in a final volume of 10 µl containing 1 µl of 10x ligation buffer and 1 unit of T4 DNA ligase, and the reaction was incubated overnight at 4°C. Positive and negative ligation control reactions were also done. A

positive ligation reaction contained no insert and 25 ng of digested vector. This reaction was performed to control the efficiency of ligation. A negative ligation reaction contained no insert and 25 ng of digested/dephosphorylated vector. This was performed to control the efficiency of the dephosphorylation.

Transformation was performed the following day as described in chapter 3.4.1.4, except that no colour selection could be applied when using pcDNA3.1(+) vector, as it does not contain the β–galactosidase gene. If the dephosphorylation and ligation worked properly, the negative control contained very few colonies when compared with the experimental ligation (at least 10 times less), and the positive control contained more colonies when compared with experimental ligation (3-4 times more). Plasmid DNA was extracted from 4-6 colonies as described in chapter 3.4.1.5 and plasmids were checked for the presence of an insert as described in chapter 3.4.1.6 using the restriction enzyme EcoRI. The orientation of insert was determined by sequencing using the T7 primer. Clones of interest were cryopreserved as described in chapter 3.4.1.7.

3.5 Sequencing

Automated DNA sequencing was performed with the ABI Prism^{TM} System 377 DNA Sequencer (Applied Biosystems) using the dideoxy termination method of Sanger (Sanger et al., 1977).

3.5.1 Sequencing of PCR-products and plasmid clones

The sequencing reaction was performed in a final volume of 10 µl. The required amount of DNA was calculated as follows:

PCR product extracted from agarose gel (ng) = length (bp)/20

Plasmid clone (ng) = [insert (bp) +vector (bp)]/4

The DNA template was mixed with 1.6 pmole of primer, the required amount of H_2O and 4 µl of Big Dye^{TM} sequencing mix. The sequencing program consisted of a pre-denaturing step at 95°C for 5 min, followed by 30 cycles of 30 sec at 94°C, 15 sec at 50°C and 4 min at 60°C. The T7 and Sp6 primers (Table 7) were used to sequence pGem®-T Easy clones, and the T7 primer was used for sequencing of pcDNA3.1(+) clones. PCR products and plasmid clones

were also sequenced using DNA specific primers. To minimise sequencing errors both strands of the DNA were sequenced.

The finished sequencing reactions were filled up to 90 µl with H_2O, and precipitated with 10 µl (0.1 volume) of 3M Na acetate pH 5.2 and 200 µl of absolute ethanol (66.67% final concentration). The samples were vortexed, put to -70°C for 10-15 min and centrifuged at 21,000 xg for 30 min at 4°C. The pellets were washed with 200 µl of 70% ethanol, centrifuged at 21,000 xg for 5 min at 4°C and dried in a vacuum centrifuge. The DNA was resuspended in a 1.5 µl mixture of formamide/loading buffer (vol/vol 5:1). After denaturing the samples for 5 min at 95°C they loaded onto a 4.5% polyacrylamide 0.167 M Urea/1x TBE gel supplemented with 0.007% ammonium peroxodisulfate (APS) and 0.03% N,N,N',N'-tetramethylethylenediamin (TEMED). The samples were separated according to size by means of electrophoresis in a 1x TBE buffer. Fluorescent signals were detected by laser, recorded by a CCD camera and transferred to the program ABI Prism^{TM} 377 Collection PE. The obtained raw sequencing data was analysed using the 377 Sequence Analysis software.

3.5.2 Sequencing of BAC clones

2.5 µg of BAC DNA was added to 8 µl of Big Dye^{TM} sequencing mix, 30 pmole of T7 or DNA specific primer in a 11 µl reaction volume. The sequencing program consisted of pre-denaturing step at 95°C for 5 min, followed by 99 cycles at 95°C for 30 sec, 20 sec at 55°C and 4 min at 60°C. The samples were precipitated and loaded as described above.

3.5.3 Sequencing analysis

The obtained sequences were analysed using the genetic computer group (GCG) package version 10 (Devereux et al., 1984).

3.6 Polymorphism detection in cattle MC1R DNA sequence

3.6.1 PCR-RFLP assays

3.6.1.1 310/311G deletion

The following PCR-RFLP was developed by Joerg et al. (1996). PCR was carried out with the primers MSHRP9 and MSHRP10 (Table 8) in a reaction volume of 25 µl containing 50-100 ng genomic DNA, 10 pmole of each primer, each dNTP at a concentration of 0.2 mM, 2.5 µl of 10x buffer and 2.5 U of Taq DNA polymerase. Genomic DNA was denatured for 5 min at 95°C, and the PCR was run for 35 cycles. One cycle consisted of denaturation step of 95°C for 30 sec, an annealing step at 56°C for 30 sec and an elongation step at 72°C for 30 sec. 35 cycles of amplification were followed by a final extension at 72°C for 7 min. 10 µl of the PCR reaction were digested with 5 units of MspI for 2 hours at 37°C in a 20 µl reaction containing 2 µl of 10x Sure/Cut buffer L. The digested DNA fragments were separated on a 1% agarose gel electrophoresis together with 100 bp DNA ladder as a standard for DNA fragment length.

3.6.1.2 T296C base substitution

The first PCR amplification was performed with the primers MSHRP9 and MSHRP10 at the conditions described above. This was diluted 1 to 100 and 1 µl was taken to the second PCR. The second PCR amplification was carried out with the primers MSHRP9 and MSHRP12 (Table 8) at the same conditions as for primers MSHRP9 and MSHRP10, except that the annealing temperature was 58°C. 5 µl of the second PCR reaction were digested with 5 units of AciI for 2 hours at 37°C in a 20 µl reaction volume containing 2 µl of 10x NE buffer 3. After 2 hours of digestion 5 more units of AciI were added to the digestion reaction and incubation was continued for 2 more hours. The digested DNA fragments were separated on a 2% agarose gel electrophoresis together with a 50 base-pair DNA ladder as a reference for DNA fragment length.

3.6.1.3 T667C base substitution

The PCR amplification was carried out with the primers MSHRP13 and MSHRP14 (Table 8) at the same conditions as for the other two RFLP assays, except that the annealing temperature

was 60°C. 10 µl of the PCR reaction were digested with 5 units of MspI for 2 hours at 37°C in a 20 µl reaction containing 2 µl of 10x Sure/Cut buffer L. The digested DNA fragments were separated on a 2% agarose gel together with 50 base-pair DNA ladder as a standard for DNA fragment length.

3.6.2 AFLP assay

3.6.2.1 651-662 duplication

PCR was carried out with the primers MSHRP15 and MSHRP16 (Table 8) at the same conditions as for the RFLP PCR with the annealing temperature of 60°C. The fragment length was analysed using the ABI Prism™ System 377 DNA Sequencer.

3.7 Rapid amplification of the 5' cDNA ends (5' RACE)

5' RACE was performed using the 5'/3' RACE kit. 5' RACE allows amplification of unknown sequences at the 5' end of the mRNA. The first strand was synthesised from total poly (A)+ RNA using a gene specific primer (SP1), and then purified from unincorporated nucleotides and primers using the High Pure PCR Product Purification kit. The terminale transferase was used to add a homopolymeric A-tail to the 3' of the cDNA. The tailed cDNA was then amplified by PCR using a gene specific primer (SP2) and the oligo dT-anchor primer (Table 7). The oligo dT-anchor primer is a mixture of primers with one non T (A, G or C) nucleotide at the 3' end, so that the oligo dT-anchor primer is forced to bind to the inner end of the poly(A)-tail. Therefore the actual length of the added poly(A)-tail is unimportant. The obtained PCR-product is reamplified by a second PCR using a nested specific primer (SP3) and the PCR anchor primer (Table 7). The obtained 5' RACE products can be cloned and sequenced.

3.7.1 First strand cDNA synthesis

The cDNA was synthesised in a reaction volume of 20 µl, containing 4 µl of cDNA synthesis buffer, dNTPs at a concentration of 2 mM each, 12.5 pmole of the cDNA synthesis primer

(SP1), 2 µg of total RNA and 20 units of AMV reverse transcriptase. The reaction was incubated for 60 min at 55°C and for 10 min at 65°C.

3.7.2 Purification of cDNA

The High Pure PCR Product Purification kit was used for purification of cDNA. 100 µl of the binding buffer were added to 20 µl of the first strand cDNA. The sample was pipetted to the upper reservoir of the High Pure filter tube combined with the collection tube. The tubes were centrifuged for 30 sec at maximum speed. The flow-through was discarded and 500 µl of the washing buffer were added to the upper reservoir and the tubes were again centrifuged for 30 sec at maximum speed. The flow-through was discarded, and 200 µl of the washing buffer were again added to the upper reservoir and the tubes centrifuged for 30 sec at maximum speed. The filter tube was inserted into a new eppendorf tube and 50 µl of 10 mM tris HCl pH 8.0 were added to the filter tube. The tubes were centrifuged for 30 sec at maximum speed. The flow-through in the eppendorf tube contained purified first strand cDNA.

3.7.3 Tailing reaction of cDNA

19 µl of purified cDNA sample, 2.5 µl of 10x reaction buffer and 2.5 µl of 2 mM dATP were mixed together and incubated at 94°C for 3 min. The mixture was then chilled on ice and briefly centrifuged. 1 µl of the terminale transferase (10 units/µl) was added to the mixture, and the reaction was incubated at 37°C for 30 min. Following the incubation, the terminal transferase was heat inactivated at 70°C.

3.7.4 PCR amplification of dA-tailed cDNA

5 µl of the dA-tailed cDNA were taken for PCR with the oligo dT-anchor primer (Table 7) and the specific primer (SP2), located upstream of SP1. PCR was performed as described in the chapter 3.2.3.

3.7.5 Second PCR amplification (nested PCR)

Nested PCR was performed using 1 µl of the PCR reaction generated in the previous chapter, diluted 1 to 20, the PCR anchor primer (Table 7) and the specific primer (SP3), located upstream of SP2. PCR products were cloned into the pGem®-T Easy vector and sequenced.

3.8 Real time PCR

RNA was extracted from the cells expressing MC1R alleles and reverse transcription was performed with 1 µg of total RNA using a TVX primer, as described in chapter 3.3.6. Real time PCR was performed with the obtained cDNA, specific MC1R primers and a TaqMan probe to confirm the expression of MC1R in the cells.

Real time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle, i.e. in real time. Real time PCR is based on the detection and quantification of the fluorescent reporter. The TaqMan real time PCR assay exploits the 5'-3' exonuclease activity of Taq Polymerase to cleave a TaqMan probe. The TaqMan probe is an oligonucleotide of 20-30 nucleotides that contains a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end. TaqMan probes are designed to anneal to an internal part of the PCR product. The 3' end of the probe is blocked to prevent extension of the probe. When the probe is intact the proximity of the reporter dye to the quencher dye results in a suppression of the reporter fluorescence. During the PCR reaction, cleavage of the probe separates the reporter dye and the quencher dye. This causes an increase of fluorescence as the amplification proceeds. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and if it is amplified during PCR (Figure 12).

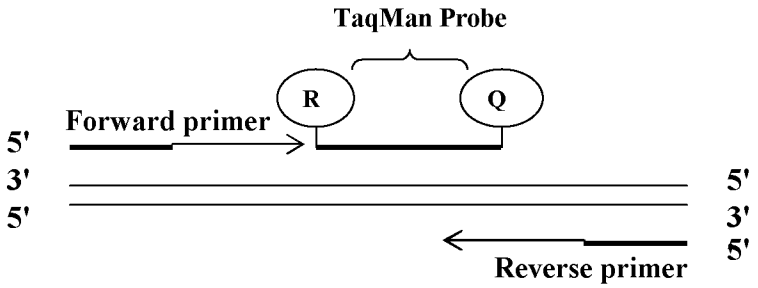


Figure 12: The primers and TaqMan probe position for real time PCR amplification

R is reporter dye and Q is quencher dye (according to TaqMan® Universal PCR Master mix manual).

The TaqMan universal PCR Master mix is used for real time PCR and contains AmpliTaq Gold DNA Polymerase, AmpErase uracil-N-glycosylase (UNG) and dNTPs with dUTPs, Passive Reference and optimised buffer conditions. AmpErase UNG prevents the reamplification of carryover-PCR products by removing any uracil in double-stranded DNA, which will no longer be used for amplification.

The Passive Reference dye is included in the TaqMan Universal PCR Master mix to provide an internal reference to which the reporter signal can be normalised during data analysis. Normalisation is necessary to correct any fluorescent fluctuations due to changes in the concentration or the volume. Normalised reporter (Rn) is a ratio of the emission intensity of the reporter dye to the emission intensity of the Passive Reference. Rn+ is the Rn value of a reaction containing all components including the template. Rn- is the Rn value of an unreacted sample. This value may be obtained from the early cycles of Real Time PCR prior to any detectable increase in the fluorescence. ΔRn is the difference between Rn+ and Rn- values. It indicates the magnitude of the signal generated by the given set of PCR conditions. The value used to compare amplification levels is a threshold cycle (C_T). C_T is a cycle number at which a statistically significant increase in ΔRn is first detected (Figure 13). However, the choice of the threshold which will determine the C_T value can be manually adjusted by the operator, which is important, particularly when several reactions performed at different occasions are compared with each other. The threshold line should be placed above any baseline activity and within the exponential increase phase. Besides being used for quantification, the C_T value can be used for qualitative analysis as a pass/fail measure.

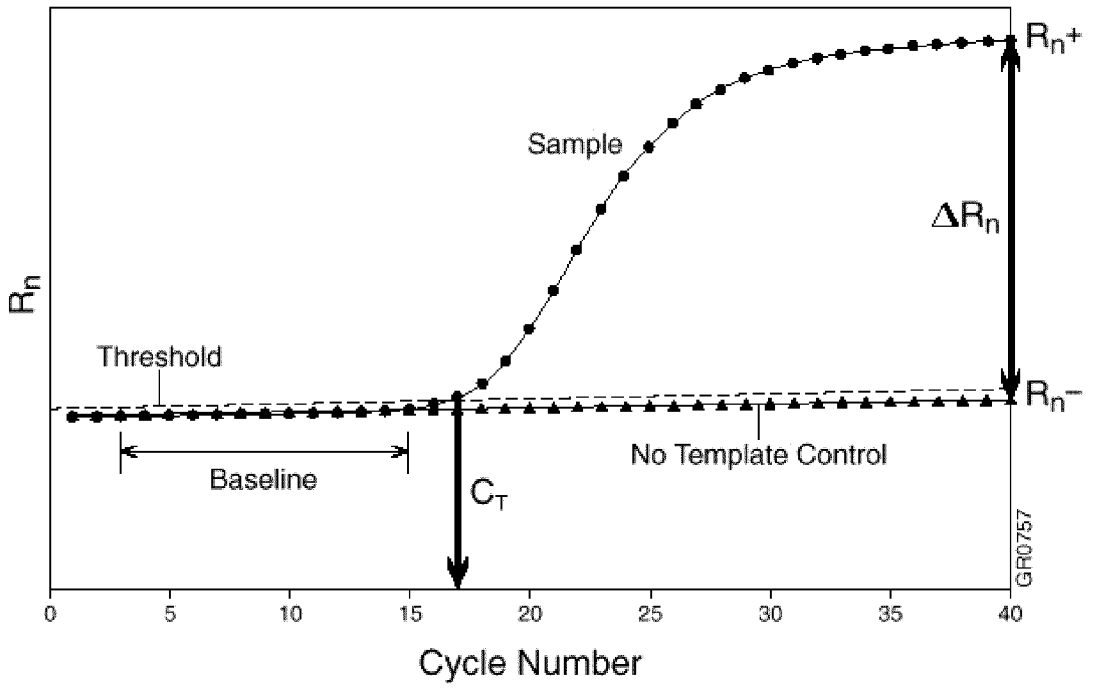


Figure 13: Definition of C_T (according to TaqMan® Universal PCR Master mix manual)

To compare the expression levels between samples the comparative C_T method can be used. One of the samples (e.g. with the lowest C_T value) is chosen as a reference. Then ΔC_T is calculated.

$$\Delta C_T = C_T(target) - C_T(reference)$$

$$comparative\ expression\ level = 2^{-\Delta C_T}$$

This means that the target sample is $2^{-\Delta C_T}$ times less expressed, then the reference sample.

3.8.1 Primers and probe design

The primers and the TaqMan probe were designed using Primer^{TM} 1.5 Express Software (Applied Biosystems).

The following criteria were used to select the probe and the primers:

1. The primers and the probe were selected in the region with a G/C nucleotide content of 20-80%.
2. The probe was selected first and the primers were designed as close as possible without overlapping with the probe, so that the resultant PCR product was 50-150 bp in length.

3. The T_m of the probe was 8-10°C higher than the T_m of the primers ensuring the full hybridisation of the probe during the primer extension phase.
4. The probe did not have a G at the 5' end, because the G adjacent to the reporter dye can influence the ΔRn after probe cleavage.
5. The probe contained more C than G, because probes with more C often produce higher normalised fluorescence values (ΔRn).

Two types of TaqMan probe were used for the real time PCR with the cattle MC1R gene. Both probes were complementary to the area of MC1R containing a deletion of a G at the position 310/311 in red animals. The probe ETH1S did not contain a deletion at this position and was used for real time PCR with the cDNA of the cells expressing the alleles of MC1R without a deletion. The probe ETH2R contained a deletion of a G and was used for an allele having a deletion of a G at the position 310/311. The ETH1S was labelled with a Fam fluorescent dye and the ETH2R was labelled with a Joe fluorescent dye at the 5' end. Both of the TaqMan probes were labelled with Tamra at the 3' end.

3.8.2 Thermal cycling parameters

Real time PCR was performed in a thermal cycler ABI Prism® 7700 Sequence Detector (Applied Biosystems) using the Micro Amp® optical tubes (Applied Biosystems). The following cycling parameters were used: 2 min at 50°C (for optimal AmpErase UNG enzyme activity), 10 min at 95°C (for activation of AmpliTaq Gold DNA Polymerase) followed by 40 cycles consisting of a denaturing step for 15 sec at 95°C and an annealing/elongation step of 1 min at 60°C.

3.8.3 Optimisation of primer and probe concentration

In order to obtain an optimal assay performance, the primer concentrations had to be optimised. Primers are always in large molar excess during the exponential phase of the PCR amplification, and by adjusting their initial concentration the effective T_m was adjusted. Three primers concentrations (50 nM, 300 nM, 900 nM) of each primer were used for the optimisation. The optimisation of the primer concentrations was performed using bovine genomic DNA as a template for amplification. The primer concentrations that provide the lowest C_T and the highest ΔRn were chosen as optimal for performance of the real time PCR

assay. The 300 nM of the forward (MSHRP21) and the reverse (MSHRP22) primers (Table 7) gave a combination of the highest ΔRn and the lowest C_T. The TaqMan probes were used at a concentration of 250 nM as recommended by TaqMan Universal PCR Master mix manual (Applied Biosystems).

3.8.4 Real time PCR reaction mix

The real time PCR was performed in a final volume of 25 µl containing the following components (Table 2):

Table 2: Reagents for real time PCR

Reaction component	Volume (µl)	Final concentration
cDNA	2.5	1/10 of 25 µl RT reaction
MSHRP21 (3 µm)	2.5	300 nM
MSHRP22 (3 µm)	2.5	300 nM
TaqMan Probe (2.5 µm)	2.5	250 nM
H_2O	2.5	
TaqMan Universal PCR Master Mix (2x)	12.5	1x
Total	25 µl	

A negative control real time PCR reaction was performed with 0.1 µg of RNA of each probe (the same amount of the starting material as in the reaction performed with cDNA) to detect traces of DNA in the sample.

The real time PCR reactions were performed in the MicroAmp® Optical tubes, which were loaded into the ABI Prism 7700 Sequence Detector. The real time amplification profiles were electronically saved and represented as a plot of cycle number against log (ΔRn). C_T values were determined from these graphs and compared between the samples.

3.9 Creation of stable cell lines expressing specific genes

The HEK 293 cell line was used in this study for stable transfection with the cattle coat colour genes cloned into the pcDNA3.1(+) mammalian expression vector. This permanent cell line was established from primary embryonic human kidney (HEK) cells which were transformed

with sheared human adenovirus type 5 DNA (Graham et al., 1977). The E1A adenovirus gene is expressed in these cells and participates in transactivation of a variety of viral and cellular promoters, which are transcribed by RNA-polymerases II and III (Flint and Shenk, 1997). In the present study 293 cells were transfected with pcDNA3.1(+) clones. Although pcDNA3.1(+) contains the SV40 origin of replication it cannot replicate autonomously in 293 cells, because they do not have the T antigen required for the initiation of DNA replication from the SV40 origin of replication (Hirschhorn and Sarve, 1998). Stable propagation of pcDNA3.1(+) therefore depends on the random integration into the host genome. The stable transfectants were selected by adding Geneticin (G418) to the culture medium. Geneticin is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin and it blocks the protein synthesis in mammalian cells by interfering with ribosome function. Expression of the bacterial aminoglycoside phosphotransferase gene (neomycin resistance gene), present in pcDNA3.1(+) results in detoxification of G418 (Southern and Berg, 1982). Cells not containing the pcDNA3.1(+) sequence in their genome can divide only once or twice in the presence of lethal concentrations of Geneticin, and afterwards they die. The human cytomegalovirus immediate-early promoter provides high-level expression of the inserted gene, which is also strengthened by presence of the E1A adenovirus gene in 293 cells.

It is also necessary to mention, that all work with the cells was performed in a sterile laminar flow hood and all reagents, glassware and plasticware were sterile and antibiotic-antomicotic agents were added to the culture medium. The cells were grown in a humidified CO_2 incubator with 5% CO_2 and 95% air in the atmosphere and at 37°C.

3.9.1 Recovery of cryopreserved 293 cells

A vial containing the frozen cells was thawed in a 37°C water bath. The contents of the vial were mixed and transferred into a T-25 cm^2 flask (Falcon®) containing 5 ml of prewarmed at 37°C complete Dulbecco's Modified Eagle Medium (DMEM). The cells were incubated in a CO_2 incubator. After 3-5 hours the cells were checked for attachment under a inverted microscope. The old medium was removed, because DMSO, included in the cryopreservation medium is toxic for living cells and 3-5 ml of fresh complete DMEM were added to the flask.

3.9.2 Maintenance of 293 cells in monolayer

Once the culture has reached 80-100% confluency the cells were transferred to a T-75 cm^2 (Falcon^{TM}) flask by trypsinisation. For this the cells were first washed with 1 ml of PBS to take away traces of the medium, because FCS included in the medium inactivates trypsin. Then 1 ml of trypsin/EDTA was added to the cells, and detachment of the cells from the bottom was facilitated by agitating the flask. The cells were observed in an inverted microscope and as soon as most of them had detached from the bottom, the action of trypsin was stopped by adding 8 ml of complete DMEM, and the cells were transferred to a new T-75 cm^2 flask.

When the cells have reached the 80-100% confluency in T-75 cm^2 flasks, they were trypsinised as described above, except that the amounts of the used solutions were adjusted: 3 ml of PBS, 1.5 ml of trypsin/EDTA and 3.5 ml (for α-MSH stimulation) or 8.5 ml (for cryopreservation) of DMEM and transferred into a falcon tube. An aliquot of approximately 100 µl was taken out and the concentration of the viable cells was counted using the trypan blue exclusion method with a hemocytometer chamber. The cells, with a determined concentration, were seeded at the required amount for α-MSH stimulation or cryopreservation. When further cell growth was required 0.1 of the cells were transferred to a new T-25 cm^2 or T-75 cm^2 flask.

3.9.3 Viable cell counts using trypan blue

The reaction of the cells with trypan blue is based on the fact that chromophore of this substance is negatively charged and does not interact with the cells unless the membrane is damaged. Therefore, all the cells which are not stained are viable. Dead cells are stained blue.

10 µl of the cell suspension, 70 µl of PBS and 20 µl of 3.8% trypan blue solution were mixed and allowed to stand for 5 min at room temperature. The hemocytometer chamber (Bürker-Türk) was prepared as follows: the slide and the cover slip were cleaned with 70% ethanol, the edges of the slide were slightly moistened and the coverslip was pressed down over counting area. The appearance of the 'Newton rings' (rainbow colours) indicated, that the cover slip had properly attached and the depth of the counting chamber is 0.1 mm. The cells were thoroughly mixed before filling the hemocytometer chamber. Viable cells were counted in the surface of 1 mm^2, which corresponds to the volume of 0.1 mm^3 or 0.1 µl. The cells that

overlap the top and left border of the orthogons in the hemocytometer chamber were counted together with the cells inside the orthogons and the cells overlapping bottom and right borders were not counted to prevent the counting of overlapping cells twice. The cell concentration was calculated as follows:

$$C\ (cells/\mu l) = cell\ counts\ in\ 1mm^2 \times dilution\ factor\ (10) \times 10$$

3.9.4 Cell cryopreservation

The confluent cells in a T-75cm^2 flask were trypsinised as described in chapter 3.9.2 and the trypsin was stopped with 8.5 ml of complete DMEM, so that the final volume was 10 ml. The cell concentration was determined as described in the chapter 3.9.2, and the total amount was calculated by multiplying cell concentration (cells/µl) by 10^4. The cells were centrifuged at 1,000 xg for 10 min at room temperature. The pellet was resuspended in the cryopreservation medium, which consisted of 10 parts of complete DMEM, 2 parts of FCS and 1 part of DMSO, so that the final cell concentration was 2-5 x 10^6 viable cells/ml and transferred to the Nunc Cryotube™ vials. Cryopreservation was achieved by putting the cells for 1 hour at 4°C, 1 hour at -20°C, 2-12 hours at -80°C and finally storing the cells in liquid nitrogen.

3.9.5 Transfection of 293 cells with pcDNA3.1(+) clones

The cells were grown in a T-25 cm^2 flask until 50-80% confluency was reached. The following solutions were prepared. Solution A**:** 1-2 µg of pcDNA3.1(+) clone and 100 µl of Opti-MEM®-I Reduced Serum Medium and solution B**:** 10 µl of LipofectAMINE™ Reagent and 100 µl Opti-MEM®-I Reduced Serum Medium. The two solutions were combined, mixed gently, and incubated at room temperature for 40 min to allow DNA-liposome complexes to form. While complexes were formed the cells were rinsed once with Opti-MEM®-I Reduced Serum Medium. 0.8 ml of Opti-MEM®-I Reduced Serum Medium were added to the each tube containing the complexes. The diluted complex solution was mixed gently and overlayed onto the rinsed cells. No antibacterial agents were added to media during transfection. The cells were incubated for 18-20 hours in a CO_2 incubator. Following incubation 1 ml of complete DMEM medium (with ABAM) and twice the normal concentration of serum were added without removing the transfection mixture. The medium was replaced with fresh

complete DMEM medium after 18-24 hours following the start of transfection. 72 hours after transfection, the cells were trypsinised as described in the chapter 3.9.2 and 10% were transferred to the DMEM medium containing 500 µg/ml Geneticin. The same procedure was performed with untransfected cells to control the toxic effect of the Geneticin to the cells not containing the pcDNA3.1(+) sequence in their genome. The medium was changed every 3-4 days. Untransfected cells died and detached from the bottom. Complete selection until only transfected cells remained and had become confluent took 3-4 weeks. The transfected cells were used for α–MSH stimulation and subsequent cAMP measurement and were cryopreserved.

3.10 Cyclic AMP measurement

3.10.1 Stimulation the cells with α-MSH

The 293 cells stably transfected with a pcDNA3.1(+) clone were grown in a T-75 cm^2 flask until they were 80% confluent. The cells were trypsinised, counted and 10^6 of the viable cells per well were seeded into a 6 well plate (Costar®) and allowed to attach overnight. The next morning 1 ml of log dilutions of α-MSH (e.g. 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M) in complete DMEM containing 0.1 mM 3-isobutyl-1-Methylxanthine (IBMX) were added to each well. IBMX is a non-specific inhibitor of phosphodiesterases (Beavo et al., 1970), the enzymes which degrade cAMP (Fimia and Sassone-Corsi, 2001). The cells were incubated for 30 min at 37°C. After the incubation the medium was removed and the cells were washed with ice-cold PBS. 1 ml of ice cold ethanol: H_20 (2:1) was added to the wells, the cells were scraped and transferred into 15 ml falcon tubes. The cell suspensions were sonicated for 1 min, the samples transferred to the eppendorf tubes, left at room temperature for 5 min and then centrifuged at 12,000 xg for 5 min. The supernatant containing cAMP was transferred to the new eppendorf tubes. At this stage the samples can be stored at -20°C. Before starting cAMP measurement the probes were centrifuged in a vacuum centrifuge until all ethanol had evaporated (2-3 hours). The obtained pellets were resuspended in 100 µl of 0.05 M Tris/EDTA buffer pH 7.5 and assayed directly with the Cyclic AMP [^{3}H] assay system.

3.10.2 Cyclic AMP Assay

The assay is based on the competition between unlabelled cAMP and a fixed amount of the tritium labelled cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of the complex protein - labelled cAMP inversely correlates with the amount of unlabelled cAMP present in the assay sample (Figure 14). Measurement of the protein-bound radioactivity enables the amount of the unlabelled cAMP in the sample to be calculated

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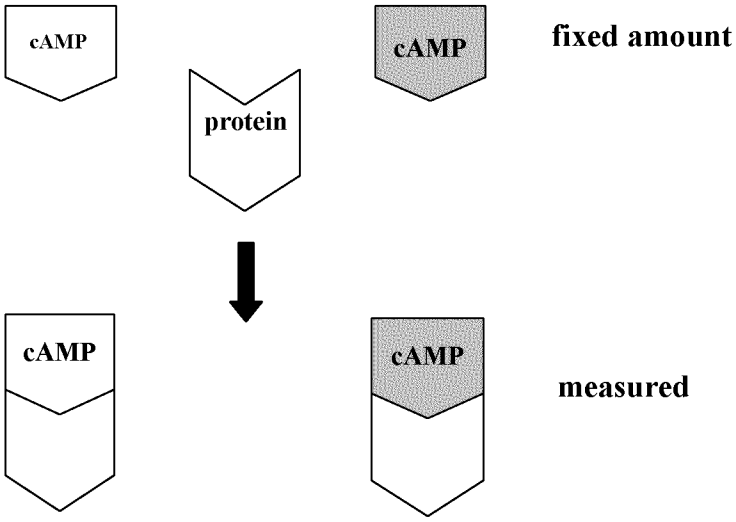

Figure 14: Scheme of competitive binding of labelled and unlabelled cAMP to the specific protein

The labelled cAMP is grey.

Separation of the cAMP bound to the protein from the unbound cyclic AMP is achieved by absorption of the free nucleotide onto coated charcoal, followed by centrifugation. An aliquot of the supernatant was then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample was then determined from a linear standard curve.

3.10.2.1 Preparation of the standards

Serial dilutions of the standard cAMP solution were prepared as follows: 0.5 ml of Tris/EDTA buffer were added to four glass tubes, marked as 8, 4, 2 and 1. 0.5 ml of the standard cAMP solution was transferred to the tube marked as 8. The solution was mixed thoroughly and 0.5 ml of this was transferred to the next tube marked as 4 and again mixed. The procedure was repeated successively with the tubes marked 2 and 1. Together with the original standard

solution five levels of cAMP standards were obtained. 50 µl from each solution gave 16, 8, 4, 2 and 1 pmole per assay tube.

3.10.2.2 Assay protocol

14 assay eppendorf tubes for the standard curve and the required amount of tubes for samples were prepared (for each sample 2 tubes were prepared). The tubes were labelled and arranged according to Table 3 and placed into the ice bath. 150 µl of the Tris/EDTA buffer were pipetted into the assay tubes 1 and 2. These tubes were for the determination of the blank counts per minute. 50 µl of the Tris/EDTA buffer were pipetted into the assay tubes 3 and 4 for determination of the binding in the absence of unlabelled cAMP. 50 µl of the prepared standard cAMP solutions starting from the lowest level (1 pmole) were put into each successive pair of assay tubes (tubes 5-14). 50 µl of each sample were pipetted into the sample tubes in duplicate (tubes 15, 16 etc.). 50 µl of labelled [^{3}H] cAMP were added to each assay and sample tube. 100 µl of the binding protein were added to each assay tube starting with the tube 3 and each sample tube.

Table 3: cAMP protocol

Tube No.	TE	Standards		Samples	[^{3}H] cAMP	binding protein
1, 2	150µl	blank counts			50 µl	
3, 4	50 µl	zero dose			50 µl	100 µl
5, 6		50 µl	1 pmole		50 µl	100 µl
7, 8		50 µl	2 pmole		50 µl	100 µl
9, 10		50 µl	4 pmole		50 µl	100 µl
11, 12		50 µl	8 pmole		50 µl	100 µl
13, 14		50 µl	16 pmole		50 µl	100 µl
15, 16, etc.				50 µl	50 µl	100 µl

All tubes were vortexed for approximately 5 sec. The tubes were kept on ice for 2-16 hours allowing the cAMP- binding protein complex to form. At least 15 min before the end of the incubation 20 ml of ice cold distilled water were added to the charcoal reagent and this was

continuously stirred in the ice bath. 100 µl of the charcoal suspension were added to all tubes. The tubes were vortexed briefly and placed on ice. Charcoal was not added to more tubes than it was possible to centrifuge in one batch. The tubes were centrifuged to sediment the charcoal. The tubes were centrifuged for not less than 1 min and not more than 6 min after addition of charcoal to the last tube in a batch. 200 µl of the supernatant were removed from each tube and placed into scintillation vials filled with 9 ml of scintillation cocktail and counted in the β-counter (Tri-Carb LSCs, with QuantaSmart™ Software for the Windows NT®; Packard BioScience S.A.) for 4 min.

3.10.2.3 Calculation of the results

The blank disintegrations per minute (dpm) were determined by calculating the average dpm for tubes 1 and 2.

$$B.D. = (D1+D2)/2 \qquad B.D. \text{ - blank dpm}$$

The blank disintegrations were subtracted from the average dpm for tubes 3 and 4. The result was the dpm bound in the absence of unlabelled cAMP ($\mathbf{D_0}$)

$$D_0 = [(D_3+D_4)/2] - B.D.$$

The average dpm was calculated for each pair of duplicates in assay and sample tubes. The blank disintegrations were subtracted from each result and gave the dpm bound to the protein in the presence of standard and unknown unlabelled cAMP ($\mathbf{D_x}$)

For example:

$$D_{5\text{-}6} = [(D_5 + D_6)/2] - B.D.$$

$\mathbf{D_0/D_x}$ was calculated for each standard and sample.

$\mathbf{D_0/D_x}$ was plotted against pmoles of cAMP present in each assay tube pair. A straight line (standard curve) was obtained with an intercept of 1 on the ordinate.

From the $\mathbf{D_0/D_x}$ values the number of pmoles of cAMP in the samples was read with the help of the standard curve.

The Microsoft Excel program (version 97 SR-1) was used for calculating the results and making graphs. Each hormone stimulation was performed in duplicate or triplicate, the mean and standard error of cAMP amount were calculated for each α-MSH concentration. The cAMP amount was plotted against α-MSH concentration for each cell line.

4 Results

4.1 MC1R

4.1.1 Cloning and sequencing of the bovine MC1R alleles

The primers MC1RF2 and MC1RR1 (Table 8) were used to amplify the complete coding sequence of bovine MC1R. These primers were designed based on the sequence published by Klungland et al. (1995; accession number Y13957). An ACG triplet was added in front of the start codon in the MC1RF2 primer, to have an A residue in position -3, which is required for proper initiation of translation (Kozak, 1987), when the coding sequence is subcloned into the pcDNA3.1(+) mammalian expression vector and expressed in mammalian cells. First the coding sequence of MC1R was amplified using DNA of a black Holstein cow, known to be a carrier of the **e** allele. The amplified DNA product, of approximately 1 kb in size was cloned into the pGem®-T Easy vector and several plasmid clones were sequenced. Two types of sequences were obtained: the **e** allele (953 bp) and the $\mathbf{E^D}$ allele (954 bp). The **e** allele contained a deletion of a G at position 310/311, which is in agreement with Klungland et al. (1995) and Joerg et al. (1996). Comparison of the **e** allele sequence with the **E+** allele, revealed two base substitutions at positions 748 and 869 (Figure 19). However, the deduced amino acid sequences of the bovine MC1R alleles showed, that the stop codon in the **e** allele lies 486 bp upstream from the stop codons in the other alleles (Figure 19). Therefore these two substitutions are located after the stop codon in the **e** allele, and thereby do not influence the protein structure. The $\mathbf{E^D}$ allele contained a missense mutation at position 296, substituting a T into a C, and subsequently a **Leu** into a **Pro** (Figures 19 and 20). This substitution was also reported by Klungland et al. (1995) in the $\mathbf{E^D}$ allele of black Norwegian cattle, indicating that the dominant black phenotype is caused by the same mutation in both Norwegian and Holstein cattle. Another base substitution was found in the sequence of the $\mathbf{E^D}$ allele at position 872. In the $\mathbf{E^D}$ sequence T encodes for **Val**, while the other alleles have a C at this position, encoding for **Ala**.

Cloning and sequencing of the MC1R coding sequence in Brown Swiss cattle revealed two other MC1R variants, designated as $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$. The $\mathbf{E^{d1}}$ variant contains a T at position 667 encoding for **Trp** at position 223, while all other variants have a C and an **Arg** at this position.

The $\mathbf{E^{d2}}$ variant has a duplication of 12 nucleotides at position 651 (Figure 19) and subsequently a duplication of four amino acids at position 218 (Figure 20).

The results from the sequencing were confirmed using RFLP and AFLP assays for the four polymorphic sites: 310/311 G deletion, **T296C**, **C667T** and duplication at position 651. The two base pair substitutions in the **e** allele were not tested with RFLP assays, as their location after the stop codon strongly indicates that they do not affect the receptor function. The **C872T** substitution was also not tested with an RFLP assay, as no suitable restriction enzyme was found for this site. Hence, it is not clear whether this substitution is really present in the $\mathbf{E^D}$ allele of MC1R, or whether it occured due to an error of Taq polymerase.

310/311 deletion: Genomic DNA was amplified using the primers MSHRP9 and MSHRP10 and the PCR product was digested with MspI. The allele containing the 310/311 G deletion was not digested with MspI and thus showed only one band of 531 bp. The allele without the deletion was digested with MspI and showed two bands of 201 bp and 331 bp. Heterozygous animals had three bands (Figure 15) of 531 bp, 331 bp and 201 bp (Joerg et al., 1996).

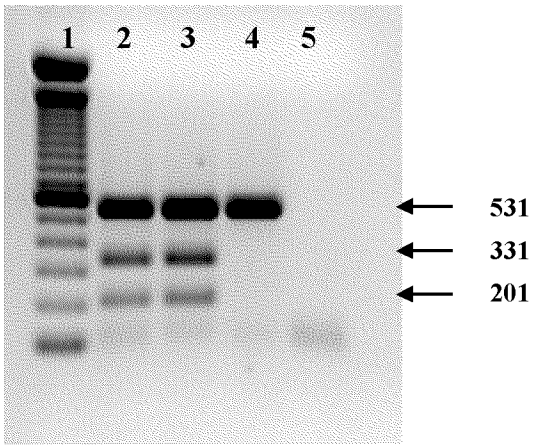

Figure 15: An example of an RFLP assay for the 310/311G deletion

Lane 1: 100 bp DNA ladder (bands start with 100 bp followed by 200 bp, 300 bp etc., with a bright band at 600 bp); **Lanes 2 and 3:** heterozygous animals **(e/-)**; **Lane 4:** a homozygoous red animal **(e/e)** having a deletion; **Lane 5:** H_2O PCR negative control.

T296C substitution: The PCR product of the MSHRP9 and MSHRP10 primers was re-amplified using MSHRP9 and MSHRP12 primers and then digested with AciI. The allele with a C ($\mathbf{E^D}$) at position 296 bp after digestion with AciI should show 3 DNA fragments of 50 bp, 90 bp and 97 bp. It was not possible to see the 7 bp difference in an agarose gel electrophoresis, therefore the fragments of 90 and 97 bp were seen as a single band of double intensity. The allele with a T at position 296 gave two DNA bands of 90 bp and 147 bp. Heterozygous animals showed three bands: 50 bp, (90+97) bp and 147 bp (Figure 16).

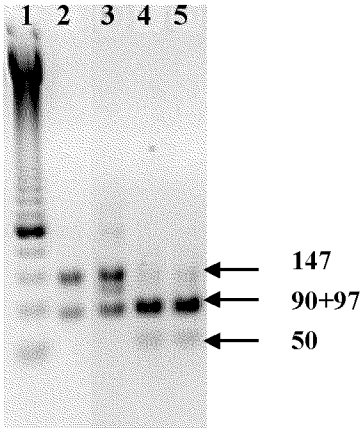

Figure 16: An example of the T296C RFLP

Lane 1: 50 bp DNA ladder (bands start with 50 bp followed by 100 bp, 150 bp etc. with a bright band at 250 bp); **Lanes 2 and 3:** homozygous animals having a T a position 296; **Lanes 4 and 5:** homozygous animals having a C at position 296 ($\mathbf{E^D/E^D}$).

C667T substitution: Genomic DNA was amplified using the primers MSHRP13 and MSHRP14, and the PCR product was digested with MspI. After digestion with MspI the variant with a C at position 667 should give two DNA fragments of 241 bp and 25 bp. The 25 bp band was, however not visible on the agarose gel. The variant with a T at position 667 ($\mathbf{E^{d1}}$) had only one band of 266 bp. Heterozygous animals have two bands of 266 bp and 241 bp (Figure 17).

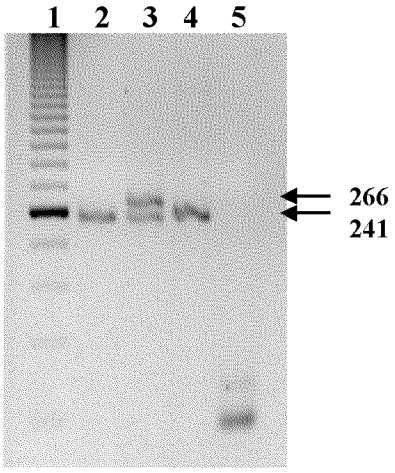

Figure 17: An example of C667T RFLP

Lane 1: 50 bp DNA ladder; **Lane 2 and 4:** a homozygous animal with a C at position 667; **Lane 3:** a C/T heterozygous animal ($\mathbf{E^{d1}}$/-); **Lane 5:** H_2O PCR negative control.

651 duplication: Genomic DNA was amplified using the MSHRP15 and MSHRP16 primers. The variant containing duplication ($\mathbf{E^{d2}}$) showed a band of 193 bp and the variant without duplication had a band of 181 bp.

These four polymorphic sites were tested in 12 unrelated Brown Swiss animals (Table 4).

None of the investigated Brown Swiss animals contained a 310/311G deletion or a C at position 296. The **C667T** base substitution and the 651 duplication were polymorphic inside the breed. According to the performed assays a third MC1R variant, containing a **C667** and no duplication is present in Brown Swiss. This variant was not sequenced, and is possibly identical to the **E+** allele reported by Klungland et al. (1995).

Four unrelated black Holstein animals were genotyped as $\mathbf{E^D/E^D}$ or $\mathbf{E^D/e}$. The **310/311G** deletion RFLP (red factor test) is routinely performed in this laboratory to reveal the carriers of the recessive red **e** allele in the Holstein breed. Red Holstein animals have always been found to be **e/e** homozygotes, which is consistent with the data of Klungland et al. (1995).

Table 4: A combination of the four polymorphic sites and predicted MC1R genotypes in Swiss cattle

Breed	Phenotype	310/311G deletion	T296C Leu99Pro	C667T Arg223Trp	Duplication	Predicted genotype
Brown Swiss	brown	-/-	TT LeuLeu	TT TrpTrp	-/-	$\mathbf{E^{d1}/E^{d1}}$
Brown Swiss	brown	-/-	TT LeuLeu	TC TrpArg	-/-	$\mathbf{E^{d1}/E+}$
Brown Swiss	brown	-/-	TT LeuLeu	TC TrpArg	du/-	$\mathbf{E^{d1}/E^{d2}}$
Brown Swiss	brown	-/-	TT LeuLeu	CC ArgArg	du/du/	$\mathbf{E^{d2}/E^{d2}}$
Holstein	black	-/-	CC ProPro	CC ArgArg	-/-	$\mathbf{E^D/E^D}$
Holstein	black	de/-	TC LeuPro	CC ArgArg	-/-	$\mathbf{E^D/e}$
Red Holstein	red	de/de	TT LeuLeu	CC ArgArg	-/-	**e/e**
Simmental	red	de/de	TT LeuLeu	CC ArgArg	-/-	**e/e**
Simmental	red	de/-	TT LeuLeu	CC ArgArg	-/-	$\mathbf{e/e^f}$

de represents the presence of the deletion

du represents the presence of duplication

Dashes (-) represent the absence of the deletion and the duplication

The modern Simmental breed consists only of red animals, therefore the red factor test is normally not performed for Simmental cattle due to the recessive mode of inheritance of the red coat colour. However, genotyping of three random animals from the Simmental breed revealed one bull (Fleuron, CH 71200607457360), which was heterozygous for the 310/311G deletion. The MC1R coding sequence of this bull was cloned into the pGEM® Easy vector and sequenced. Two types of clones were obtained: clones containing the 310/311G deletion and clones without the deletion, containing a **T890C** base substitution**.** No suitable restriction enzyme was found to perform an RFLP assay at this polymorphic site. Therefore, the existing polymorphism was confirmed by direct sequencing of the PCR product, which was amplified with the primers MSHRP16 and MC1RR1 and using the High Fidelity PCR system with a decreased error rate (Figure 18). The 310/311G deletion RFLP test was also applied to the mother (Fichte CH 71007004949760) and the father (Remo CH 71282006638960) of Fleuron. This test revealed that Remo was homozygous for the deletion and Fichte was heterozygous. Sequencing of the MSHRP16-MC1RR1 PCR products amplified from DNA of Fichte and Remo showed that Remo was homozygous for **T890** and Fichte was heterozygous for **T890C**. Cloning and sequencing of the complete MC1R coding sequence in Fichte showed that she has the **T890C** variant of MC1R identical to Fleuron. Thus, a new MC1R variant was found in Simmental containing the **T890C** substitution in the DNA sequence and **Ile297Thre** in the deduced amino acid sequence. This variant was designated as $\mathbf{e}^{\mathbf{f}}$, where **e** stands for recessive red and **f** stands for Fleuron, the first animal where it was found. The $\mathbf{e}^{\mathbf{f}}$ also contained one base substitution **T663C**, which is the third residue of the codon and thus does not change the amino acid.

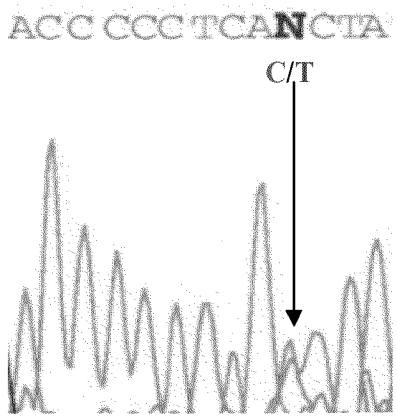

Figure 18: Part of the sequencing image of the PCR fragment of Fleurons MC1R gene

An arrow points the **T890C** heterozygous position in the Fleuron MC1R gene.

The DNA and deduced amino acid sequences were aligned to all known bovine MC1R variants: **E+**, **E^D**, **E^{d1}**, **E^{d2}**, **e** and **e^f** (Figure 19 and 20). The location of the transmembrane domains (TM), extracellular and intracellular loops (EL and IL) were determined by comparison with the mouse and human MC1R sequences. The **E+** allele amino acid sequence was compared to the MC1R of other species to reveal the conservative and identical amino acids (Figure 21). **Leu99** and **Ile297** are conserved among the wild type receptors of the mouse, human, horse, sheep, fox, dog, pig and cattle, indicating that amino acids at these positions can be important for the function of the receptor. The **Arg223** is conservative in all species, except mice, which has a **Gln** at this position. Arginine belongs to the class of acidic side chain amino acids, while glutamine belongs to the class of uncharged polar side chain amino acids, and tryptophan, which is located at position 223 in the **E^{d1}** variant of cattle, is an aromatic amino acid with uncharged polar side chains. These observations indicate, that the type of amino acid located at this position is not important for the receptor function. The **ARGI** motive in the IL3 is present in the dog, fox, sheep, horse and cattle, and it is changed into **GRHI** in the pig, **AQGI** in the human and **VQGI** in the mouse. Thus, it is difficult to predict if the duplication of ARGI motive in the **E^{d2}** cattle variant can influence the function of MC1R.

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1   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50 Ed1
1   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50 E+
1   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50 ED
1   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50 ef
1   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50 e
1   ATGCCTGCAC TTGGCTCCCA GAGGCGGCTG CTGGGTTCCC TTAACTGCAC  50 Ed2

51  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100 Ed1
51  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100 E+
51  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100 ED
51  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100 ef
51  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100 e
51  GCCCCCAGCC ACCCTCCCCT TCACCCTGGC CCCCAACCGG ACGGGGCCCC 100 Ed2

101 AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150 Ed1
101 AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150 E+
101 AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150 ED
101 AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150 ef
101 AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150 e
101 AGTGCCTGGA GGTGTCCATC CCTGACGGGC TCTTTCTCAG CCTGGGGCTG 150 Ed2

151 GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200 Ed1
151 GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200 E+
151 GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200 ED
151 GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200 ef
151 GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200 e
151 GTGAGTCTCG TGGAGAACGT GCTGGTAGTG GCTGCCATTG CCAAGAACCG 200 Ed2
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201 CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG  250 E+
201 CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG  250 E^D
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201 CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG  250 e
201 CAACCTGCAC TCCCCCATGT ACTACTTTAT CTGCTGCCTG GCTGTGTCTG  250 E^d2

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251 ACTTGCTGGT GAGCGTCAGC AACGTGCTGG AGACGGCAGT CATGCCGCTG  300 E^D
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251 ACTTGCTGGT GAGCGTCAGC AACGTGCTGG AGACGGCAGT CATGCTGCTG  300 e
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301 CTGGAGGCCG GTGTCCTGGC CACCCAGGCG GCCGTGGTGC AGCAGCTGGA  350 E^D
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351 CAATGTCATC GACGTGCTCA TCTGCGGATC CATGGTGTCC AGCCTCTGCT  400 E^D
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401 TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG  450 E^D
401 TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG  450 e^f
400 TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG  449 e
401 TCCTGGGTGC CATTGCTGTG GACCGCTACA TCTCCATCTT CTACGCCCTG  450 E^d2

451 CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC  500 E^d1
451 CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC  500 E+
451 CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC  500 E^D
451 CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC  500 e^f
450 CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC  499 e
451 CGGTACCACA GTGTTGTGAC ACTGCCCCGA GCGTGGAGGA TCATTGCGGC  500 E^d2

501 CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA  550 E^d1
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501 CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA  550 E^D
501 CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA  550 e^f
500 CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA  549 e
501 CATCTGGGTG GCCAGCATCC TCACCAGCCT GCTCTTCATC ACCTACTACA  550 E^d2

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551 ACCACAAGGT CATCCTGCTG TGCCTCGTTG GCCTCTTCAT AGCTATGCTG  600 E^d2

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601 GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA  650 E^D
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600 GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA  649 e
601 GCCCTGATGG CCGTCCTCTA CGTCCACATG CTGGCCCGGG CCTGCCAGCA  650 E^d2
```

```
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651 .......... ..TGCCCGGG GCATTGCCCG GCTCCAGAAG AGGCAGCGCC  688 E+
651 .......... ..TGCCCGGG GCATTGCCCG GCTCCAGAAG AGGCAGCGCC  688 ED
651 .......... ..TGCCCGGG GCATCGCCCG GCTCCAGAAG AGGCAGCGCC  688 ef
650 .......... ..TGCCCGGG GCATTGCCCG GCTCCAGAAG AGGCAGCGCC  687 e
651 TGCCCGGGGC ATTGCCCGGG GCATTGCCCG GCTCCAGAAG AGGCAGCGCC  700 Ed2

689 CCATTCATCA GGGCTTTGGC CTCAAGGGCG CTGCCACCCT CACCATCCTG  738 Ed1
689 CCATTCATCA GGGCTTTGGC CTCAAGGGCG CTGCCACCCT CACCATCCTG  738 E+
689 CCATTCATCA GGGCTTTGGC CTCAAGGGCG CTGCCACCCT CACCATCCTG  738 ED
689 CCATTCATCA GGGCTTTGGC CTCAAGGGCG CTGCCACCCT CACCATCCTG  738 ef
688 CCATTCATCA GGGCTTTGGC CTCAAGGGCG CTGCCACCCT CACCATCCTG  737 e
701 CCATTCATCA GGGCTTTGGC CTCAAGGGCG CTGCCACCCT CACCATCCTG  750 Ed2

739 CTGGGCGTCT TCTTCCTCTG CTGGGGCCCC TTCTTCCTGC ACCTCTCGCT  788 Ed1
739 CTGGGCGTCT TCTTCCTCTG CTGGGGCCCC TTCTTCCTGC ACCTCTCGCT  788 E+
739 CTGGGCGTCT TCTTCCTCTG CTGGGGCCCC TTCTTCCTGC ACCTCTCGCT  788 ED
739 CTGGGCGTCT TCTTCCTCTG CTGGGGCCCC TTCTTCCTGC ACCTCTCGCT  788 ef
738 CTGGGCGTCT CCTTCCTCTG CTGGGGCCCC TTCTTCCTGC ACCTCTCGCT  787 e
751 CTGGGCGTCT TCTTCCTCTG CTGGGGCCCC TTCTTCCTGC ACCTCTCGCT  800 Ed2

789 CATCGTCCTC TGCCCCCAGC ACCCCACCTG TGGCTGCATC TTCAAGAACT  838 Ed1
789 CATCGTCCTC TGCCCCCAGC ACCCCACCTG TGGCTGCATC TTCAAGAACT  838 E+
789 CATCGTCCTC TGCCCCCAGC ACCCCACCTG TGGCTGCATC TTCAAGAACT  838 ED
789 CATCGTCCTC TGCCCCCAGC ACCCCACCTG TGGCTGCATC TTCAAGAACT  838 ef
788 CATCGTCCTC TGCCCCCAGC ACCCCACCTG TGGCTGCATC TTCAAGAACT  837 e
801 CATCGTCCTC TGCCCCCAGC ACCCCACCTG TGGCTGCATC TTCAAGAACT  850 Ed2

839 TCAACCTCTT CCTGGCCCTC ATCATTTGCA ACGCCATTGT GGACCCCCTC  888 Ed1
839 TCAACCTCTT CCTGGCCCTC ATCATTTGCA ACGCCATTGT GGACCCCCTC  888 E+
839 TCAACCTCTT CCTGGCCCTC ATCATTTGCA ACGTCATTGT GGACCCCCTC  888 ED
839 TCAACCTCTT CCTGGCCCTC ATCATTTGCA ACGCCATTGT GGACCCCCTC  888 ef
838 TCAACCTCTT CCTGGCCCTC ATCATTTGCA ATGCCATTGT GGACCCCCTC  887 e
851 TCAACCTCTT CCTGGCCCTC ATCATTTGCA ACGCCATTGT GGACCCCCTC  900 Ed2

889 ATCTATGCCT TCCGCAGCCA GGAGCTCCGG AAGACGCTCC AAGAGGTGCT  938 Ed1
889 ATCTATGCCT TCCGCAGCCA GGAGCTCCGG AAGACGCTCC AAGAGGTGCT  938 E+
889 ATCTATGCCT TCCGCAGCCA GGAGCTCCGG AAGACGCTCC AAGAGGTGCT  938 ED
889 ACCTATGCCT TCCGCAGCCA GGAGCTCCGG AAGACGCTCC AAGAGGTGCT  938 ef
888 ATCTATGCCT TCCGCAGCCA GGAGCTCCGG AAGACGCTCC AAGAGGTGCT  937 e
901 ATCTATGCCT TCCGCAGCCA GGAGCTCCGG AAGACGCTCC AAGAGGTGCT  950 Ed2

939 GCAGTGCTCC TGGTGA  954 Ed1
939 GCAGTGCTCC TGGTGA  954 E+
939 GCAGTGCTCC TGGTGA  954 ED
939 GCAGTGCTCC TGGTGA  954 ef
938 GCAGTGCTCC TGGTGA  953 e
951 GCAGTGCTCC TGGTGA  966 Ed2
```

Figure 19: Alignment of the coding sequences of the five MC1R variants characterised in this study $\mathbf{E}^{D}$, **e**, $\mathbf{E}^{d1}$, $\mathbf{E}^{d2}$ and $\mathbf{e}^{f}$ as well as **E**+ allele reported by Klungland et al. (1995, accession number Y13957)

Non-identical and non-synonymous residues are shown in grey squares. The non-identical, but synonymous change in the $\mathbf{e}^{f}$ allele is shown in a white square.

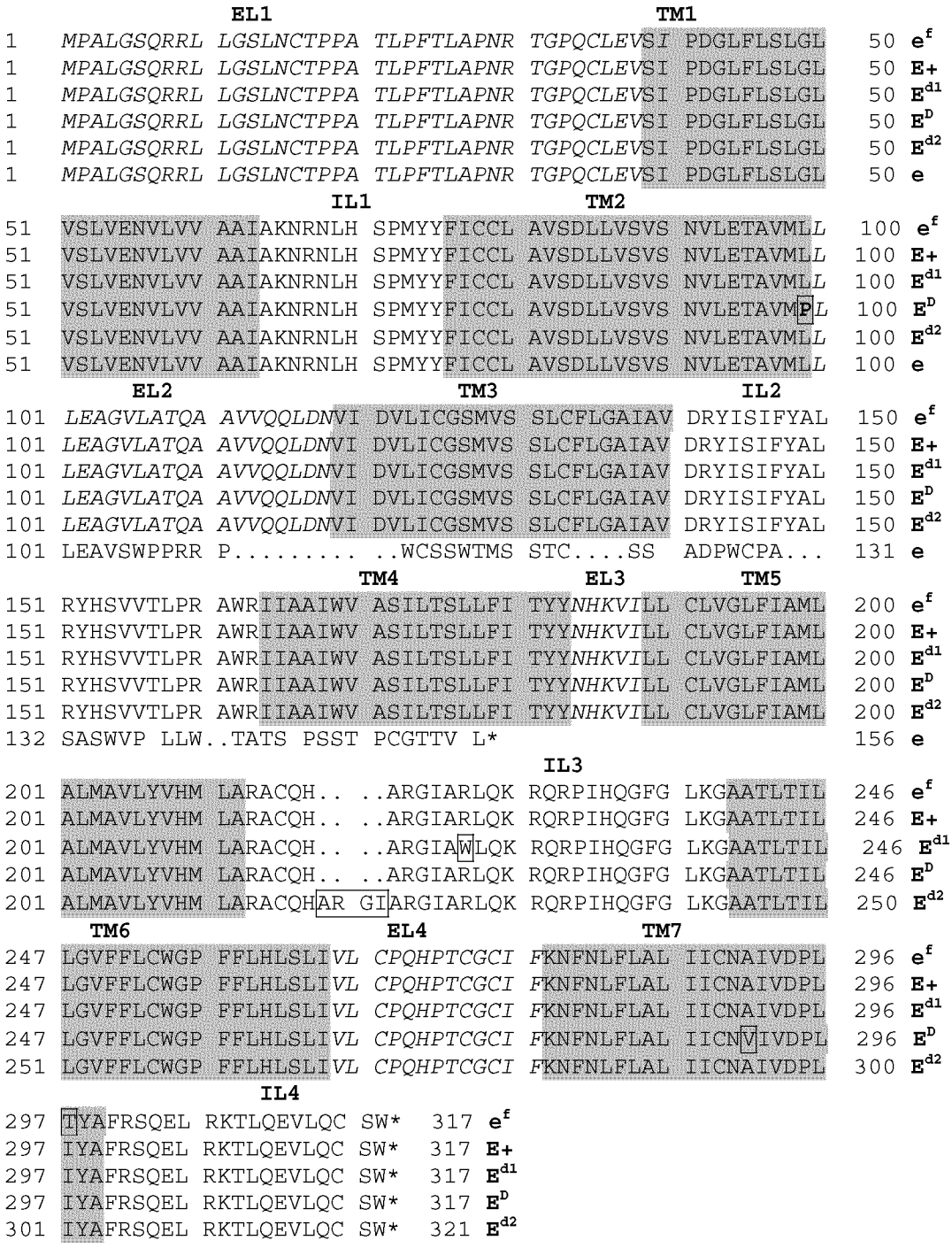

Figure 20: Comparison of the deduced amino acid sequences of six bovine MC1R variants ($\mathbf{E^D}$, **E+**, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$, **e** and $\mathbf{e^f}$)

Non-identical amino acids are shown in squares, except for the e allele. Stop codons are shown with stars. Putative transmebrane domains (TM) are shown in grey. Extracellular loops (EL) are shown in cursive, and intracellular loops (IL) are shown in normal letters.

```
cattle          1   MPALGSQRRL LGSLNCTP-- PATLPFTLA- --PNRTGPQC LEVSIPDGLF
dog             1   MVWQGPQRRL LGSLNGTS-- PATPHFELA- --ANQTGPRC LEVSIPNGLF
fox             1   MSGQGPQRRL LGSPNATS-- PTTPHFKLA- --ANQTGPRC LEVSIPNGLF
sheep           1   MPVLGSQRRL LGSLNCTP-- PATLPLTLA- --PNRTGPQC LEVSIPDGLF
horse           1   MPLQGPQRRL LGSLNSTL-- PATPYLGLT- --TNQTEPPC LEVSIPDGLF
pig             1   MPVLGPERRL LASLS--Sap PAAPRLGLAa nqTNQTGPQC LEVSIPDGLF
man             1   MAVQGSQRRL LGSLNSTP-- TAIPQLGLA- --ANQTGARC LEVSISDGLF
mouse           1   MSTQEPQKSL LGSLN--S-- NATSHLGLA- --TNQSEPWC LYVSIPDGLF

cattle         46   LSLGLVSLVE NVLVVAAIAK NRNLHSPMYY FICCLAVSDL LVSVSNVLET
dog            46   LSLGLVSVVE NVLVVAAIAK NRNLHSPMYY FIGCLAVSDL LVSVTNVLET
fox            46   LSLGLVSVVE NVLVVAAIAK NRNLHSPMYY FIGCLAVSDL LVSVTNVLET
sheep          46   LSLGLVSLVE NVLVVAAIAK NRNLHSPMYY FICCLAMSDL LVSVSNVLET
horse          46   LSLGLVSLVE NVLVVTAIAK NRNLHSPMYY FICCLAVSDL LVSMSNVLEM
pig            49   LSLGLVSLVE NVLVVAAIAK NRNLHSPMYY FVCCLAVSDL LVSVSNVLET
man            46   LSLGLVSLVE NALVVATIAK NRNLHSPMYC FICCLALSDL LVSGTNVLET
mouse          44   LSLGLVSLVE NVLVVIAITK NRNLHSPMYY FICCLALSDL MVSVSIVLET

cattle         96   AVMLLLEAGV LATQAAVVQQ LDNVIDVLIC GSMVSSLCFL GAIAVDRYIS
dog            96   AVMLLVEAGA LAAQAAVVQQ LDDIIDVLIC GSMVSSLCFL GAIAVDRYLS
fox            96   AVMLLVEAGA LAAQAAVVQQ LDDIIDVLIC GSMVSSLCFL GAIAVDRYLS
sheep          96   AVMLLLEAGV LATRAAVVQQ LDNVIDVLIC SSMVSSLCFL GAIAVDRYIS
horse          96   AILLLLEAGV LATQASVLQQ LDNIIDVLIC GSMVSSLCFL GSIAVDRYIS
pig            99   AVLLLLEAGA LAAQAAVVQQ LDNVMDVLIC GSMVSSLCFL GAIAVDRYVS
man            96   AVILLLEAGA LVARAAVLQQ LDNVIDVITC SSMLSSLCFL GAIAVDRYIS
mouse          94   TIILLLEVGI LVARVALVQQ LDNLIDVLIC GSMVSSLCFL GIIAIDRYIS

cattle        146   IFYALRYHSV VTLPRAWRII AAIWVASILT SLLFITYYNH KVILLCLVGL
dog           146   IFYALRYHSI VTLPRAWRAI SAIWVASVLS STLFIAYYNH TAVLLCLVSF
fox           146   IFYALRYHSI VTLPRAWRAI SAIWVASVLS STLFIAYYNH TAVLLCLVSF
sheep         146   IFYALRYHSV VTLPRAWRII AAIWVASILT SVLSITYYNH TVVLLCLVGF
horse         146   IFYALRYHSI MMLPRVWRAI VAIWVVSVLS STLFIAYYNH TAVLLCLVTF
pig           149   IFYALRYHSI VTLPRAGRAI AAIWAGSVLS STLFIAYYHH TAVLLGLVSF
man           146   IFYALRYHSI VTLPRAPRAV AAIWVASVVF STLFIAYYDH VAVLLCLVVF
mouse         144   IFYALRYHSI VTLPRARRAV VGIWMVSIVS STLFITYYKH TAVLLCLVTF

cattle        196   FIAMLALMAV LYVHMLARAC QHARGIARLQ KRQRPIHQGF GLKGAATLTI
dog           196   FVAMLVLMAV LYVHMLARAR QHARGIARLR KRQHSVHQGF GLKGAATLTI
fox           196   FVAMLVLMAV LYVHMLARAR QHARGIARLR KRQHSVHQGF GLKGAATLTI
sheep         196   FIAMLALMAV LYVHMLARAC QHARGIARLQ KRQRPIHQGF GLKGAATLTI
horse         196   FVAMLVLMAV LYVHMLARAC QHARGIARLH KRQHPIHQGF GLKGAATLTI
pig           199   FVAMLALMAV LYVHMLARAC QHGRHIARLH KIQHPTRQGC GLKGAATLTI
man           196   FLAMLVLMAV LYVHMLARAC QHAQGIARLH KRQRPVHQGF GLKGAVTLTI
mouse         194   FLAMLALMAI LYAHMFTRAC QHVQGIAQLH KRRRSIRQGF CLKGAATLTI

cattle        246   LLGVFFLCWG PFFLHLSLIV LCPQHPTCGC IFKNFNLFLA LIICNAIVDP
dog           246   LLGIFFLCWG PFFLHLSLMV LCPQHPICGC VFQNFNLFLT LIICNSIIDP
fox           246   LLGIFFLCWG PFFLHLSLMV LCPQHPICGC VFQNFNLFLT LIICNSIIDP
sheep         246   LLGVFFLCWG PFFLHLSLIV LCPQHPTCGC IFKNFNLFLA LIICNAIVDP
horse         246   LLGVFFLCWG PFFLHLSLLI LCPQHPTCGC VFKNFKLFLT LILCSAIVDP
pig           249   LLGVFLLCWA PFFLHLSLVV LCPQHPTCGC VFKNVNLFLA LVICNSIVDP
man           246   LLGIFFLCWG PFFLHLTLIV LCPEHPTCGC IFKNFNLFLA LIICNAIIDP
mouse         244   LLGIFFLCWG PFFLHLLLIV LCPQHPTCSC IFKNFNLFLL LILSSTVDP
```

```
cattle      296   LIYAFRSQEL RKTLQEVLQC SW*
dog         296   FIYAFRSQEL RKTLQEVVLC SW*
fox         296   FIYAFRSQEL RKTLQEVVLC SW*
sheep       296   LIYAFRSQEL RKTLQEVLQC SW*
horse       296   LIYAFRSQEL RKTLQEVLLC SW*
pig         299   LIYAFRSQEL RKTLQEVLQC SW*
man         296   LIYAFHSQEL RRTLKEVLTC SW*
mouse       294   LIYAFRSQEL RMTLKEVLLC SW*
```

Figure 21: Comparison of the bovine MC1R amino acid sequence with that of the other mammalian species human (X65634), mouse (X65635), pig (AF326520), sheep (Y13965), horse (AF288357), dog (AF064455) and fox (X90844)

Non-identical amino acids are shown in black. Boxed sequences show transmembrane domains in the mouse sequence according to Cone et al. (1996). Stop codons are shown with stars.

4.1.2 Creation of the stable lines expressing MC1R alleles

The coding sequences of five MC1R variants ($\mathbf{E^D}$, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$, **e** and $\mathbf{e^f}$) were cloned into the mammalian expression vector pcDNA3.1(+). The pcDNA3.1(+) clones were transfected into HEK 293 cells and the cells stably expressing MC1R alleles were selected by resistance to G418. Two independent transfections were performed for the $\mathbf{e^f}$ variant: one clone was taken from the mother Fichte and one clone from the son Fleuron. Thus, two cell lines expressing $\mathbf{e^f}$ ($\mathbf{e^f}$-Fichte and $\mathbf{e^f}$-Fleuron) MC1R allele were established, although these clones have identical DNA sequences.

4.1.2.1 Expression of bovine MC1R variants in the transfected cell lines

To confirm the expression of MC1R in the 293 cells, reverse transcription with subsequent real time PCR was performed with RNA extracted from the cells. RT was done with 1 µg of RNA using the TVX primer (Table 7). Real time PCR was performed with 2.5 µl of RT reaction using the MSHRP21 and MSHRP22 primers and the Taq-Man ETH1S probe for the $\mathbf{E^D}$, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$, $\mathbf{e^f}$-Fleuron and $\mathbf{e^f}$-Fichte cell lines and Taq-Man ETH2R probe for the **e** cell line. 0.1 µg of RNA was taken directly to the real time PCR to detect DNA contamination present in the RNA sample.

The threshold line for determination of the C_T value was manually adjusted and put at the same level in all samples. The C_T values for real time PCR amplification of RT-products and the 0.1 µg of RNA are shown in the Table 5.

Table 5: C_T values for real time PCR amplification of RT-products and RNA of cell lines expressing MC1R variants

Cell line	C_T for RT product	C_T for contaminant DNA in 0.1 µg of RNA
$\mathbf{E^D}$	25.46	35.30
$\mathbf{E^{d1}}$	23.54	35.68
$\mathbf{E^{d2}}$	27.7	34.63
$\mathbf{e^f}$-Fleuron	22.05	34.67
$\mathbf{e^f}$-Fichte	29.92	35.66
e	34.08	40.00

DNA was present in all RNA samples at low levels, despite of DNase treatment. In the cell lines $\mathbf{E^D}$, $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$, $\mathbf{e^f}$-Fleuron and $\mathbf{e^f}$-Fichte DNA was present at comparable levels with C_T varying from 34.63 to 35.68. In the **e** cell line the curve of contaminant DNA was also present, but at the lower level and it did not reach the threshold line (Figure 22).

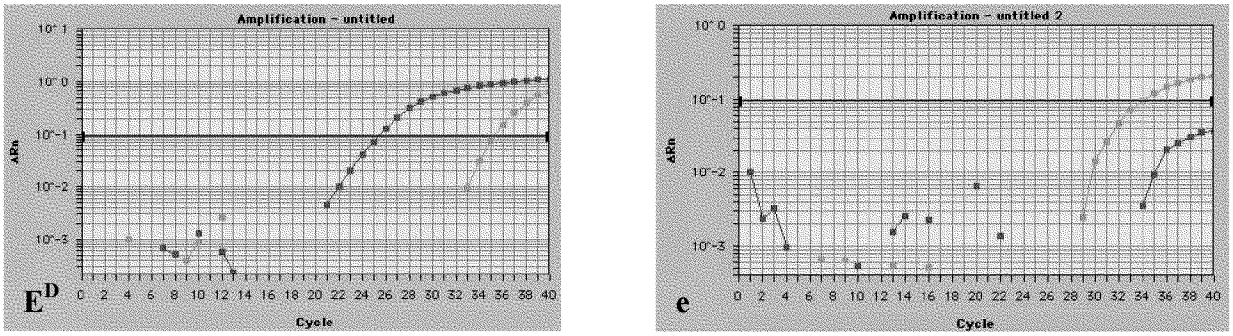

Figure 22: Amplification plots for the $\mathbf{E^D}$ and **e** cell lines

ΔRn is plotted against the cycle number. The threshold line is adjusted manually at 10^{-1}. RT-product is red and contaminant DNA is green in the $\mathbf{E^D}$ sample, and vice versa in the **e** sample.

The real time amplification curves for RT-products were also shown for all cell lines with the C_T values considerably higher than for contaminant DNA, indicating that MC1R RNA was expressed in each cell line. The C_T values varied between samples, with a maximum of 34.08 for the **e** cell line and a minimum of 22.05 for the cell line $\mathbf{e^f}$-Fleuron.

The big difference of the **e** cell line threshold cycle values for both RT-products and contaminant DNA can be explained by the different TaqMan probes used for this cell line, and

thus the C_T values for it can not be compared with the other cell lines. However, for all other cell lines where the same TaqMan probe was used, the C_T values for contaminant DNA were within the same range and the C_T values for RT products considerably varied (the minimum 22.05 for the **e**^{f} -Fleuron and the maximum 29.92 for the **e**^{f}-Fichte).

The purpose of performing the real time PCR was to confirm the expression of the MC1R alleles in the transfected cell lines, but not to quantify the level of RNA expression. Therefore each real time PCR reaction was performed only once, while usually it should be performed in triplicate and the mean and the standard deviation should be calculated to compare the level of expression between the samples. Comparison of the C_T values showed an approximately 250 times higher expression level in the Fleuron cell line than in the Fichte cell line. Even so, as mentioned above the aim of this experiment was to verify the expression of the MC1R in the transfected cell lines, and not to compare the expression levels between the cell lines.

4.1.3 Pharmacological characterisation of the bovine MC1R alleles

Intracellular cAMP was measured as a response to α-MSH stimulation in the cell lines expressing five variants of cattle MC1R and in the HEK 293 cells as a negative control. The HEK 293 cells have the cAMP level close to zero at all concentrations of α-MSH. The **E**^{D} and **e** receptor alleles were unresponsive to a wide range of α-MSH concentrations with the same level of intracellular cAMP at about 1 pmole per $5x10^5$ cells (Figure 23).

The **E**^{d1} and **E**^{d2} receptor variants responded to α–MSH stimulation in a dose-dependent manner. The median effective concentration (EC_{50}) was at approximately at the same level ($10^{-6.75}$) for both variants, but the **E**^{d2} variant appeared to have a higher ability to elevate intracellular cAMP in response to α-MSH, achieving the maximum levels of approximately 100 pmoles of cAMP per $5x10^5$ cells, while maximum level of the intracellular cAMP for the **E**^{d1} variant was approximately 30 pmoles per $5x10^5$ cells (Figure 24).

Measuring the intracellular cAMP of the **e**^{f} variant in the **e**^{f}-Fleuron cell culture revealed that the stimulation curve did not achieve the plateau level at the concentration of 10^{-5} M of α–MSH, while the curves of the **E**^{d1} and **E**^{d2} receptor variants achieved a maximum stimulation level already at the 10^{-6} M of α-MSH. Therefore additional transfection was performed with the **e**^{f} MC1R allele and thus one more cell line (**e**^{f}-Fichte) expressing the the **e**^{f} variant was produced. The intracellular cAMP for both **e**^{f} cell lines was measured at additional concentrations of α-MSH $10^{-6.5}$, $10^{-5.5}$, $10^{-4.5}$ and 10^{-4} M. The obtained stimulation curve for

the $\mathbf{e^f}$ receptor allele (taken as an average of the Fleuron and Fichte cell lines curves) was shifted to the right and had higher EC_{50} value ($10^{-5.25}$) when compared to the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ curves. However, the maximum of cAMP were the same for the $\mathbf{e^f}$ and the $\mathbf{E^{d1}}$ variants (Figure 24).

Taking in account, that the measuring of cAMP for the $\mathbf{e^f}$ allele was performed using two different cell lines, the curves for both of them are shown (Figure 25). The Fleuron cell line had lower cAMP level at the α-MSH concentrations of 10^{-5} and $10^{-5.5}$ M. However, it can not be argued that this difference occurs due to the lower level of MC1R expression in Fleuron cell line, because according to real time PCR results, the RNA expression level was much higher in the Fleuron cell line. Most probably the differences in intracellular cAMP production levels between the Fleuron and Fichte cell lines are not significant, lying within the error of experiment and not depending on the level of RNA expression. This difference of the Fleuron and Fichte curves does not affect the shifting to the right of the average curve relatively to the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ receptor curves.

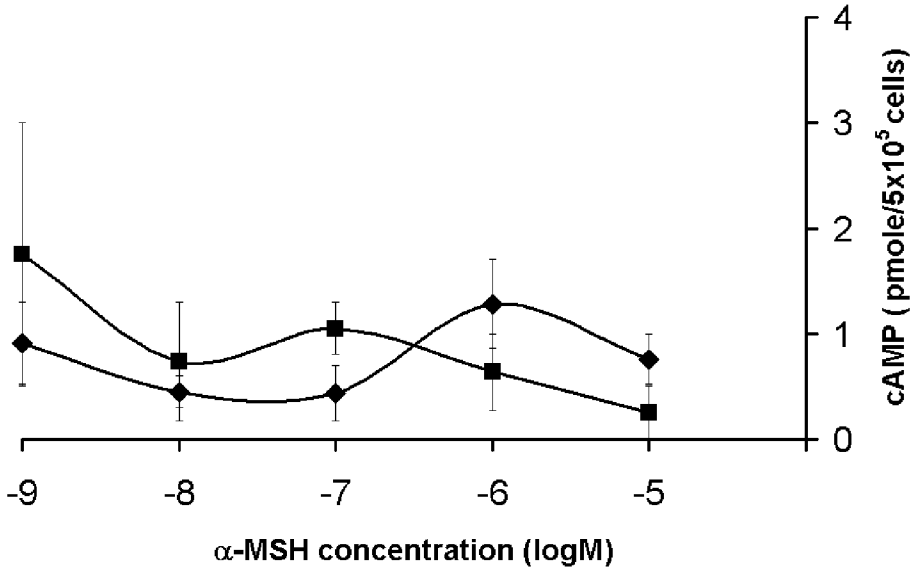

Figure 23: Accumulation of intracellular cAMP in 293 cells transfected with **e** (■) and $\mathbf{E^D}$ (◆) MC1R variants in response to increasing concentrations of α-MSH

Data points indicate the means of two independent stimulation experiments and bars indicate the standard errors.

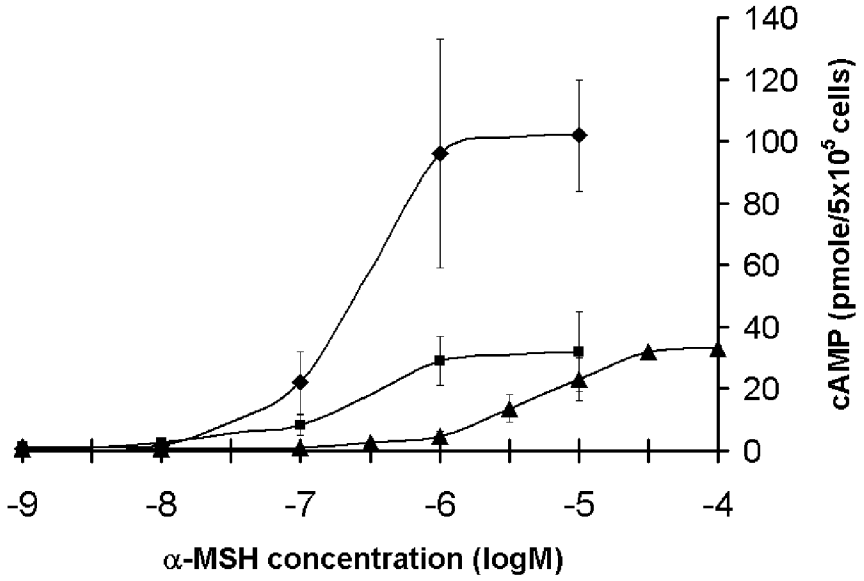

Figure 24: Accumulation of intracellular cAMP in 293 cells transfected with E^{d1}(■), E^{d2}(◆) and e^{f} (▲) MC1R variantss in response to increasing concentrations of α-MSH

Data points indicate the mean of the three independent stimulation experiments and bars indicate the standard errors.

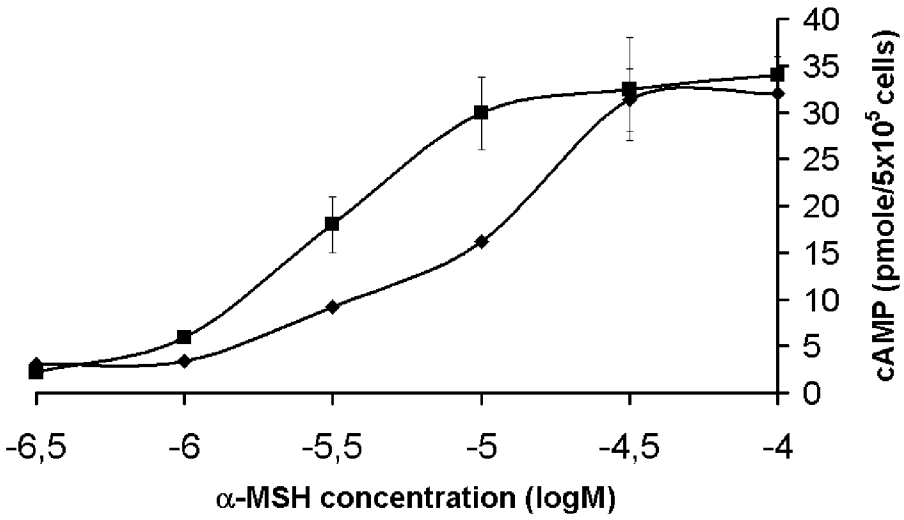

Figure 25: Accumulation of intracellular cAMP in the two cell lines of Fleuron(◆) and Fichte(■) transfected with the e^{f} MC1R variant in response to increasing concentrations of α-MSH

Data points indicate the mean of two measurements of the same stimulation sample and bars indicate the standard errors.

4.2 Agouti

4.2.1 Sequencing of the bovine agouti coding sequence

The bovine agouti complete coding sequence, containing coding exons 1, 2 and 3 [exons 2, 3 and 4 respectively according to the mouse nomenclature (Siracusa, 1994)] was amplified using primers AgF1 and AgR1 (Table 9) designed based on the bovine GenBank sequence (accession number X99692). An AGG triplet was added before the start codon in the AgF1 primer, to have an A residue in position -3, which is required for the proper initiation of translation (Kozak, 1987), when the coding sequence is subcloned into the pcDNA3.1(+) mammalian expression vector and expressed in mammalian cells. To obtain cDNA for amplification reverse transcription was performed with RNA isolated from the testes of Simmental and Brown Swiss bulls, and liver of Brown Swiss and Holstein cows using TVX primer (Table 7). PCR with the primers MgP3 and MgP4 (Table 10) for the attractin gene which were positive for both testes and kidney cDNA was performed as a positive control of reverse transcription. However, it was only possible to amplify the agouti coding sequence from cDNA transcribed from RNA of Simmental bull testes, and there was no visible signal of amplification from the cDNA of Brown Swiss bull testes and Brown Swiss and Holstein cow's livers. The obtained fragment of the Simmental testes agouti coding sequence of size 405 bp was cloned into the pGem®-T Easy vector. Three of the obtained clones were sequenced, and all of them contained sequence showing 100% identity to the sequence from the GenBank (accession number X99692).

As it was not possible to amplify the coding sequence of agouti from Holstein and Brown Swiss cDNA, new primers lying in the introns closely adjacent to the exon sequences were designed based on the genomic DNA sequence of the bovine agouti (accession number X99691) for amplification of the each coding exon separately. Thus the coding exon 1 was amplified using primers AgF1 and AgI1R1, the coding exon 2 was amplified using primers AgI1F1 and AgI2R1 and the coding exon 3 was amplified using primers AgI2F1 and AgR1. Genomic DNA of a Brown Swiss bull and a Holstein cow was used for amplification. The obtained fragments of the sizes 185 bp (exon 1), 150 bp (exon 2) and 213 bp (exon 3) were cloned into the pGem®-T Easy vector. Two clones of each exon in both Holstein and Brown Swiss breeds were sequenced, and no differences were found within the coding sequence when compared to the agouti coding sequence of Simmental breed.

The deduced amino acid sequence of bovine agouti was compared with the agouti amino acid sequences of mouse, human, fox, sheep, horse and pig (Figure 26).

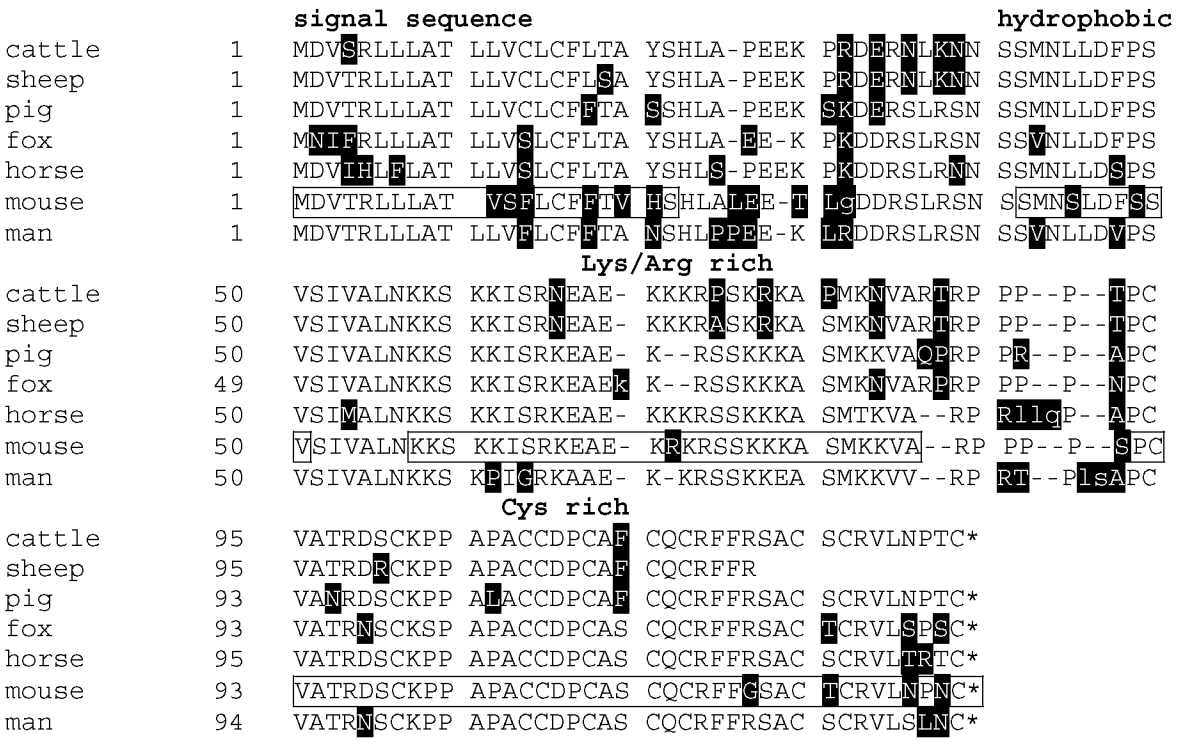

Figure 26: Comparison of the ASIP deduced amino acid sequences of cattle, sheep (Parsons et al., 1999), pig (AJ251837), fox (Y09877), horse (AF288358), mouse (L06941) and human (NM 001672)

Non identical amino acids are shown in black. Boxed sequences show functional domains in the mouse sequence according to Virador et al. (2000). Stop-codons are shown with stars.

The **R-F-F** (118-120 in cattle) motive in the carboxyl terminus of ASIP, which was shown to be critical for the affinity of the mouse ASIP to MC1R, MC3R and MC4R (Kiefer et al., 1998) is conserved in all compared species. The **KVARP** motive (82-86 in cattle) in a Lys/Arg rich region, shown in in the mouse to be minimal functional domain in this region (Virador et al., 2000), is however not conserved in all species. It is changed into **NVART** in the cattle and sheep, **KVAQP** in the pig, **NVARP** in the fox and **KVVRP** in the human. Comparison of bovine ASIP and AGRP showed a relatively low similarity of 37% with the highest homology in the carboxyl terminus, especially between cysteine residues (Figure 27).

```
AGRP   1 MLTAVLLSCALLLAMPPLQGAQMGPAPLEGIGRPEEALFLELQGLS...L 47
         :| | || |   |        :  |     :         |:   .|   |
ASIP   6 LLLATLLVCLCFLTAYSHLAPEEKPRDERNLKNNSSMNLLDFPSVSIVAL 55

AGRP  48 QPSLKRITEEQAEESLLQEAEAKALAEVLDPEGRKPRSPRRCVRLHESCL 97
             |:|.  :||.   ..  .|  | . .    :|  |  ||   :||
ASIP  56 NKKSKKISRNEAEK...KKRPSKRKAPMKNVARTRPPPPTPCVATRDSCK 102

AGRP  98 GHQVPCCDPCATCYCRFFNAFCYCRKLGTT 127
               |||||| | |||| . | || |  |
ASIP 103 PPAPACCDPCAFCQCRFFRSACSCRVLNPT 132
```

Figure 27: Comparison of the amino acid sequences of bovine ASIP and AGRP (AJ002025)

4.2.2 Pharmacological characterisation of the bovine ASIP

The coding sequence of bovine ASIP was cloned into the mammalian expression vector pcDNA3.1(+) and transfected into the HEK 293 cells. $5x10^5$ cells expressing ASIP were simultaneously seeded with $5x10^5$ cells expressing either the $\mathbf{E^{d1}}$ or the $\mathbf{E^{d2}}$ MC1R variant and stimulate with α-MSH at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M. As a control $5x10^5$ cells expressing the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ MC1R variants were stimulated with the same concentrations of α–MSH. Total intracellular cAMP from the whole culture was measured. cAMP present in the cells expressing agouti was considered to be negligible when compared to the cells expressing MC1R due to the close to zero levels of cAMP in the HEK 293 cells, stimulated with α-MSH. A significant reduction of the cAMP production was observed in the cells expressing MC1R, which were cultured together with the cells expressing ASIP, when compared to the cells expressing MC1R cultured alone (Figures 28 and 29).

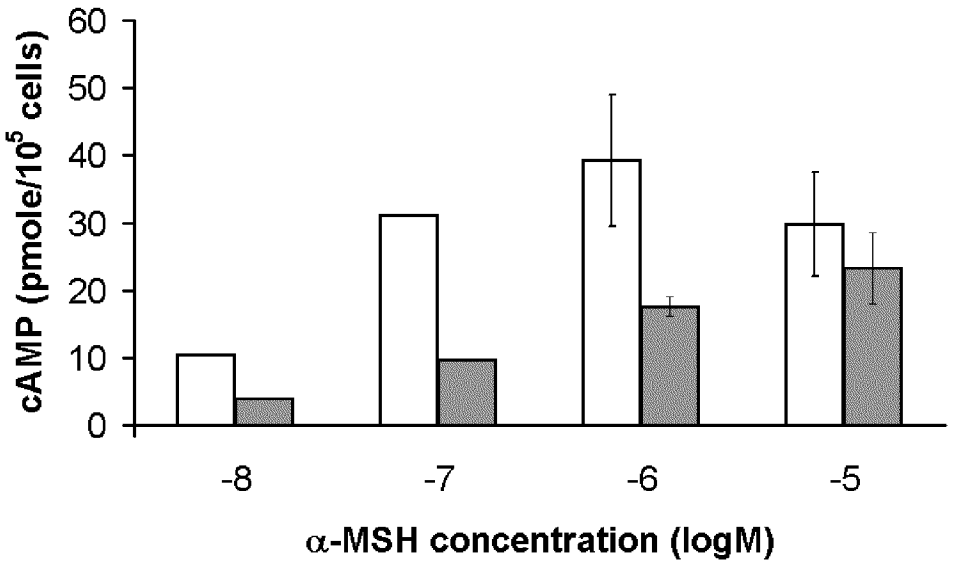

Figure 28: Accumulation of intracellular cAMP in the cells transfected with $\mathbf{E^{d1}}$ MC1R variant co-incubated with the cells transfected with ASIP (grey columns) and the cells transfected with $\mathbf{E^{d1}}$ MC1R variant alone (white columns)

Bars indicate the standard errors of two measurements of the same stimulation sample.

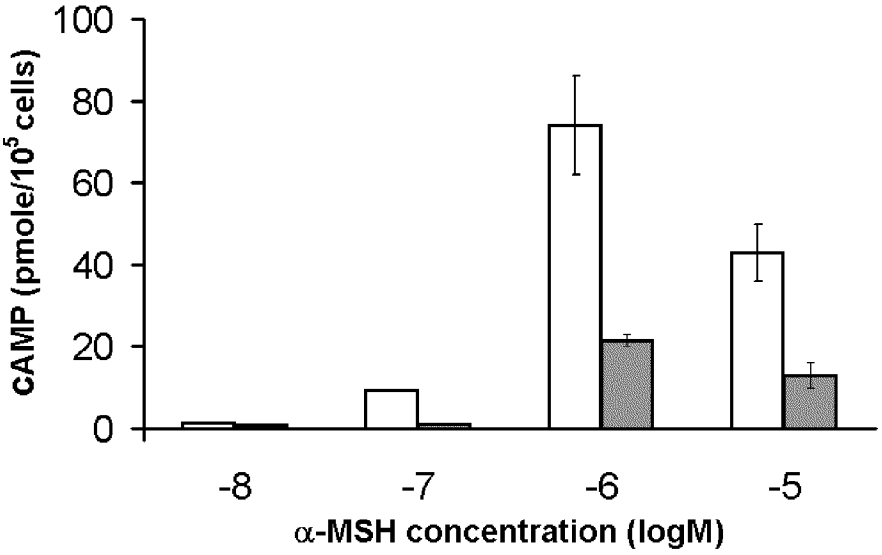

Figure 29: Accumulation of intracellular cAMP in the cells transfected with $\mathbf{E^{d2}}$ MC1R variant co-incubated with the cells transfected with ASIP (grey columns) and the cells transfected with $\mathbf{E^{d2}}$ MC1R variant alone (white columns)

Bars indicate the standard errors of two measurements of the same stimulation sample.

4.3 Attractin

4.3.1 Amplification of the bovine attractin coding sequence

The mouse mahogany protein mRNA sequence (AF116897) and the human attractin mRNA (AF034957) were compared and the regions of 100% homology were chosen for designing primers. cDNA reverse transcribed from RNA extracted of Brown Swiss liver with the TVX primer (Table 7) was used for amplification of the coding sequence of bovine attractin. First two fragments, that were not overlapping were amplified using the primer pairs MgP1-MgP2 (~1.3 kb) and MgP3-MgP4 (~0.8 kb) (Table 10). The fragment (~1.3 kb) overlapping with the previous two fragments was amplified using the primers MgP7 and MgP8, designed using the sequence of the MgP1-MgP2 and MgP3-MgP4 fragments (Figure 30). The MgPend primer was designed in the area of the highest homology of the 3' UTR sequences between the mouse mahogany protein mRNA sequence (AF116897) and human transmembrane form attractin (AF218915). A fragment of ~1.5 kb was amplified using the MgP9 primer and the cross-species MgPend primer (Figure 30). The obtained fragments were cloned into the pGem®-T Easy vector and sequenced.

4.3.2 5' end of the bovine attractin

Amplification of the cDNA from the 5' region using cross-species primers did not produce any PCR products, probably due to the lower homology between species in this area. Therefore the 5' RACE method was applied to amplify the 5' end of the bovine attractin gene. Reverse primers were designed in the 5' end of the already known sequence and used for reverse transcription of RNA from the liver of Brown Swiss. MgP14 (Table 10) was the optimal primer for RT, and the primer MgR1 together with the oligo dT-anchor primer (Table 7) was used for the first amplification of cDNA. Nested PCR with the PCR product using MgR2 primer (Table 10) together with the PCR anchor primer (Table 7) produced two fragments of 250 bp and 400 bp, which were cloned into a pGem®-T Easy vector. Sequencing of the clones containing the 250 bp fragment showed that it was an unspecific sequence. However, sequencing of the clones containing 400 bp fragment has revealed a sequence homological to the 5' end of the previously sequenced part of the bovine attractin. Thus, with the help of the 5' RACE approximately 100 bp of the new 5' end sequence of the bovine attractin were obtained

(Figure 30). Search for the putative translation start in the bovine attractin sequence did not find any ATG which would initiate translation into a full-length protein, leading to the assumption that the reverse transcription was not full processive until the very beginning of the gene mRNA. 5' RACE with the reverse primers lying closer to the 5' end of the bovine attractin generated only unspecific sequences. To obtain the full coding sequence of the bovine attractin, a bovine BAC genomic library produced from fibroblasts of a Holstein male foetus (http://www-dga.jouy.inra.fr/grafra/) was screened. Exon-intron boundaries were defined in the cDNA of bovine atrractin by comparison with the human genomic sequence of attractin (AF218889). Primer pairs were designed in the exons 1 and 3. Screening the BAC library by PCR with the primer pair Mgex3F1 and Mgex3R1 from the exon 3 gave a positive signal, and thus the BAC clone Mgex3 was isolated. The BAC clone was sequenced using the reverse primer Mgex1R1 from the first exon. Thus, the remaining sequence from the 5' end of the coding sequence of the bovine attractin was obtained. The next step was to amplify the full coding sequence of the bovine attractin. However, designing the primers at the 5' UTR end, as well as at the beginning of the coding sequence (Figure 35, Appendix) did not provide satisfactory results. Amplification of the cDNA transcribed from the RNA of Brown Swiss and Holstein liver and Brown Swiss and Simmental testes, using four forward primers from the 5' end and the MgPend did not produce any PCR products. An attempt to amplify shorter fragments of the sizes ranging from 200 to 1500 bp using the same forward primers and reverse primers closer to the 5' end also was not successful, producing either no PCR product, or unspecific products. Amplification of the genomic DNA of Simmental, Brown Swiss and Holstein cattle using the same forward primers and Mgex1R1 also produced unspecific fragments. The reason of the amplification failure of the 5' end of the bovine attractin might be low processivity of the reverse transcription, i.e. the inability of the enzyme to transcribe a complete cDNA sequence. However, a control PCR with the cDNA of all samples and the primers MgexF1 and MgP2 have amplified a specific fragment.

4.3.3 Sequencing of the attractin coding sequence from different cattle breeds

The overlapping fragments of the MgP1-MgP2, MgP7-MgP8, MgP3-MgP4 and MgP9-MgPend (Figure 30) were amplified using cDNA reverse transcribed from RNA of Holstein liver, Brown Swiss liver and testes and Simmental testes. The obtained PCR fragments were cloned into the pGem®-T Easy vector and subsequently sequenced. No

differences were found between the sequences of the clones from different breeds, except for few single nucleotide polymorphisms, which were not checked with any SNP test and thus may be either errors of the Taq-polymerase or actual SNPs.

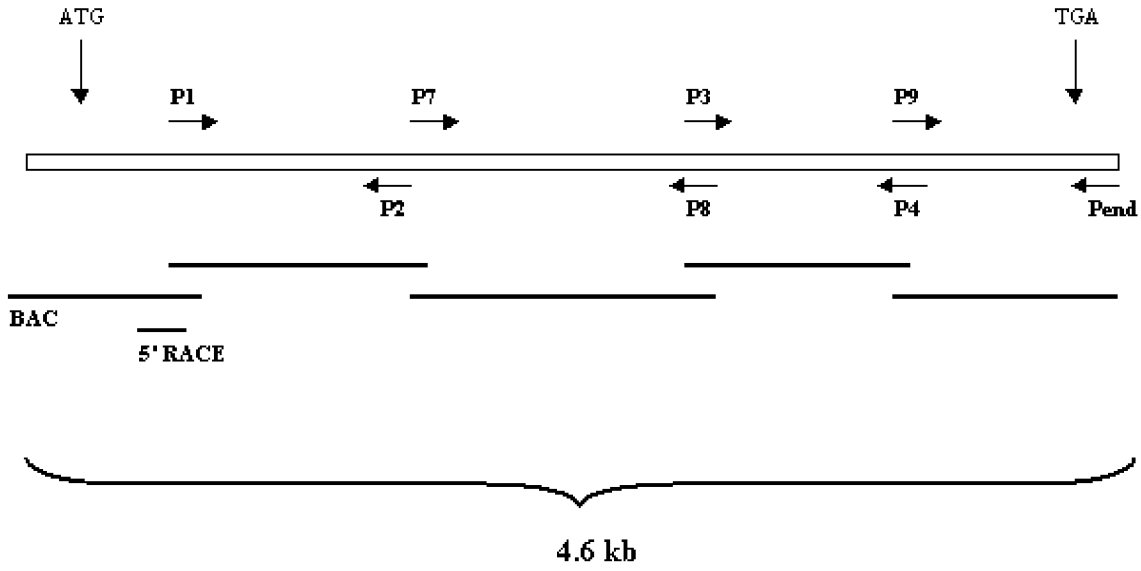

Figure 30: Scheme of the amplification and sequencing of the bovine attractin

4.3.4 Sequence analysis of the bovine attractin

The location of the putative start codon (ATG) was determined by comparison with the mouse attractin sequence (AF116897). The DNA sequence was deduced into a protein sequence of 1415 amino acids (Figure 35, Appendix). Attractin protein sequences of the cattle, mouse, rat and two secreted and one membrane form of the human were compared (Figure 31).

```
cattle          1   mv-----AAA TEARL-RRTV VTPAPTSRNG RRCRDWDATR AGRPGLRTGL
mouse           1   mv-AVAAAAA TEARLRGSTT TTAAPAGRKG RQHRPCTATG AWRPGPRARL
man1            1   M---VAAAAA TEARLRRRTA ATAALAGRSG GPH------- ----------
man2            1   M---VAAAAA TEARLRRRTA ATAALAGRSG GPHWDWDVTR AGRPGLGAGL
man3            1   M---VAAAAA TEARLRRRTA ATAALAGRSG GPHWDWDVTR AGRPGLGAGL
rat             1   mvaAAAAAEA TEARLRGYTT ATAAPAGWKE RQHRPCAATG AWRPWPRAGL

cattle         45   RLPRrwrr-- -----LLL-- -LLLPP---L LLLPWAAEAA AAAASVSGSA
mouse          50   CLPRVLSRAL PPPPLLPL-- -LFS---LLL LPLPREAEAA AVAAAVSGSA
man1           31   ---------- ---------- ---------- ---------- ----------
man2           48   RLPRLLSPPL RPRLLLLL-- -LLLPPPLLL LLLPCEAEAA AAAAAVSGSA
man3           48   RLPRLLSPPL RPRLLLLL-- -LLLSPPLLL LLLPCEAEAA AAAAAVSGSA
rat            51   CLPRVLSRAL SPPPLLPLlp lLFS---LLL LPLPREAEAA AVAAAVSGSA
```

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                                  EGF
cattle        82   AAEAKECDRP CVNGGRCNPG TGQCVCPAGW VGEQCQHCGG RFRLTGSSGF
mouse         94   AAEAKECDRP CVNGGRCNPG TGQCVCPTGW VGEQCQHCGG RFRLTGSSGF
man1          31   ---------- CVNGGRCNPG TGQCVCPAGW VGEQCQHCGG RFRLTGSSGF
man2          95   AAEAKECDRP CVNGGRCNPG TGQCVCPAGW VGEQCQHCGG RFRLTGSSGF
man3          95   AAEAKECDRP CVNGGRCNPG TGQCVCPAGW VGEQCQHCGG RFRLTGSSGF
rat           98   AAEAKECDRP CVNGGRCNPG TGQCVCPTGW VGEQCQHCGG RFRLTGSSGF

cattle       132   ITDGPGNYKY KTKCTWLIEG QPNRIMRLRF NHFATECSWD HLYVYDGDSI
mouse        144   VTDGPGNYKY KTKCTWLIEG QPNRIMRLRF NHFATECSWD HLYVYDGDSI
man1          71   VTDGPGNYKY KTKCTWLIEG QPNRIMRLRF NHFATECSWD HLYVYDGDSI
man2         145   VTDGPGNYKY KTKCTWLIEG QPNRIMRLRF NHFATECSWD HLYVYDGDSI
man3         145   VTDGPGNYKY KTKCTWLIEG QPNRIMRLRF NHFATECSWD HLYVYDGDSI
rat          148   VTDGPGNYKY KTKCTWLIEG QPNKIMRLRF NHFATECSWD HLYVYDGDSI
                                        CUB
cattle       182   YAPLVAAFSG LIVPERDGNE TVPEVVATSG YALLHFFSDA AYNLTGFNIT
mouse        194   YAPLIAAFSG LIVPERDGNE TAPEVTVTSG YALLHFFSDA AYNLTGFNIT
man1         121   YAPLVAAFSG LIVPERDGNE TVPEVVATSG YALLHFFSDA AYNLTGFNIT
man2         195   YAPLVAAFSG LIVPERDGNE TVPEVVATSG YALLHFFSDA AYNLTGFNIT
man3         195   YAPLVAAFSG LIVPERDGNE TVPEVVATSG YALLHFFSDA AYNLTGFNIT
rat          198   YAPLIAAFSG LIVPERDGNE TAPEVTVTSG YALLHFFSDA AYNLTGFNIT
                                        EGF
cattle       232   YNFDMCPNNC SGRGECKISN SSNTVQCECS ENWKGEACDI PHCVNNCGFP
mouse        244   YNFDMCPNNC SGRGECKSSN SSSAVECECS ENWKGESCDI PHCTDNCGFP
man1         171   YSFDMCPNNC SGRGECKISN SSDTVECECS ENWKGEACDI PHCTDNCGFP
man2         245   YSFDMCPNNC SGRGECKISN SSETVECECS ENWKGEACDI PHCTDNCGFP
man3         245   YSFDMCPNNC SGRGECKISN SSDTVECECS ENWKGEACDI PHCTDNCGFP
rat          248   YNFDMCPNNC SGRGECKSSN SSSTVECECS ENWKGESCDI PHCTDNCGFP

cattle       282   HRGICNSSDV RGCSCFSEWQ GPGCSVPVPA NQSFWTREEY SDLKLPRASH
mouse        294   HRGICNASDT RGCSCFPHWQ GPGCSIPVPA NQSFWTREEY SDLKLPRASH
man1         221   HRGICNSSDV RGCSCFSDWQ GPGCSVPVPA NQSFWTREEY SNLKLPRASH
man2         295   HRGICNSSDV RGCSCFSDWQ GPGCSVPVPA NQSFWTREEY SNLKLPRASH
man3         295   HRGICNSSDV RGCSCFSDWQ GPGCSVPVPA NQSFWTREEY SNLKLPRASH
rat          298   HRGICNASDT RGCSCFPHWQ GPGCSIPVPA NQSFWTREEY SDLKLPRASH

cattle       332   KAVVNGNIMW VVGGYMFNHS DYNMVLAYDL ASREWLALNR SVNSVVVRYG
mouse        344   KAVVNGNIMW VVGGYMFNHS DYSMVLAYDL TSREWLPLNH SVNSVVVRYG
man1         271   KAVVNGNIMW VVGGYMFNHS DYNMVLAYDL ASREWLPLNR SVNNVVVRYG
man2         345   KAVVNGNIMW VVGGYMFNHS DYNMVLAYDL ASREWLPLNR SVNNVVVRYG
man3         345   KAVVNGNIMW VVGGYMFNHS DYNMVLAYDL ASREWLPLNR SVNNVVVRYG
rat          348   KAVVNGNIMW VVGGYMFNHS DYSMVLAYDL ASREWLSLNH SVNSVVVRYG

cattle       382   HSLALYQDKI YMYGGKIDST GNVTNELRVF HIHNESWVLL SPKAKEQYAV
mouse        394   HSLALHKDKI YMYGGKIDST GNVTNELRVF HIHNESWVLL TPKAKDQYAV
man1         321   HSLALYKDKI YMYGGKIDST GNVTNELRVF HIHNESWVLL TPKAKEQYAV
man2         395   HSLALYKDKI YMYGGKIDPT GNVTNELRVF HIHNESWVLL TPKAKEQYAV
man3         395   HSLALYKDKI YMYGGKIDST GNVTNELRVF HIHNESWVLL TPKAKEQYAV
rat          398   HSLALHKDKI YMYGGKIDST GNVTNELRVF HIHNESWVLL TPKAKDQYAV

cattle       432   VGHSAHIVTL KNGRVVMLVI FGHCPLYGYI SSVQEYDLDK NTWSILHTQG
mouse        444   VGHSAHIVTL ASGRVVMLVI FGHCPLYGYI SVVQEYDLEK NTWSILHTQG
man1         371   VGHSAHIVTL KNGRVVMLVI FGHCPLYGYI SNVQEYDLDK NTWSILHTQG
man2         445   VGHSAHIVTL KNGRVVMLVI FGHCPLYGYI SNVQEYDLDK NTWSILHTQG
man3         445   VGHSAHIVTL KNGRVVMLVI FGHCPLYGYI SNVQEYDLDK NTWSILHTQG
rat          448   VGHSAHIVTL SSGRVVMLVI FGHCPLYGYI SVVQEYDLEK NTWSILQTQG
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cattle      482   ALVQGGYGHS SVYDDRTKAL YIHGGYKAFS ANKYRLADDL YRYDVDTQMW
mouse       494   ALVQGGYGHS SVYDDRTKAL YVHGGYKAFS ANKYRLADDL YRYDVDTQMW
man1        421   ALVQGGYGHS SVYDHRTRAL YVHGGYKAFS ANKYRLADDL YRYDVDTQMW
man2        495   ALVQGGYGHS SVYDHRTRAL YVHGGYKAFS ANKYRLADDL YRYDVDTQMW
man3        495   ALVQGGYGHS SVYDHRTRAL YVHGGYKAFS ANKYRLADDL YRYDVDTQMW
rat         498   ALVQGGYGHS SVYDHRTKAL YVHGGYKAFS ANKYRLADDL YRYHVDTQMW

cattle      532   TILKDSRFFR YLHTAVIVSG TMLVFGGNTH NDTSMSHGAK CFSSDFMAYD
mouse       544   TILKDSRFFR YLHTAVIVSG TMLVFGGNTH NDTSMSHGAK CFSSDFMAYD
man1        471   TILKDSRFFR YLHTAVIVSG TMLVFGGNTH NDTSMSHGAK CFSSDFMAYD
man2        545   TILKDSRFFR YLHTAVIVSG TMLVFGGNTH NDTSMSHGAK CFSSDFMAYD
man3        545   TILKDSRFFR YLHTAVIVSG TMLVFGGNTH NDTSMSHGAK CFSSDFMAYD
rat         548   TILKDSRFFR YLHTAVIVSG TMLVFGGNTH NDTSMSHGAK CFSSDFMAYD

cattle      582   IDCDRWSVLP RPDLHHDVNR FGHSAVLYNR TMYVFGGFNS LLLSDILVFT
mouse       594   IACDRWSVLP RPELHHDVNR FGHSAVLYNS TMYVFGGFNS LLLSDVLVFT
man1        521   IACDRWSVLP RPDstmmstd laipAVLHNS TMYVFGGFNS LLLSDILVFT
man2        595   IACDRWSVLP RPDLHHDVNR FGHSAVLHNS TMYVFGGFNS LLLSDILVFT
man3        595   IACDRWSVLP RPDLHHDVNR FGHSAVLHNS TMYVFGGFNS LLLSDILVFT
rat         598   IACDRWSVLP RPELHHDVNR FGHSAVLHNS TMYVFGGFNS LLLSDVLVFT

cattle      632   SEQCEAHQSE AACLAAGPGV RCMWDTGSSQ CVSWELAQ-E AQEKIKSECF
mouse       644   SEQCDAHRSE AACVAAGPGI RCLWDTQSSR CTSWELATEE QAEKLKSECF
man1        571   SEQCDAHRSE AACLAAGPGI RCVWNTGSSQ CISWALATDE QEEKLKSECF
man2        645   SEQCDAHRSE AACLAAGPGI RCVWNTGSSQ CISWALATDE QEEKLKSECF
man3        645   SEQCDAHRSE AACLAAGPGI RCVWNTGSSQ CISWALATDE QEEKLKSECF
rat         648   SEQCDAHRSE AACVAAGPGI RCLWDTQSSR CTSWELATEE QAEKLKSECF

cattle      681   SKRIFDHDRC DQLTDCYSCT ANTNgCQWCN DHCVPTNHSC TEGQISIFKY
mouse       694   SKRTLDHDRC DQHTDCYSCT ANTNDCHWCN DHCVPVNHSC TEGQISIAKY
man1        621   SKRTLDHDRC DQHTDCYSCT ANTNDCHWCN DHCVPRNHSC SEGQISIFRY
man2        695   SKRTLDHDRC DQHTDCYSCT ANTNDCHWCN DHCVPRNHSC SEGQISIFRY
man3        695   SKRTLDHDRC DQHTDCYSCT ANTNDCHWCN DHCVPRNHSC SEGQISIFRY
rat         698   SKRTLDHDRC DQHTDCYSCT ANTNDCHWCN DHCVPVNHSC TEGQISIAKY

cattle      731   DHCPKDNPMY YCNKKTSCRS CALDQNCQWE PRNQECIALP ENICGIGWHL
mouse       744   ESCPKDNPMY YCNKKTSCRS CALDQNCQWE PRNQECIALP ENICGNGWHL
man1        671   ENCPKDNPMY YCNKKTSCRS CALDQNCQWE PRNQECIALP ENICGIGWHL
man2        745   ENCPKDNPMY YCNKKTSCRS CALDQNCQWE PRNQECIALP ENICGIGWHL
man3        745   ENCPKDNPMY YCNKKTSCRS CALDQNCQWE PRNQECIALP ENICGIGWHL
rat         748   DNCPKDNPMY YCNKKTSCRS CALDQNCQWE PRNQECIALP ENICGIGWHL

cattle      781   VGNSCLKITT ARETYDNAKL SCRNHNAFLA SLTTQKKVEF VLKQLRIMQS
mouse       794   VGNSCLKITT AKENYDNAKL SCRNHNAFLA SLTSQKKVEF VLKQLRLMQS
man1        721   VGNSCLKITT AKENYDNAKL FCRNHNALLA SLTTQKKVEF VLKQLRIMQS
man2        795   VGNSCLKITT AKENYDNAKL FCRNHNALLA SLTTQKKVEF VLKQLRIMQS
man3        795   VGNSCLKITT AKENYDNAKL FCRNHNALLA SLTTQKKVEF VLKQLRIMQS
rat         798   VGNSCLKITT AKENYDNAKL SCRNHNAFLA SLTSQKKVEF VLKQLRLMQS
                                        C-type lectin
cattle      831   SQSMSKFTLT PWVGLRKINV SYWCWEDMSP FTNSSLQWMP SEPSDAGFCG
mouse       844   SQSMSKLTLT PWVGLRKINV SYWCWEDMSP FTNSLLQWMP SEPSDAGFCG
man1        771   SQSMSKLTLT PWVGLRKINV SYWCWEDMSP FTNSLLQWMP SEPSDAGFCG
man2        845   SQSMSKLTLT PWVGLRKINV SYWCWEDMSP FTNSLLQWMP SEPSDAGFCG
man3        845   SQSMSKLTLT PWVGLRKINV SYWCWEDMSP FTNSLLQWMP SEPSDAGFCG
rat         848   SQSTSKLTLT PWVGLRKINV SYWCWEDMSP FTNSLLQWMP SEPSDAGFCG

cattle      881   ILSEPSTRGL KAATCINPLN GSVCERPANH SAKQCRTPCA LRTACGECTS
mouse       894   ILSEPSTRGL KAATCINPLN GSVCERPANH SAKQCRTPCA LRTACGECTS
man1        821   ILSEPSTRGL KAATCINPLN GSVCERPANH SAKQCRTPCA LRTACGDCTS
man2        895   ILSEPSTRGL KAATCINPLN GSVCERPANH SAKQCRTPCA LRTACGDCTS
man3        895   ILSEPSTRGL KAATCINPLN GSVCERPANH SAKQCRTPCA LRTACGDCTS
rat         898   ILSEPSTRGL KAATCINPLN GSVCERPANH SAKQCRTPCA LRTACGECTS
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cattle      931   GSSECMWCSN MKQCVDSNAY VASFPFGQCM EWYTMSSCPP ENCSGYCTCS
mouse       944   SSSECMWCSN MKQCVDSNAY VASFPFGQCM EWYTMSSCPP ENCSGYCTCS
man1        871   GSSECMWCSN MKQCVDSNAY VASFPFGQCM EWYTMSTCPP ENCSGYCTCS
man2        945   GSSECMWCSN MKQCVDSNAY VASFPFGQCM EWYTMSTCPP ENCSGYCTCS
man3        945   GSSECMWCSN MKQCVDSNAY VASFPFGQCM EWYTMSTCPP ENCSGYCTCS
rat         948   SSSECMWCSN MKQCVDSNAY VASFPFGQCM EWYTMSSCPP ENCSGYCTCS

cattle      981   HCLEQPGCGW CTDPSNTGKG KCIEGSYKGP VKMPSQGPTG NSYPQPLLNS
mouse       994   HCLEQPGCGW CTDPSNTGKG KCIEGSYKGP VKMPSQASAG NVYPQPLLNS
man1        921   HCLEQPGCGW CTDPSNTGKG KCIEGSYKGP VKMPSQAPTG NFYPQPLLNS
man2        995   HCLEQPGCGW CTDPSNTGKG KCIEGSYKGP VKMPSQAPTG NFYPQPLLNS
man3        995   HCLEQPGCGW CTDPSNTGKG KCIEGSYKGP VKMPSQAPTG NFYPQPLLNS
rat         998   HCLEQPGCGW CTDPSNTGKG KCIEGSYKGP VKMPSHASTG NVYPQPLLNS
                                                  L-type EGF
cattle     1031   SMCLEDSRYN WSFIHCPACQ CNGHSKCINQ SICEKCENLT TGKHCETCIS
mouse      1044   SMCLEDSRYN WSFIHCPACQ CNGHSKCINQ SICEKCEDLT TGKHCETCIS
man1        971   SMCLEDSRYN WSFIHCPACQ CNGHSKCINQ SICEKCENLT TGKHCETCIS
man2       1045   SMCLEDSRYN WSFIHCPACQ CNGHSKCINQ SICEKCENLT TGKHCETCIS
man3       1045   SMCLEDSRYN WSFIHCPACQ CNGHSKCINQ SICEKCENLT TGKHCETCIS
rat        1048   SMCLEDSRYN WSFIHCPACQ CNGHSKCINQ SICEKCEDLT TGKHCETCIS
                                                  L-type EGF
cattle     1081   GFYGDPTNGG KCQPCRCNGH ASLCNTNTGK CFCTTKGVKG DECQLCEVEN
mouse      1094   GFYGDPTNGG KCQPCKCNGH ASLCNTNTGK CFCTTKGVKG DECQLCEVEN
man1       1021   GFYGDPTNGG KCQPCKCNGH ASLCNTNTGK CFCTTKGVKG DECQLCEVEN
man2       1095   GFYGDPTNGG KCQPCKCNGH ASLCNTNTGK CFCTTKGVKG DECQLCEVEN
man3       1095   GFYGDPTNGG KCQPCKCNGH ASLCNTNTGK CFCTTKGVKG DECQLCEVEN
rat        1098   GFYGDPTNGG KCQPCKCNGH ASLCNTNTGK CFCTTKGVKG EECQLCEVEN

cattle     1131   RYQGNPLKGT CYYTLLIDYQ FTFSLSQEDD RYYTAINFVA TPDEQNRDLD
mouse      1144   RYQGNPLKGT CYYTLLIDYQ FTFSLSQEDD RYYTAINFVA TPDEQNRDLD
man1       1071   RYQGNPLRGT CYYTLLIDYQ FTFSLSQEDD RYYTAINFVA TPDEQNRDLD
man2       1145   RYQGNPLRGT CYYTLLIDYQ FTFSLSQEDD RYYTAINFVA TPDEQNRDLD
man3       1145   RYQGNPLRGT CYYTLLIDYQ FTFSLSQKDD RYYTAINFVA TPDEQNRDLD
rat        1148   RYQGNPLKGT CYYTLLIDYQ FTFSLSQEDD RYYTAINFVA TPDEQNRDLD

cattle     1181   MFINASKNFN LNITWAASFS AGTQAGEEMP VVSKTNIKEY KDSFSNEKFD
mouse      1194   MFINASKNFN LNITWATSFP AGTQTGEEVP VVSKTNIKEY KDSFSNEKFD
man1       1121   MFINASKNFN LNITWAASFS AGTQAGEEMP VVSKTNIKEY KDSFSNEKFD
man2       1195   MFINASKNFN LNITWAASFS AGTQAGEEMP VVSKTNIKEY KDSFSNEKFD
man3       1195   MFINASKNFN LNITWAASFS AGTQAGEEMP VVSKTNIKEY KDSFSNEKFD
rat        1198   MFINASKNFN LNITWATSFP AGTQTGEEVP VVSKTNIKEY KDSFSNEKFD
                                                            TM
cattle     1231   FRNHPNITFF VYVSNFTWPI KIQIAFSQHS NFMDLVQFFV TFFSCFLSLL
mouse      1244   FRNHPNITFF VYVSNFTWPI KIQIAFSQHS NFMDLVQFFV TFFSCFLSLL
man1       1171   FRNHPNITFF VYVSNFTWPI KIQVQTEQ*- ---------- ----------
man2       1245   FRNHPNITFF VYVSNFTWPI KIQVQTEQ*- ---------- ----------
man3       1245   FRNHPNITFF VYVSNFTWPI KIQIAFSQHS NFMDLVQFFV TFFSCFLSLL
rat        1248   FRNHPNITFF VYVSNFTWPI KIQIAFSQHS NFMDLVQFFV TFFSCFLSLL

cattle     1281   LVAAVVWKIK QSCWASRRRE QLLREMQQMA SRPFASVNVA LETDEEPPDL
mouse      1294   LVAAVVWKIK QSCWASRRRE QLLREMQQMA SRPFASVNVA LETDEEPPDL
man1       1199   ---------- ---------- ---------- ---------- ----------
man2       1273   ---------- ---------- ---------- ---------- ----------
man3       1295   LVAAVVWKIK QSCWASRRRE QLLREMQQMA SRPFASVNVA LETDEEPPDL
rat        1298   LVAAVVWKIK QSCWASRRRE QLLREMQQMA SRPFASVNVA LETDEEPPDL

cattle     1331   IGGSIKTVPK PIALEPCFGN KAAVLSVFVR LPRGLGGIPP PGQSGLAVAS
mouse      1344   IGGSIKTVPK PIALEPCFGN KAAVLSVFVR LPRGLGGIPP PGQSrsrcgq
man1       1199   ---------- ---------- ---------- ---------- ----------
man2       1273   ---------- ---------- ---------- ---------- ----------
man3       1345   IGGSIKTVPK PIALEPCFGN KAAVLSVFVR LPRGLGGIPP PGQSGLAVAS
rat        1348   IGGSIKTVPK PIALEPCFGN KAAVLSVFVR LPRGLGGIPP PGQSGLAVAS
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cattle     1381   ALVDISQQMP VVYKEKSGAV RNRKQQPPAQ PGTCI*---- ----------
mouse      1394   cpgghfsada nsvqgevrsc kkpeaaaact awnlhlilgq efaltegvws
man1       1199   ---------- ---------- ---------- ---------- ----------
man2       1273   ---------- ---------- ---------- ---------- ----------
man3       1395   ALVDISQQMP IVYKEKSGAV RNRKQQPPAQ PGTCI*---- ----------
rat        1398   ALVDISQQMP IVYKEKSGAV RNRKQQPPAQ PGTCI*---- ----------

cattle     1416   ---------- ---------- ---------
mouse      1444   tklstgeaag rkqawgrrlg tleasnsca*
man1       1199   ---------- ---------- ---------
man2       1273   ---------- ---------- ---------
man3       1430   ---------- ---------- ---------
rat        1433   ---------- ---------- ---------
```

Figure 31: Comparison of the attractin deduced amino acid sequences of cattle, mouse (AF116897), two secreted forms of human attractin "man1" (AF034957) and "man2" (AF106861), membrane form of human attractin "man3" (AF218915) and rat (AB049248)

Non-identical amino acids are shown in black. The boxed sequences show the functional domains in the mouse sequence according to Gunn et al. (1999). Stop codons are indicated by stars.

The protein sequences of attractin are relatively well conserved between species, especially in the middle part of the protein containing functional domains. The N terminus before the first EGF domain is not as conserved as the middle part. The C-terminus after the transmembrane domain is completely homological between bovine, rat and the membrane form of human attractin, except for **Ile1391Val** substitution in the bovine sequence. The sequence of the mouse C-terminus entirely differs from the bovine, rat and human sequences after position 1387 (Figure 31).

Comparison of the nucleotide sequences of the bovine attractin sequenced in this study with the exons 26-29 of bovine attractin submitted to Genbank (accession number AF301008) have revealed three mismatches (Figure 31), however all of them were the third nucleotide of a codon, and thus did not change amino acid sequence.

```
AF3010089    1 GCACAGCAACTTCATGGACCTGGTACAGTTCTTCGTGACCTTCTTCAGTT 50
               ||||||||| || |||||||| ||||||||||||||||||||||||||||
          3945 GCACAGCAATTTTATGGACCTAGTACAGTTCTTCGTGACCTTCTTCAGTT 3994

AF3010089 51 GTTTCCTCTCTCTGCTCCTGGTGGCTGCTGTGGTTTGGAAGATCAAACAA 100
               ||||||||||||||||||||||||||||||||||||||||||||||||||
         3995 GTTTCCTCTCTCTGCTCCTGGTGGCTGCTGTGGTTTGGAAGATCAAACAA 4044
```

```
AF3010089 101 AGTTGTTGGGCTTCCAGACGTAGAGAGCAACTTCTTCGAGAGATGCAGCA 150
              ||||||||||||||||||||||||||||||||||||||||||||||||||
          4045 AGTTGTTGGGCTTCCAGACGTAGAGAGCAACTTCTTCGAGAGATGCAGCA 4094

AF3010089 151 GATGGCCAGTCGTCCCTTTGCCTCTGTAAATGTCGCCTTGGAAACAGATG 200
              ||||||||||||||||||||||||||||||||||||||||||||||||||
          4095 GATGGCCAGTCGTCCCTTTGCCTCTGTAAATGTCGCCTTGGAAACAGATG 4144

AF3010089 201 AAGAGCCTCCTGATCTTATTGGGGGGAGTATAAAGACTGTTCCCAAGCCC 250
              ||||||||||||||||||||||||||||||||||||||||||||||||||
          4145 AAGAGCCTCCTGATCTTATTGGGGGGAGTATAAAGACTGTTCCCAAGCCC 4194

AF3010089 251 ATTGCCCTGGAGCCGTGTTTTGGCAACAAAGCTGCTGTCCTCTCTGTGTT 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||
          4195 ATTGCCCTGGAGCCGTGTTTTGGCAACAAAGCTGCTGTCCTCTCTGTGTT 4244

AF3010089 301 TGTGAGGCTCCCTCG 315
              |||||||||||||||
          4245 TGTGAGGCTCCCTCG 4259
```

Figure 32: Comparison of the bovine attractin DNA sequence from the Genbank (AF3010089) and of the bovine attractin sequenced in this study

5 Discussion

5.1 MC1R

Five variants of the cattle MC1R were characterised in this study and the relation of their DNA sequences to the coat colour as well as the differences in their responsiveness to α-MSH (as determined by cAMP production) were analysed.

5.1.1 Dominant black MC1R allele (E^D)

The $\mathbf{E^D}$ allele was only found in the black animals, which are represented by the Holstein Friesian breed in Switzerland. The $\mathbf{E^D}$ allele has an amino acid substitution from leucine to proline at the very end of the TM2. The same mutation has been reported to be associated with the black coat colour in Norwegian cattle (Klungland et al., 1995). The black colour in the previously red Norwegian cattle had been introduced mainly through the import of European Friesian and American Holstein animals (Klungland et al., 1995). Thus it is not surprising, that the dominant black colour in Norwegian cattle and Holstein Friesian breed is due to the same dominant allele of MC1R. The $\mathbf{E^D}$ allele has also been found in the black animals of the Eringer breed (Gaillard, personal communication). The dominant inheritance of the black coat colour in cattle suggests that the $\mathbf{E^D}$ allele can encode for a constitutively active receptor. The $\mathbf{E^D}$ allele was unresponsive to the wide range of α-MSH concentrations, and its pharmacological profile was indistinguishable from the **e** allele, which has a frameshift mutation and thus encodes for a truncated receptor (Figure 23).

The dominant black alleles of MC1R were found in the mouse, pig, dog, fox and sheep. So far no dominant black alleles have been reported in humans and the horse. The constitutive activation of the MC1R were shown for **Glu92Lys** and **Leu99Pro** MC1R variants of mice, the **Cys125Arg** variant of the fox and the double mutant variant **Met71Lys; Asp119Asn** variant found in sheep (Table 6). Only one dominant black allele in mouse **Ser69Leu** was shown to be not constitutively active (Robbins et al., 1993). No pharmacological data are available for dominant black alleles of the pig and the dog.

The **Leu99Pro** variant in cattle is identical to the $\mathbf{E^{D1}}$ allele found in pigs (Kijas et al., 1998) and similar to the mouse $\mathbf{E^{so}}$ allele (Robbins et al., 1993; Cone et al., 1996). In the mouse $\mathbf{E^{so}}$

allele a Leu-Leu-Leu sequence is changed to Leu-Pro-Leu, whereas in the bovine $\mathbf{E^D}$ allele, the corresponding Leu-Leu-Leu is changed into Pro-Leu-Leu. The $\mathbf{E^{so}}$ allele was shown to be a constitutively active receptor which is unresponsive to α-MSH stimulation (Cone et al., 1996). The pharmacological data of the $\mathbf{E^{so}}$ allele is not shown, however it is said, that it is very similar to the $\mathbf{E^{so\text{-}3J}}$ mouse allele (**Glu92Lys**) (Cone et al., 1996). The $\mathbf{E^{so\text{-}3J}}$ mouse variant is also a constitutively active receptor, and its activity is significantly higher than the basal activity of the **E**+ MC1R murine allele (Robbins et al., 1993; Cone et al., 1996). However, this was not the case of the bovine $\mathbf{E^D}$ MC1R allele, where the activity of the receptor is similar to the basal activities of the $\mathbf{E^{d1}}$, $\mathbf{E^{d2}}$ and the $\mathbf{e^f}$ receptors and was the same as the **e** receptor.

Table 6: Dominant black MC1R alleles in different mammalian species

Species	Amino acid	Location	Constitutive activity
Mouse ($\mathbf{E^{so\text{-}3J}}$)	**Glu92Lys**	TM2	Shown by Robbins et al. (1993) and Cone et al. (1996)
Mouse ($\mathbf{E^{so}}$)	**Leu98Pro**	TM2	Shown by Cone et al., 1996
Mouse ($\mathbf{E^{tob}}$)	**Ser69Leu**	IL1	Not constitutively active (Robbins et al., 1993)
Pig ($\mathbf{E^{D1}}$)	**Leu99Pro**	TM2	Not investigated
Pig ($\mathbf{E^{D2}}$)	**Asp121Asn**	TM3	Not investigated
Dog	**Ser90Gly**	TM2	Not investigated
Fox ($\mathbf{E^{A}}$)	**Cys125ARg**	TM3	Shown by Vage et al., 1997
Sheep	**Met73Lys; Asp121Asn**	IL1; TM3	Shown by Vage et al., 1999
Cattle ($\mathbf{E^{D}}$)	**Leu99Pro**	TM2	Shown in this work?

It can be argued, that a reduced activity of the $\mathbf{E^D}$ receptor variant results from **Ala291Val** substitution located in TM7, which possibly is not present in the natural $\mathbf{E^D}$ allele and was introduced into the clone by an amplification error. However, it is not very likely, that the **Ala291Val** substitution can affect the function of MC1R, as alanine (nonpolar side chain) at

this position is not conserved, having **Ser** (uncharged polar side chain) in the mouse, dog and fox. Furthermore alanine and valine belong to the same class of nonpolar side chain amino acids.

It is remarkable, that the pharmacological characteristics of the cattle $\mathbf{E^D}$ receptor are similar to the sheep double mutant (**Met73Lys**; **Asp119Asn**) allele. When these two mutations were introduced together into the mouse MC1R (**Met71Lys** and **Asp121Asn**), the response of the receptor to α-MSH was reduced to undetectable levels. The mutated receptor could be activated by the high concentrations of NDP-MSH, even though no ligand binding could be detected (Vage et al., 1999). Receptor-binding experiments were not performed in this study, thus it is not known, whether the $\mathbf{E^D}$ receptor can bind to its ligands. Also the $\mathbf{E^D}$ receptor was not stimulated by NDP-MSH, which was shown, in contrast to α-MSH, to activate the constitutive active MC1Rs of the mouse, fox and sheep (Cone et al., 1996; Vage et al., 1997; Vage et al., 1999), indicating that the mechanism of MC1R activation by α-MSH and NDP-MSH is not exactly the same and that a NDP-MSH is more potent agonist of MC1R. This observation was also confirmed by Lu et al. (1998), who showed that for some *in vitro* mutated constitutively active MC1 receptors agonist potency could be shown only with the use of NDP-MSH. In view of this, it would be interesting to perform binding studies with the bovine $\mathbf{E^D}$ MC1R variant with both α-MSH and NDP-MSH, and measure intracellular cAMP in response to NDP-MSH stimulation.

Vage et al. (1997) showed by competition binding studies that the fox $\mathbf{E^A}$ allele (**Cys125Arg**) and the analogous murine MC1R variant are expressed at a very low copy number when stabely transfected into HEK 293 cells. They speculated that it can happen due to a potent down-regulation of the constitutive active receptor. The real-time RT-PCR results obtained in this study, however, indicate that bovine $\mathbf{E^D}$ MC1 receptor is expressed at comparable levels with other MC1R variants. Thus, if any down-regulation took place for the $\mathbf{E^D}$ receptor, it did not occur at the transcription level.

The unresponsiveness of bovine $\mathbf{E^D}$ MC1R to α-MSH stimulation in conjunction with the dominant inheritance of this allele strongly support the hypothesis, that the $\mathbf{E^D}$ is a constitutively active receptor. The mouse and fox constitutive active receptors have a significantly higher receptor ability to couple to adenylyl cyclase, even in absence of ligand stimulation, when compared with the basal activity of the wild type receptor. However, the sheep constitutively active MC1R had a very low ability to activate adenylyl cyclase at a wide range of α-MSH concentrations, as it also has been shown for bovine $\mathbf{E^D}$ allele. The location of the **Leu99Pro** substitution near the conjunction of the TM2 and EL2 also supports the

hypothesis of constitutive activation of this variant, as TM2 was shown to be involved in the agonist binding (Cone et al., 1996; Haskell-Luevano et al., 1996; Yang et al., 1997). The **Leu99Pro** could change the TM2 structure and thus convert the receptor confirmation into the active form. This mutation can also change the $\mathbf{E^D}$ receptor affinity to the ligand. However, the absence or presence of ligand binding does not show if receptor is constitutively active or not. The location of the **Leu99Pro** change in cattle near the **Leu98Pro** substitution of mice, which was shown to encode for a constitutively active receptor, also supports the constitutive activation of the bovine $\mathbf{E^D}$ MC1R allele.

5.1.2 Recessive red MC1R alleles (e and e^f)

The **e** allele, which in its homozygous form is associated with red coat colour (Klungland et al., 1995; Joerg et al., 1996), has the single nucleotide deletion 310/311G, which results in a frameshift of the open reading frame. The stop codon in the **e** allele is located at the position 156 of the amino acid sequence, and the protein structure of the **e** receptor variant is completely changed after **Ala103** in EL2. Thus the **e** MC1R variant encodes for a truncated receptor, which was hypothesised to be non-functional. The pharmacological characterisation of the **e** variant has confirmed this hypothesis, as the **e** allele was unable to couple to adenylyl cyclase at various concentrations of α-MSH (Figure 24).

The $\mathbf{e^f}$ allele (**Ile297Thr**) was found in the Simmental breed, which consists only of red animals. Thus, although this allele was found in a compound heterozygous form in two $\mathbf{e/e^f}$ animals, it should be associated with the red coat colour. However, while the Simmental population was not investigated for the presence of the $\mathbf{e^f}$ allele, it is not known if the homozygous animals for this allele exist.

The $\mathbf{e^f}$ allele was responsive to α-MSH stimulation, and the stimulation curve of the $\mathbf{e^f}$ receptor allele was shifted to the right relatively to the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ receptor variants with the median effective concentration being more then ten times higher (Figure 24). The shifting to the right of the stimulation curve indicates that the $\mathbf{e^f}$ allele is a partial loss-of-function MC1R variant with pharmacological properties similar to the **Ile40Thr** and **Val122Met** found in humans with fair skin (Jimenez-Servantes et al., 2001). The location of the **Ile297Thr** in TM7 does not distinguish, whether this mutation affects the receptor-ligand binding or G-protein coupling. The TM7 is considered to be involved in the ligand binding, and **Phe280** and **Asn281** are believed to interact with the His-Phe-Arg-Trp core sequence of melanocortins (Cone et al., 1996; Haskell-Luevano et al., 1996). In humans the **Asp294His** variant was found to be

associated with red hair and fair skin (Smith et al., 1999). This variant had been shown to have a similar affinity to α-MSH and NDP-MSH, as the wild type receptor, but it was unable to couple with G-proteins and activate adenylyl cyclase (Schiöth et al., 1999). The **Ile297Thr** mutation can affect cAMP signalling pathway through either a decreased α-MSH affinity, or a diminished G-protein coupling ability. This assumptions can be analysed by performing binding studies with this receptor variant. Isoleucine belongs to the class of non-polar side chain amino acids, while threonine has an uncharged polar side chain. Thus it is possible, that the **Ile297Thr** change affects the structure of the TM7 in such a way that it decreases the receptor activity, but is not sufficient to abolish it completely. The pheomelenin production in $\mathbf{e^f/e}$ cattle can thus result from a reduction of the receptor activity caused by the **Ile297Thr** mutation solely, or a possible dominant agouti allele can be involved. The existence of homozygous $\mathbf{e^f/e^f}$ animals is a matter for further investigation, and if they exist, the red pelage is expected, which could also be darker red, because all animals of the modern Simmental breed are red.

It is unlikely, that this mutation spontaneously arose in Fichte and then was transferred to Fleuron. The $\mathbf{e^f}$ allele does not contain a deletion, which means, that the $\mathbf{e^f}$ allele most probably originates from the wild type MC1R (**E+**). If the $\mathbf{e^f}$ variant spontaneously arose in the modern Simmental population, there should be animals possessing the **E+** allele with a phenotype distinct from red, however this is not the case. Thus the $\mathbf{e^f}$ allele should have an older origin and this is in agreement with the fact that in the 19^{th} century some strains of the Swiss Simmental breed had brown pied and black pied animals, which were later bred out from the Swiss Simmental breed (Felius, 1995).

However, it is remarkable, that the **e** allele to date has been found in many different breeds, red Norwegian cattle (Klungland et al., 1995), Red Holstein (Joerg et al., 1996), Simmental (this study) and different French cattle exhibiting more or less strong red coat colour [Blonde d'Aquitane, Charolaise, Limousine and Salers, (Rouzaud et al., 2000)], indicating that it is older in origin, than the $\mathbf{e^f}$ variant. The $\mathbf{e^f}$ variant has so far only been found up to date only in the Simmental breed. Thus it is possible to speculate, that the $\mathbf{e^f}$ variant arose in the Simmental breed and the **e** allele was introduced into Simmental breed by cross breeding with other red breeds. However, it would be interesting to test for presense of the $\mathbf{e^f}$ allele in other red breeds, especially those exhibiting a dark red coat colour.

5.1.3 MC1R alleles similar to the wild type (E^{d1} and E^{d2})

Two MC1R variants were found in the Brown Swiss breed $\mathbf{E^{d1}}$ (**Arg223Trp**) and $\mathbf{E^{d2}}$ (**218ARGI** duplication). According to the performed RFLPs and AFLP the **E+** allele, identical to the allele sequenced by Klungland et al. (1995), is also present in the Brown Swiss breed. However, the putative **E+** allele was neither sequenced, nor characterised pharmacologically.

Both the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ variants responded to α-MSH in a dose dependent manner with similar E_{50} values (Figure 24). Nevertheless, the $\mathbf{E^{d2}}$ variant had a greater ability to stimulate adenylyl cyclase and produce cAMP. This difference between the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ stimulation curves can not be explained by the differences in RNA expression, because according to real time PCR results (Table 5), the $\mathbf{E^{d1}}$ cell line has a lower C_T value and thus higher RNA expression level, than $\mathbf{E^{d2}}$**.** It is difficult to analyse if this feature of the $\mathbf{E^{d2}}$ allele could somehow influence the eumelanin/pheomelanin production ratio, as the differences of the coat colour shades inside Brown Swiss breed have not been categorised.

The amino acids of the C-terminal part of the IL3 were shown to be involved in G-protein coupling and particularly **Lys226-Arg227-Gln228-Arg229** are essential for this process (Frandeberg et al., 1998). The **218ARGI** duplication and **Arg223Trp** are located closer to the N-terminal of the IL3 and moreover **Arg223Ala** site-directed mutant human MC1R was shown to have a similar potency as the wild type receptor. Thus the **Arg223Trp** variant ($\mathbf{E^{d1}}$) of Brown Swiss is most probably a neutral mutation, not affecting the receptor function.

The $\mathbf{E^{d2}}$ MC1R variant was also found in the Brown Swiss breed by Kriegesmann et al. (2001) and was postulated to be a Brown Swiss specific allele. However, this allele was also found in Aubrac (brownish) and Gasconne (light grey) breeds in France in both the homozygous and the **E+** compound heterozygous forms, whose phenotypes are distinct from that of the Brown Swiss (Rouzard et al., 2000).

Thus, it can be speculated, that the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ alleles have similar properties with the **E+** (wild type) allele, and although the $\mathbf{E^{d2}}$ allele is capable of more effectively coupling to adenylyl cyclase pathway, this does not have a direct influence on the phenotype. To better analyse the difference between the $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ alleles, it would be of interest to perform a cAMP assay for the cells transfected with the **E+** allele. The difference of the phenotypes between Brown Swiss, Aubrac and Gasconne breeds are most likely due to the differences in the other pigmentation loci.

5.2 Agouti

5.2.1 Agouti coding sequence and expression

No differences were found in the agouti coding sequences in the three cattle colour variants Simmental (red), Holstein (black) and Brown Swiss (brown-grey). No frameshift or nonsense mutations were found in the bovine agouti coding sequence when compared to other species.
It was possible to amplify agouti from cDNA of Simmental testes, and it was not possible to amplify agouti from cDNA of Holstein and Brown Swiss liver and from the Simmental testes. In the mouse wild type agouti mRNA is expressed in neonatal skin and testes. However, in the human agouti, RNA is expressed in the adipose tissue, heart, ovary, testes and at lower levels in the kidney, liver and foreskin (Kwon et al., 1994; Wilson et al., 1995). Oulmouden et al. (1996, unpublished, accession number X99692) amplified the bovine agouti coding sequence using RNA from the kidney, showing that the expression pattern of agouti in cattle differs from that observed in the mouse. RT-PCR results in the present study indicate that differences in the agouti expression may exist in different cattle coat colour variants and in different tissues. As no agouti phenotype (black hair with a terminal or sub-terminal yellow band) has been observed in both cattle and human, it would be logical to suggest, that the agouti expression in cattle resembles the wide expression pattern of agouti in humans. Another possibility, however, would be that agouti is not so widely expressed in cattle, like in the human, and expression of agouti in the kidney is due to the mutations in the regulation sequences of agouti, like it is observed in mice with ubiquitous pattern of agouti expression (Blutman et al., 1992; Miller et al., 1993). Expression experiments (i.e. Northern blot hybridisation, RNase protection assay, real-time RT-PCR) with various tissues would help to determine the expression pattern of bovine ASIP.

5.2.2 Agouti function

Agouti may play a functional role in pigmentation also in species without the wild type agouti phenotype. Human ASIP was shown to suppress black pigmentation in transgenic mice and decrease cAMP production in B16 melanoma cells (Wilson et al., 1995). Mutations associated with the recessive black colour found in the agouti gene of the horse (Rieder et al., 2001) and fox (Vage et al., 1997) were found, even so no wild type agouti phenotype is observed in these

species. Adalsteinsson et al. (1995) and Klungland et al. (1995) propose the existence of recessive black agouti alleles in cattle, as some animals in black Norwegian and Icelandic cattle were genotyped for MC1R as **E+/E+** or **E+/e**. In the Holstein breed the black coat colour is inherited in a dominant fashion, indicating that agouti does not have an influence on black colour in this breed. The red coat colour in Holstein is inherited as a recessive trait and no red animals that are not homozygous for the **e** allele have been found to date which excludes the influence of agouti in this breed. However, hormone responsive MC1R alleles are present in Brown Swiss and Simmental breeds, which can be affected by agouti.

To test if the bovine agouti can affect pigmentation, an *in vitro* cell system was created, where the interactions of agouti and MC1R were investigated (Figure 33). Two cell lines one expressing α-MSH responsive MC1R allele ($\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$) and another expressing ASIP were incubated together. ASIP should be secreted to the culturing medium and act as antagonist of MC1R and thereby inhibit α-MSH stimulation.

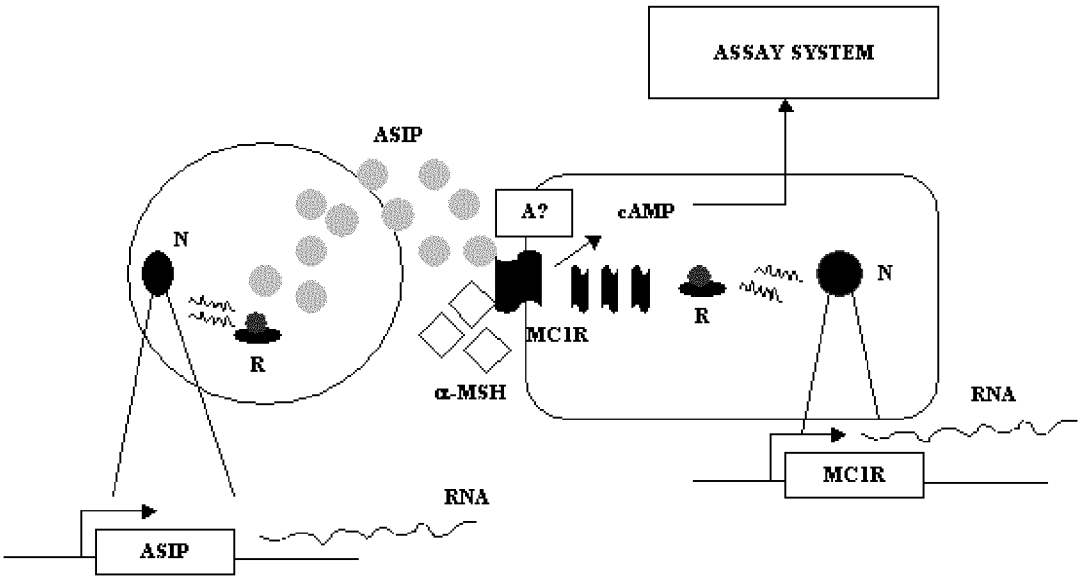

Figure 33: In vitro cell system created to investigate MC1R and ASIP functioning

N is the nucleus, R is the ribosome, A? indicates attractin, possibly expressed in HEK 293 cells. MC1R is transcribed, translated and expressed on the cell surface. ASIP is transcribed, translated and secreted to the culture medium and binds to MC1R, antagonising α-MSH, which is added in the culture medium. Intracellular cAMP of the cells expressing MC1R was measured.

A reduction of cAMP production in the cells expressing MC1R co-incubated with the cells expressing ASIP (Figures 28 and 29) gives strong evidence, that cattle express a functional agouti protein. Ability of ASIP to down-regulate cAMP production in HEK 293 cells, expressing MC1R indicates, that functional attractin is endogenously expressed in these cells, as, it was shown in the mouse, that ASIP can down-regulate MC1R only in the presence of functional attractin (He et al., 2001). High homology of the carboxyl terminus between mouse and bovine ASIP (Figure 26), which was shown to be sufficient for the MC1R down-regulation (Eberle, et al., 2001) also supports the presence of functional agouti in cattle. The experiment performed in this study could show the antagonist activity of the bovine ASIP, which means that agouti can compete with α-MSH to bind to MC1R and thus down-regulate the receptor signalling, i.e. cAMP synthesis. The ability of the bovine agouti to act as an inverse agonist remains unclear, as the created MC1R expression system is not sensitive to measure differences in cAMP production in the absense of α-MSH stimulation.

Existence of functional ASIP in cattle, expression of which was detected in Simmental testes raises the question, whether dominant agouti alleles similar to those ubiquitously expressed in mice are also present in cattle. An ubiquitous expression of ASIP in the mouse is associated with obesity and is caused by rearrangements in the 5' regulation sequences (Blutmam et al., 1992; Siracusa, 1994). No such striking obese phenotypes has been observed in cattle as observed in mice carrying the dominant agouti alleles reported in cattle, indicating that probably there are no cattle breeds overexpressing agouti. It is also possible that ubiquitous expression of ASIP in cattle would not cause the same strong phenotype as observed in mice. The body composition traits undoubtedly vary within different cattle breeds. However, the dairy breeds Holstein and Brown Swiss are similar to the dual purpose Simmental breed in the lean-to-fat ratio as well as in marbling (Marshall, 1999).

As only the coding region of ASIP was sequenced in this study and no differences were found in between red, black and brown coat colour variants, it can be proposed, that if any mutations, affecting the function of agouti exist in cattle, they are located outside the coding sequence. Agouti can exhibit its pigmentation effects only on α–MSH responsive alleles of MC1R, such as $\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$ in Brown Swiss and $\mathbf{e^{f}}$ in Simmental. It is improbable, that Brown Swiss express dominant alleles of agouti, as ASIP was not amplified by RT-PCR from testes and liver of this breed, and the brown coat colour represents the wild type of pigmentation regulated by the wild type MC1R ($\mathbf{E^{d1}}$ and $\mathbf{E^{d2}}$) and agouti (**A+**) alleles. However, the red coat colour in $\mathbf{e^{f}/e}$ animals found in this study can be caused either, only by a reduction of the $\mathbf{e^{f}}$ receptor allele signalling, regulated by α–MSH and wild type ASIP, or by a dominant agouti

allele (**A**) possibly present in this breed and down regulating the $\mathbf{e^f}$ MC1R allele activity, causing red coat colour.

To investigate agouti alleles in cattle it would be advisable first to perform expression studies with various tissues from different breeds. If any differences are found in the expression patterns, this would indicate, that different alleles of agouti with mutations in the regulation sequences exist, which can subsequently be sequenced. It would be advisable to take the following breeds: black Norwegian cattle genotyped **E+/E+**, the Simmental breed which is red and has a hormone responsive $\mathbf{e^f}$ allele, Holstein cattle, which change from red to black or less commonly from black to red as they age from calf to adult (http://sask.usask.ca/~schmutz/colors.html), and thus probably possessing agouti alleles controlled by a temporally regulated promoter and breeds such as Brown Swiss, which express coat colour intermediate between red and black (i.e. brown) and therefore most probably have wild type alleles of agouti.

5.3 Attractin

The complete coding sequence and small parts of the 5' and 3' UTR of the bovine attractin were sequenced in this study. However, for some reason it was not possible to amplify the 5' end of the bovine attractin gene from cDNA reverse transcribed of RNA from the Brown Swiss liver and testes, Holstein liver and Simmental testes. One possible explanation of the failure to amplify the 5' end of the bovine attractin gene is an insufficient processivity of the reverse transcription, i.e. inability of the reverse transcriptase to synthesise full length cDNA. The most upstream lying primer which was successfully used for amplification of cDNA was Mgex1F1, amplification of cDNA using the forward primers Mgex1F6, Mgex1F3, Mgex1F4 and Mgex1F5 lying 150 bp, 237 bp, 260bp and 325 bp upstream from the Mgex1F1 respectively (Figure 35, Appendix) did not produce any specific amplification products. Another explanation may be the alternative splicing taking place at the 5' end of the bovine attractin mRNA. In the human two isoforms of soluble attractin were found differing by a 222 bp G-C rich fragment at the 5' end (accession numbers AF034957 and AF106861), the isoform missing the 222 bp fragment at the 5' end has a deletion of 74 residues in the deduced amino acid sequence (Figure 31). However, the reason for this difference was not reported, and can either be a different mRNA splicing, or possibly even a different gene, as the sequence of the isoform with a deletion has a completely different piece at the position 534-545 (Figure 31). If the difference between human sequences is due to the alternative splicing, it can be speculated, that bovine attractin is also differently spliced at the 5' end and the second isoform is

preferably expressed in the liver and testes, tissues investigated in this study. In this case the start codon in the alternatively spliced mRNA should lie upstream from the start codon, shown here (Figure 35, Appendix), in the 5' part, which was not sequenced in this study. To investigate the possible splicing variants of bovine attractin, Northern hybridisation can be performed with RNA extracted from different tissues. The different sizes of the hybridisation signals would indicate splicing variants. Northern hybridisation analysis will also help to find out if bovine attractin has both soluble and membrane forms, resulting from the alternative splicing as reported in the human and rat, (Kuramoto et al., 2001; Tang et al., 2001), or only a membrane form as found in the mouse and hamster (Gunn et al., 1999; Kuramoto et al., 2002). It would be of interest to describe the complete genomic sequence of the bovine attractin. For this purpose the obtained BAC clone Mgex3 can be sequenced.

To investigate the function of attractin in the *in vitro* cell culture system, the cells not expressing endogenous attractin should be used. The HEK 293 cells used in this study most probably express attractin, as the mouse kidney was shown to express attractin (Gunn et al., 1999) and bovine agouti could suppress cAMP production in the HEK 293 cells expressing MC1R. Furthermore was shown in the mouse, that agouti can down-regulate MC1R only in the presence of functional attractin (He et al., 2001). However, it was found that the murine uterus, spleen and muscle do not express attractin (Gunn et al., 1999), therefore it would be advisable to use the cell lines established from these tissues.

5.4 In vitro cell culture gene expression technique in livestock species

Genetic improvement is one of the most effective strategies available for altering the performance of farm animals. Rates of genetic improvement depend on several factors, one of them is accuracy with which genetic merit of the trait of interest can be predicted. Knowledge about the genes and its functional alleles involved in the forming of the trait of interest would be of a great advantage for genetic improvement. To date, most selection in livestock has been practised with little or no knowledge of what is happening at the genes level. Selection has been on the effects of the genes (phenotype), rather then on genes themselves. However, novel molecular genetic methods allow to show the association with variations on DNA level with phenotypic variations. Proposing a candidate gene for a trait of interest in livestock is possible using information on physiological homology, comparative mapping and homology in amino acid and DNA sequence from mouse and human. Cloning, mapping and sequencing of a

candidate gene and search for the mutations is a next step in characterisation of a trait. *In vitro* cell culture gene expression technique can be applied to investigate if the found mutations is causative for a specific phenotype. In case if a mutation causes a functional differences between alleles, the selection for a preferred allele can be performed.

In this study cell lines expressing five alleles of bovine MC1R and also agouti gene were established. Based on the knowledge about the biochemical pathways in which MC1R and ASIP are involved it was possible to show the functional differences between MC1R alleles and to prove the function of agouti in cattle. The technique of gene expression *in vitro* can be used to examine other genes, for example candidate genes for genetic diseases or genes involved in forming of economic important traits.

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7 Appendix

7.1 Primers

Table 7: Universal and kit primers

Primer name	Sequence 5'→3'	Ta (°C)
TVX	17TVN	
T7	TAATACGACTCACTATAGGG	58°C
Sp6	ATTTAGGTGACACTATAG	58°C
Oligo dT-anchor	GACCACGGTATCGATGTCGAC16TV	
PCR anchor primer	GACCACGGTATCGATGTCGAC	

Ta (°C) -annealing temperature

V = A, G. C

N = A, G, C, T

All gene specific primers were synthesised by Mycrosynth GmbH (Balgach, Switzerland)

Table 8: Bovine MC1R specific primers

Primer name	Sequence 5'→3'	Position in the coding sequence	Ta (°C)
MC1-RF2	ACGATGCCTGCACTTGGCTCCCAG	-3- 21	58°C
MC1-RR1	CCTCACCAGGAGCACTGCAGCAC	954- 934	58°C
MSHR-P9	GGAGGTGTCCATCTCTGACGG	108-128	56-60°C
MSHR-P10	CCGGGCCAGCATGTGGACGTA	639-618	56°C
MSHR-P12	CTGCTGACCACGG*A*CGCCTG*	345-325	60°C
MSHR-P13	CCTGCCAGCATGC*A*CGGGGC**	641-660	58°C
MSHR-P14	GGCTGCGGAAGGCATAGATGAG	907-886	58°C
MSHR-P15	GCCAGCATCCTCACCAGCCTG	510-531	60°C
MSHR-P16	JoeTGGGGCGCTGCCTCTTCTGG***	691-670	60°C
MSHR-P21	TACTTTATCTGCTGCCTGGCT	223-243	60°C
MSHR-P22	ATGACATTGTCCAGCTGC	359-342	60°C
Taq-ManETH1R	FamCTGGAGGCCGGTGTTCCTGGCCATamra	301-323	
Taq-ManETH2S	JoeCTGGAGGCCGTGTTCCTGGCCATamra	301-322	

* G in the original MSHR sequence is replaced in the primer sequence by A (*cursive*) to erase the AciI digestion site in the PCR product

** C in the original MSHR sequence is replaced in the primer sequence by A (*cursive*) to erase the MspI digestion site in the PCR product

*** The primer MSHR-P16 was labelled at 5' end with the fluorescent dye Joe

Table 9: Bovine agouti specific primers

Primer name	Sequence 5'→3'	Position in genomic sequence	Ta (°C)
AgF1	AGGATGGATGTCAGCCGCCTCC	-3-19	58°C
AgI1R1	GGCCGCAGGCCAGGCAACTC	182-163	58°C
AgI1F1	GCACTTCACTGAAGTTGGACC	1414-1436	58°C
AgI2R1	CGACAATGTGAACTCCCAGGCC	1565-1544	58°C
AgI2F1	CGTAGATGGCTCGGGCAGCC	4925-49445	58°C
AgR1	TCAGCAGGTGGGGTTGAGCACG	5139-5118	58°C

Table 10: Bovine attractin specific primers

Primer name	Sequence 5'→3'	Position in the coding sequence	Ta (°C)
MgP1	CGAGCAATGCCAGCACTGCGGGG	339-361	58°C
MgP2	TGGTTCCACTCACTATCACAGC	1656-1636	58°C
MgP3	GTGAAAGGCCTGCTAACCACAC	2711-2732	58°C
MgP4	CATTGATGAACATGTCCAAATCCC	3553-3530	58°C
MgP7	GATGATCTCTACAGATATGATGTGG	1555-1579	56°C
MgP8	CACGTAGGCATTGGAGTCCACG	2853-2823	56°C
MgP9	GCAATGGACATGCGTCGCTATGC	3290-3312	58°C
MgPend	CAGAAGTAAAAGGTTAGATGCTGG	4442-4419	58°C
MgP14	GTCAGAATATTCCTCTCGTGTCC	969-947	used fo RT
MgR1	CGTCTCTCTCTCTCAGGAACAATGAG	595-574	55°C
MgR2	GGTGCATAAATTGAGTCCCCG	551-531	56°C
Mgex1F1	CGGGCTCAGCCGCAGCCGAG	233-252	60°C
Mgex1R8	GCTGGCATTGCTCGCCCACCC	352-332	60°C
Mgex3F1	GCCAAATAGAATAATGAGACTTC	456-478	58°C
Mgex3F1	CTAAAGGCAGCAACTAGGGGTGC	569-547	58°C
Mgex1F3	GAAATGGTGGCGGCGGCGAC	-3-17	did not work
Mgex1F4	CACGGGACACCGTCTCAGCC	-27--8	did not work
Mgex1F5	CCAGGCGAAGCTGAGCCGG	-90--72	did not work
Mgex1F6	ACTGGGACGCGACTAGAGCTGG	86-107	did not work

7.2 Solutions and Media

α–MSH	10^{-3} M
Ampicillin	50 mg/ml
DEPC (Diethyl pyrocarbonate) treated H_2O	
DEPC	0.05% (v/v)
water is stirred vigorously with magnetic stirrer for 20-30 min and then autoclaved to destroy DEPC	
Dithiothretol	1 mM
DMEM complete	
Non-Essential Amino Acids (NEAA)	0.1 mM
ABAM	5 ml/L
Sodium pyruvate	0.001M
FCS (heat inactivated at 56°C 30 min)	10%
DNA loading dye	
Orange G	0.25% (w/v)
Xylene cyanol	25% (w/v)
Glycerol	30%
Ethidium bromide	10 mg/ml
FSB	
Potassium acetate pH 7.5	10mM
$MnCl_2$	45 mM
$CaCl_2$	10 mM
KCl	100 mM
Hexammineocobalt chloride	3 mM
Glycerol	10% (v/v)
pH of the solution is adjusted to 6.4 with 0.1 N HCl	
autoclaved	
Geneticin	0.1g/ml in H_2O
IBMX	10 mg/ml (45 mM) in absolute ethanol
IPTG	2.5% (w/v)

LB medium (Luria-Bertram medium)

Bacto-tryptone	10 g/l
Bacto-yeast extract	5 g/l
NaCl	10 g/l
5 N NaOH	0.2 ml/l

autoclaved

LB-ampicillin agar

Agar	20 g/l in LB medium

autoclaved

Ampicillin is added to a concentration of 50 µg/ml

Methylen blue 0.02% (w/v) in 10 mM trisHCl pH 7.5

MOPS 10x

Morpholino-propane-sulfonic-acid	0.4 M
Sodium acetate	0.1M
EDTA	10 mM

autoclaved and gets yellow colour

NE buffer

NaCl	10 mM
EDTA pH 8.0	10 mM

autoclaved

PBS 9.55g/l

autoclaved

Phenol-chloroform-isoamyl alcohol

Phenol	50% (w/v)
Chloroform	48% (v/v)
Isoamyl alcohol	2% (v/v)

The solution is saturated with 1M tris HCl pH 8.0, until pH ~7.8 and stored under 100 mM tris HCl pH 8.0

Proteinase K 20 mg/ml in H_2O

RNA loading dye

Bromphenol blue	0.04% (w/v)
EDTA pH 8.0	1 mM
Glycerol	61.5% (v/v)

SOB medium

Bacto-tryptone	20 g/l
Bacto-yeast extract	5 g/l
NaCl	0.5 g/l
250 mM KCl	10 ml/l
2 M $MgCl_2$	5 ml/l
5 N NaOH	0.2 ml/l
autoclaved	

SOC medium

Glucose	20 mM in SOB medium

Sodium chloride (NaCl) saturated solution

NaCl is dissolved in water until the pellet starts to fall out

Sodium puryvate	0.1M in H2O
sterilised by filtration	

Sperm cells lysis buffer

NaOH	200 mM
SDS	1%

TBE 10x

Trizma-base	107.8 g/l
Boric acid	55 g/l
EDTA	7.4 g/l
autoclaved	

TE pH 8.0

Tris HCl pH 8.0	10 mM
EDTA pH 8.0	1 mM

TNE buffer

Tris HCl pH 8.0	10 mM
NaCl	150 mM
EDTA	10 mM
Trypan blue	3.8% (w/v)

Trypsin/EDTA

Trypsin (0.25% in PBS without Ca^{2+}, Mg^{2+})	100 ml
EDTA (1% in PBS)	1.5 ml

X-Gal	2.5% (w/v in dimethylformamide)

7.3 Reagents

3-Isobutyl-1-Methylxanthine (IBMX) Calbiochem® (410957)

5'/3' RACE kit Roche (1734792)

293-F Human embryonic kidney cells Gibco (11625)

50 Base-Pair Ladder Pharmacia Biotech (27-4005-01)

100 Base-Pair Ladder Pharmacia Biotech (27-4001)

α-MSH Sigma (M4135)

β-mercaptoethanol (14.3 M) Sigma (M-6250)

ABAM Gibco (15240-096).

AciI (C↓CGC) New England Biolabs (R0551S)

supplied with 10x reaction buffer NE Buffer 3 (100 mM NaCl, 50 mM tris HCl, 10 mM $MgCl_2$, 1 mM dithiothretol, pH 7.9 at 25°C)

Agarose Gibco (1551-027)

Ampicillin Gibco (11593-019)

AMV Reverse transcriptase Promega (M510A)

supplied with 5x reaction buffer (250 mM tris HCl pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine and 50 50 mM DTT)

APS Fluka (09915)

Bacto-tryptone Difco (3-01-1)

Bacto-yeast extract Gibco (30393-029)

Big Dye™ Terminator version 3.0 Applied Biosystems (439024807006)

Boric acid Fluka (15660)

$CaCl_2$ Fluka (21079)

Calf intestinal alkaline phosphatase Pharmacia Biotech (27-0620-01)

Chloroform Fluka (25690)

Cyclic AMP (^3H) assay system Amersham (TRK 432)

including:

Tris/EDTA buffer

Binding protein, purified from bovine muscle

[8-^3H] Adenosine 3',5'-cyclic phosphate

Adenosine 3',5'-cyclic phosphate standard, 1600 pmole

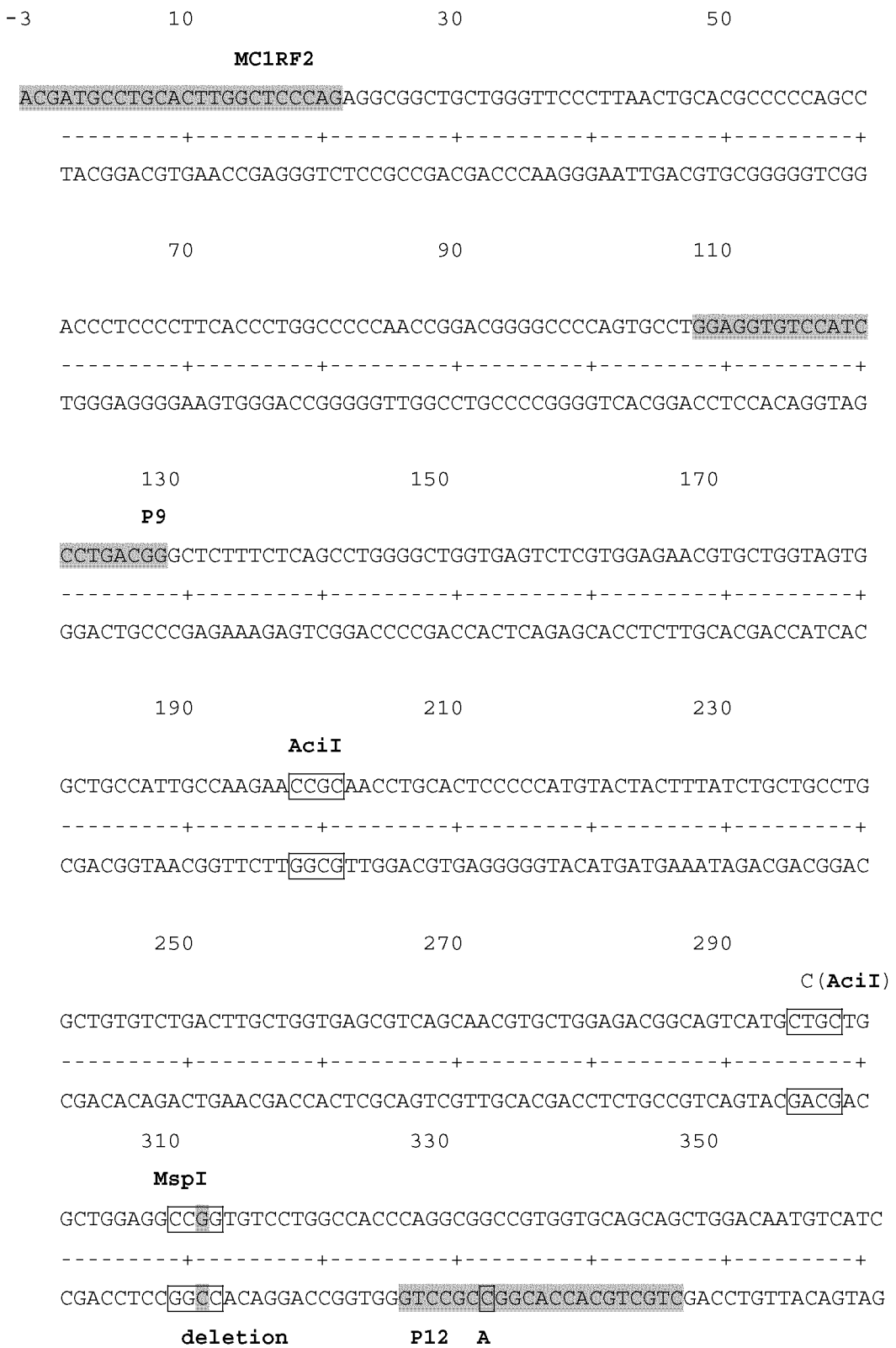
Charcoal adsorbent

DEPC	Fluka (32490)
DTT	Sigma (D-5545)
DMSO	Fluka (41640)
DMEM	Gibco (41965)
EcoRI (G↓AATTC)	Pharmacia Biotech (E 1040Y)
supplied with 10x reaction buffer H (500 mM tris HCl pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothretol, 1000 mM NaCl)	
Epicurian Coli® XL10-Gold™ ultracompetent cells	Stratagene (#211188)
Ethanol absolute	Merck (1.00983)
Ethidium bromide	Sigma (E-8751)
EDTA	Sigma (E-5134)
Expand™ High Fidelity PCR System	Boehringer Mannheim (1 732 650)
Fetal calf serum	Seromed (0113)
Formaldehyde	Fluka (47629)
Formamide	Fluka (47670)
Glycerol	Merck (1.04094)
Geneticin® (G-418 Sulfate)	Gibco (11811)
Hexaminocobalt chloride	Fluka (52740)
High Pure PCR Product Purification kit	Roche (1732668)
IPTG	Boehringer Mannheim (724815)
Isoamyl alcohol	Fluka (65980)
Kanamycin	Seromed (A 2510)
KiloBase™ DNA Marker	Pharmacia Biotech (27-4004-01)
LipofectAMINE™ Reagent	Gibco (18324-012)
Loading buffer	Applied Biosystems (36097407008)
Magnesium chloride ($MgCl_2$)	Fluka (63063)
Manganese chloride tetrahydrate ($MnCl_2$ x $4H_2O$)	Sigma (M-3634)
Methylen blue	Fluka (66720)
MOPS	Fluka (69949)
MspI (C↓CGG)	Boehringer Mannheim (633518)
supplied with 10x Sure/Cut reaction Buffer L (100 mMtris HCl, 100 mM $MgCl_2$, 10 mM dithioerythriol, pH 7.5 at 37°C)	
Non-Essential Amino Acids (100x)	Gibco (11140)
Opti-MEM®-I Reduced Serum Medium	Gibco (31985)

Orange G	Chroma Gesselschaft (1A 116)
PBS (without Ca^{2+} and Mg^{2+})	Seromed (L182-10)
pcDNA3.1(+)	Invitrogen (V790-20)
pGem®-T Easy vector system	Promega (A 1360)
including:	
pGem®-T Easy vector	
control insert DNA	
T4 DNA ligase	
2 X Rapid Ligation Buffer (60 mM Tris HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol)	
Phase Lock Gel II Heavy	5 PRIME 3→PRIME, INC.® (5301-174163)
Phenol	Fluka (77610)
Potassium acetate	Fluka (60034)
Potassium chloride (KCl)	Merck (1.04936)
Proteinase K	Sigma (P-0390)
T4 DNA ligase	Boehringer Mannheim (481 220)
supplied with 10X buffer (660 mM Tris HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5)	
Recombinant RNasin® ribonuclease inhibitor	Promega (N251A)
RNeasy kit	Qiagen (75162)
RNase free DNase Set	Qiagen (79254)
Scintilation cocktail Emulsifier SafeTM	Packard (601 3389)
SDS	Fluka (71729)
Sodium acetate trihydrate	Fluka (71188)
Sodium chloride (NaCl)	Merck (1.06404)
Sodium hydroxide (NaOH)	Merck (6498)
tetra-Sodium pyrophosphate decahydrate	Fluka (71514)
Sodium pyruvate	Fluka (15990)
Taq DNA polymerase	Pharmacia (27-0799-03)
TaqMan Universal PCR Master Mix	Applied Biosystems (P/N 4304437)
TEMED	Amresco (110-18-9)
Trizma base	Sigma (T-1503)
Trypan blue	Sigma (T 8154)

Trypsin (0.25% in PBS without Ca^{2+}, Mg^{2+})	Biochrom KG (L2123)
QIAEX II Agarose Gel Extraction kit	Qiagen (20051)
QIAquick PCR Purification kit	Qiagen (28104)
Qiagen Plasmid Maxi kit	Qiagen (12162)
X-Gal	Fluka (16665)
Xylene cyanol FF	Fluka (95600)

7.4 Sequences



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       370                 390                 410

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---------+---------+---------+---------+---------+---------+
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       430                 450                 470

GACCGCTACATCTCCATCTTCTACGCCCTGCGGTACCACAGTGTTGTGACACTGCCCCGA
---------+---------+---------+---------+---------+---------+
CTGGCGATGTAGAGGTAGAAGATGCGGGACGCCATGGTGTCACAACACTGTGACGGGGCT

       490                 510                 530
                                                P15
GCGTGGAGGATCATTGCGGCCATCTGGGTGGCCAGCATCCTCACCAGCCTGCTCTTCATC
---------+---------+---------+---------+---------+---------+
CGCACCTCCTAGTAACGCCGGTAGACCCACCGGTCGTAGGAGTGGTCGGACGAGAAGTAG

       550                 570                 590

ACCTACTACAACCACAAGGTCATCCTGCTGTGCCTCGTTGGCCTCTTCATAGCTATGCTG
---------+---------+---------+---------+---------+---------+
TGGATGATGTTGGTGTTCCAGTAGGACGACACGGAGCAACCGGAGAAGTATCGATACGAC

       610                 630                 650
                                                      P13
GCCCTGATGGCCGTCCTCTACGTCCACATGCTGGCCCGGGCCTGCCAGCATGCCCGGGGGC
---------+---------+---------+---------+---------+---------+
CGGGACTACCGGCAGGAGATGCAGGTGTACGACCGGGCCCGGACGGTCGTACGGGCCCCG
                 P10                                  duplication
       670                 690                 710
      T MspI
ATTGCCCGGCTCCAGAAGAGGCAGCGCCCCATTCATCAGGGCTTTGGCCTCAAGGGCGCT
---------+---------+---------+---------+---------+---------+
TAACGGGCCGAGGTCTTCTCCGTCGCGGGGTAAGTAGTCCCGAAACCGGAGTTCCCGCGA
           P16
       730                 750                 770

GCCACCCTCACCATCCTGCTGGGCGTCTTCTTCCTCTGCTGGGGCCCCTTCTTCCTGCAC
---------+---------+---------+---------+---------+---------+
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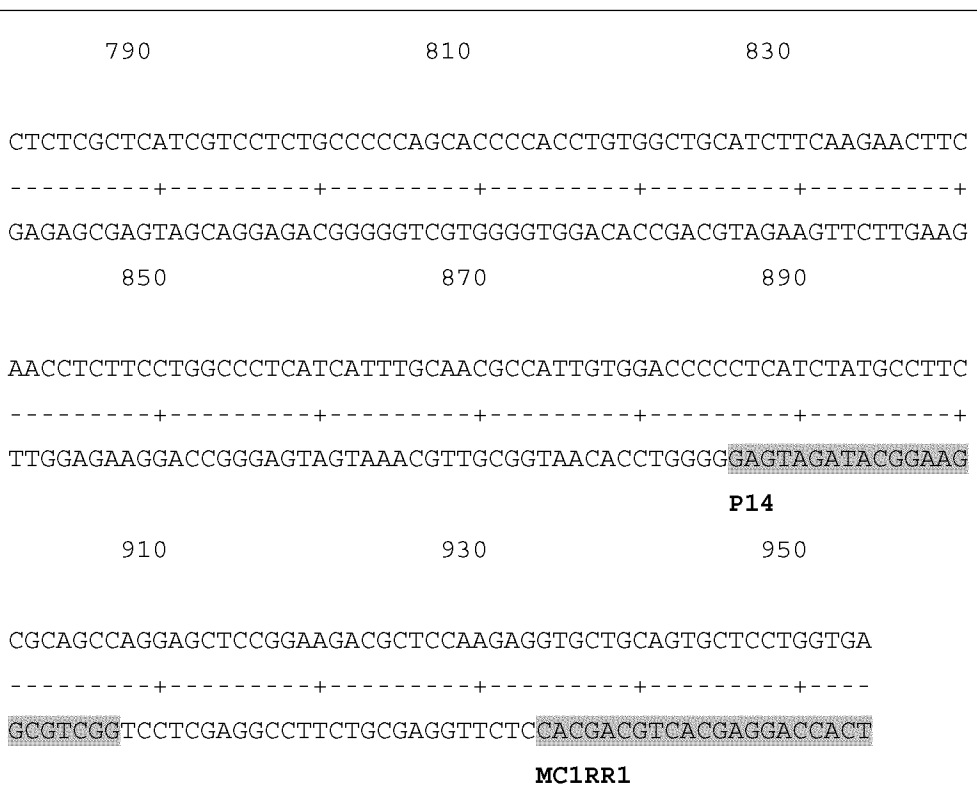
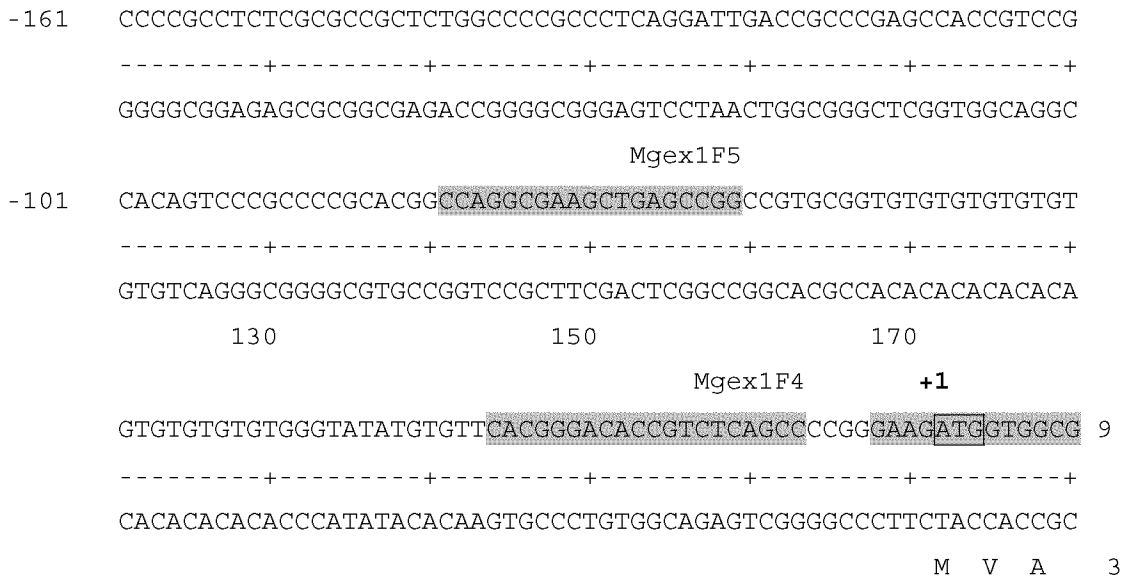
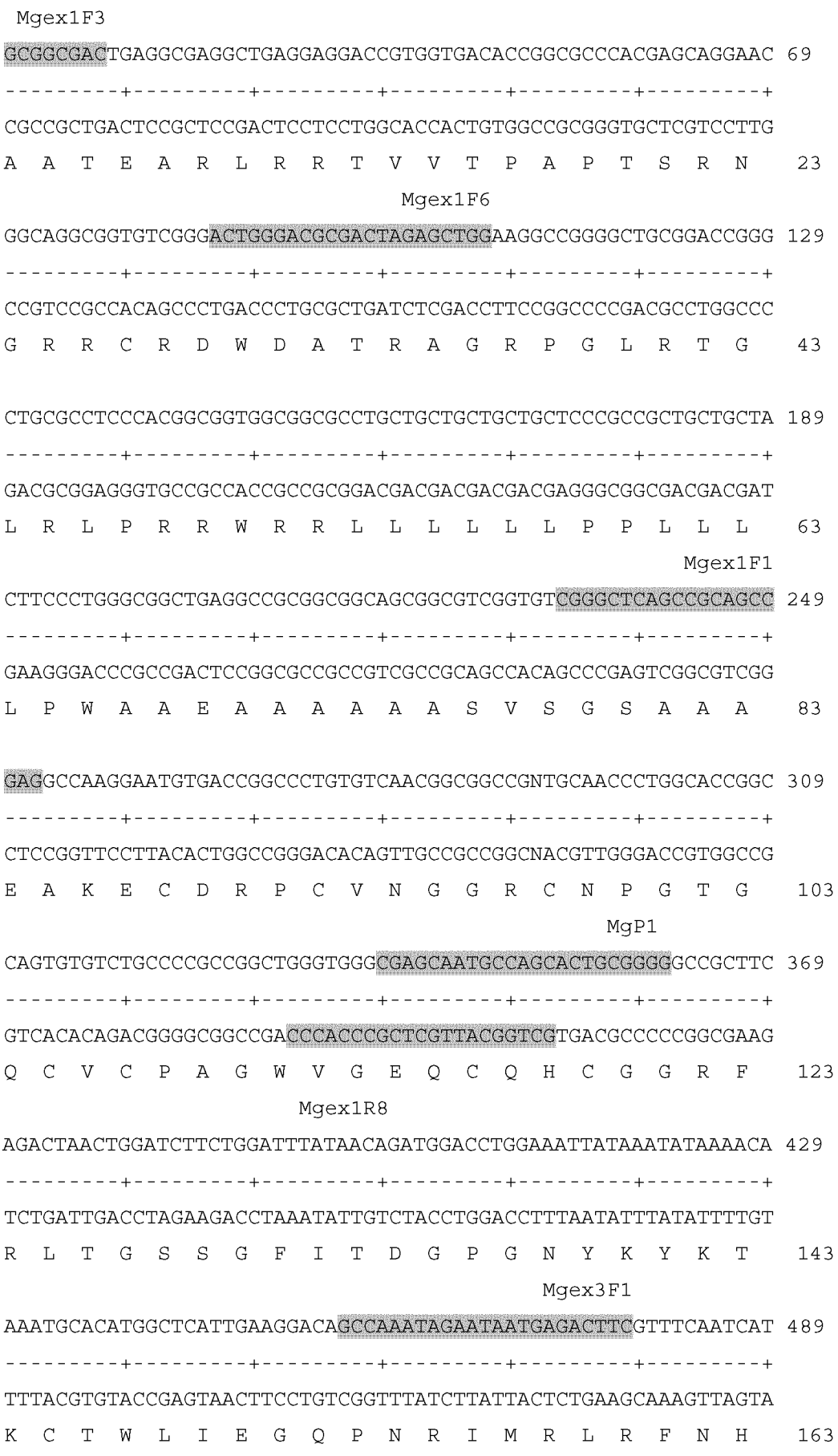


Figure 34: Location of the primers, restriction enzyme sites and polymorphic sites on bovine MC1R sequence

Primers are shown in grey. Restriction enzyme sites are shown in boxes. The polymorphic sites used for RFLPs are shown with letters above the substituted residues. Duplication is underlined and shown in cursive letters.




Mgex1F3
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CGCCGCTGACTCCGCTCCGACTCCTCCTGGCACCACTGTGGCCGCGGGTGCTCGTCCTTG
A A T E A R L R R T V V T P A P T S R N 23
Mgex1F6
GGCAGGCGGTGTCGGGACTGGGACGCGACTAGAGCTGGAAGGCCGGGGCTGCGGACCGGG 129
CCGTCCGCCACAGCCCTGACCCTGCGCTGATCTCGACCTTCCGGCCCCGACGCCTGGCCC
G R R C R D W D A T R A G R P G L R T G 43
CTGCGCCTCCCACGGCGGTGGCGGCGCCTGCTGCTGCTGCTGCTCCCGCCGCTGCTGCTA 189
GACGCGGAGGGTGCCGCCACCGCCGCGGACGACGACGACGACGAGGGCGGCGACGACGAT
L R L P R R W R R L L L L L L P P L L L 63
Mgex1F1
CTTCCCTGGGCGGCTGAGGCCGCGGCGGCAGCGGCGTCGGTGTCGGGCTCAGCCGCAGCC 249
GAAGGGACCCGCCGACTCCGGCGCCGCCGTCGCCGCAGCCACAGCCCGAGTCGGCGTCGG
L P W A A E A A A A A A S V S G S A A A 83
GAGGCCAAGGAATGTGACCGGCCCTGTGTCAACGGCGGCCGNTGCAACCCTGGCACCGGC 309
CTCCGGTTCCTTACACTGGCCGGGACACAGTTGCCGCCGGCNACGTTGGGACCGTGGCCG
E A K E C D R P C V N G G R C N P G T G 103
MgP1
CAGTGTGTCTGCCCCGCCGGCTGGGTGGGCGAGCAATGCCAGCACTGCGGGGGCCGCTTC 369
GTCACACAGACGGGGCGGCCGACCCACCCGCTCGTTACGGTCGTGACGCCCCCGGCGAAG
Q C V C P A G W V G E Q C Q H C G G R F 123
Mgex1R8
AGACTAACTGGATCTTCTGGATTTATAACAGATGGACCTGGAAATTATAAATATAAAACA 429
TCTGATTGACCTAGAAGACCTAAATATTGTCTACCTGGACCTTTAATATTTATATTTTGT
R L T G S S G F I T D G P G N Y K Y K T 143
Mgex3F1
AAATGCACATGGCTCATTGAAGGACAGCCAAATAGAATAATGAGACTTCGTTTCAATCAT 489
TTTACGTGTACCGAGTAACTTCCTGTCGGTTTATCTTATTACTCTGAAGCAAAGTTAGTA
K C T W L I E G Q P N R I M R L R F N H 163

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---------+---------+---------+---------+---------+---------+
AAACGATGTCTCACATCAACCCTGGTAAATATACAAATACTGCCCCTGAGTTAAATACGT
F  A  T  E  C  S  W  D  H  L  Y  V  Y  D  G  D  S  I  Y  A    183
                                          MgR2
CCCCTAGTTGCTGCCTTTAGCGGCCTCATTGTTCCTGAGAGAGACGGCAATGAGACTGTC 609
---------+---------+---------+---------+---------+---------+
GGGGATCAACGACGGAAATCGCCGGAGTAACAAGGACTCTCTCTGCCGTTACTCTGACAG
P  L  V  A  A  F  S  G  L  I  V  P  E  R  D  G  N  E  T  V    203
Mgex3F1                 MgR1

CCAGAGGTTGTCGCCACATCAGGTTATGCCCTGCTGCATTTTTTTAGTGATGCTGCTTAT 669
---------+---------+---------+---------+---------+---------+
GGTCTCCAACAGCGGTGTAGTCCAATACGGGACGACGTAAAAAAATCACTACGACGAATA
P  E  V  V  A  T  S  G  Y  A  L  L  H  F  F  S  D  A  A  Y    223

AACTTGACTGGATTTAATATCACTTACAATTTTGACATGTGTCCAAATAATTGCTCAGGC 729
---------+---------+---------+---------+---------+---------+
TTGAACTGACCTAAATTATAGTGAATGTTAAAACTGTACACAGGTTTATTAACGAGTCCG
N  L  T  G  F  N  I  T  Y  N  F  D  M  C  P  N  N  C  S  G    243

CGAGGAGAATGTAAGATCAGTAATAGCAGCAACACTGTTCAATGTGAATGTTCTGAAAAC 789
---------+---------+---------+---------+---------+---------+
GCTCCTCTTACATTCTAGTCATTATCGTCGTTGTGACAAGTTACACTTACAAGACTTTTG
R  G  E  C  K  I  S  N  S  S  N  T  V  Q  C  E  C  S  E  N    263

TGGAAAGGTGAAGCATGTGACATTCCTCACTGTGTAAATAACTGTGGTTTTCCTCATCGA 849
---------+---------+---------+---------+---------+---------+
ACCTTTCCACTTCGTACACTGTAAGGAGTGACACATTTATTGACACCAAAAGGAGTAGCT
W  K  G  E  A  C  D  I  P  H  C  V  N  N  C  G  F  P  H  R    283

GGCATCTGCAATTCAAGTGATGTCAGAGGATGCTCCTGCTTCTCAGAGTGGCAAGGTCCT 909
---------+---------+---------+---------+---------+---------+
CCGTAGACGTTAAGTTCACTACAGTCTCCTACGAGGACGAAGAGTCTCACCGTTCCAGGA
G  I  C  N  S  S  D  V  R  G  C  S  C  F  S  E  W  Q  G  P    303

GGATGTTCAGTTCCTGTACCAGCTAACCAGTCATTTTGGACACGAGAGGAATATTCTGAC 969
---------+---------+---------+---------+---------+---------+
CCTACAAGTCAAGGACATGGTCGATTGGTCAGTAAAACCTGTGCTCTCCTTATAAGACTG
G  C  S  V  P  V  P  A  N  Q  S  F  W  T  R  E  E  Y  S  D    323
                                       MgP14
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```
CTAAAGCTCCCCAGAGCCTCTCACAAAGCTGTGGTCAATGGAAATATAATGTGGGTTGTT 1029
---------+---------+---------+---------+---------+---------+
GATTTCGAGGGGTCTCGGAGAGTGTTTCGACACCAGTTACCTTTATATTACACCCAACAA
L  K  L  P  R  A  S  H  K  A  V  V  N  G  N  I  M  W  V  V    343

GGCGGATATATGTTCAACCACTCAGATTACAACATGGTTCTAGCGTATGACCTTGCTTCT 1089
---------+---------+---------+---------+---------+---------+
CCGCCTATATACAAGTTGGTGAGTCTAATGTTGTACCAAGATCGCATACTGGAACGAAGA
G  G  Y  M  F  N  H  S  D  Y  N  M  V  L  A  Y  D  L  A  S    363

AGGGAGTGGCTTGCACTGAACCGTTCTGTGAACAGTGTGGTTGTTAGATATGGCCATTCT 1149
---------+---------+---------+---------+---------+---------+
TCCCTCACCGAACGTGACTTGGCAAGACACTTGTCACACCAACAATCTATACCGGTAAGA
R  E  W  L  A  L  N  R  S  V  N  S  V  V  V  R  Y  G  H  S    383

TTAGCATTATACCAGGATAAAATTTACATGTATGGAGGAAAAATTGATTCAACAGGGAAT 1209
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AATCGTAATATGGTCCTATTTTAAATGTACATACCTCCTTTTTAACTAAGTTGTCCCTTA
L  A  L  Y  Q  D  K  I  Y  M  Y  G  G  K  I  D  S  T  G  N    403

GTGACCAATGAGTTGAGAGTGTTTCATATTCATAATGAATCATGGGTGTTGTTGTCCCCC 1269
---------+---------+---------+---------+---------+---------+
CACTGGTTACTCAACTCTCACAAAGTATAAGTATTACTTAGTACCCACAACAACAGGGGG
V  T  N  E  L  R  V  F  H  I  H  N  E  S  W  V  L  L  S  P    423

AAAGCAAAGGAGCAGTATGCAGTGGTTGGGCACTCGGCGCACATTGTCACGTTGAAAAAT 1329
---------+---------+---------+---------+---------+---------+
TTTCGTTTCCTCGTCATACGTCACCAACCCGTGAGCCGCGTGTAACAGTGCAACTTTTTA
K  A  K  E  Q  Y  A  V  V  G  H  S  A  H  I  V  T  L  K  N    443

GGCCGAGTGGTCATGCTGGTTATCTTTGGTCACTGCCCTCTCTATGGATATATAAGCAGT 1389
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CCGGCTCACCAGTACGACCAATAGAAACCAGTGACGGGAGAGATACCTATATATTCGTCA
G  R  V  V  M  L  V  I  F  G  H  C  P  L  Y  G  Y  I  S  S    463

GTGCAGGAATATGACTTGGATAAGAACACATGGAGTATATTACACACTCAGGGTGCCCTT 1449
---------+---------+---------+---------+---------+---------+
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V  Q  E  Y  D  L  D  K  N  T  W  S  I  L  H  T  Q  G  A  L    483
```

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V  Q  G  G  Y  G  H  S  S  V  Y  D  D  R  T  K  A  L  Y  I    503

CACGGAGGCTACAAGGCTTTCAGTGCCAATAAATACCGGCTTGCAGATGATCTCTACAGA 1569
---------+---------+---------+---------+---------+---------+
GTGCCTCCGATGTTCCGAAAGTCACGGTTATTTATGGCCGAACGTCTACTAGAGATGTCT
H  G  G  Y  K  A  F  S  A  N  K  Y  R  L  A  D  D  L  Y  R    523
      MgP7
TATGATGTGGACACCCAGATGTGGACCATTCTTAAGGACAGCCGATTTTTCCGTTACTTG 1629
---------+---------+---------+---------+---------+---------+
ATACTACACCTGTGGGTCTACACCTGGTAAGAATTCCTGTCGGCTAAAAAGGCAATGAAC
Y  D  V  D  T  Q  M  W  T  I  L  K  D  S  R  F  F  R  Y  L    543

CACACAGCTGTGATAGTGAGTGGAACCATGCTGGTGTTTGGAGGAAACACACACAATGAC 1689
---------+---------+---------+---------+---------+---------+
GTGTGTCGACACTATCACTCACCTTGGTACGACCACAAACCTCCTTTGTGTGTGTTACTG
H  T  A  V  I  V  S  G  T  M  L  V  F  G  G  N  T  H  N  D    563
      MgP2

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---------+---------+---------+---------+---------+---------+
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T  S  M  S  H  G  A  K  C  F  S  S  D  F  M  A  Y  D  I  D    583

TGTGACCGATGGTCAGTGCTTCCCAGACCTGATCTCCACCATGATGTCAACAGATTTGGC 1809
---------+---------+---------+---------+---------+---------+
ACACTGGCTACCAGTCACGAAGGGTCTGGACTAGAGGTGGTACTACAGTTGTCTAAACCG
C  D  R  W  S  V  L  P  R  P  D  L  H  H  D  V  N  R  F  G    603

CACTCAGCGGTCTTATACAACAGAACCATGTATGTATTTGGTGGTTTTAATAGTCTCCTC 1869
---------+---------+---------+---------+---------+---------+
GTGAGTCGCCAGAATATGTTGTCTTGGTACATACATAAACCACCAAAATTATCAGAGGAG
H  S  A  V  L  Y  N  R  T  M  Y  V  F  G  G  F  N  S  L  L    623

CTCAGTGACATCCTAGTATTCACATCGGAACAGTGTGAAGCACACCAGAGTGAAGCTGCT 1929
---------+---------+---------+---------+---------+---------+
GAGTCACTGTAGGATCATAAGTGTAGCCTTGTCACACTTCGTGTGGTCTCACTTCGACGA
L  S  D  I  L  V  F  T  S  E  Q  C  E  A  H  Q  S  E  A  A    643
```

```
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C  L  A  A  G  P  G  V  R  C  M  W  D  T  G  S  S  Q  C  V    663

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---------+---------+---------+---------+---------+---------+
AGGACCCTCGACCGTGGACTTCGTGTTCTTTTCTATTTCAGTCTTACAAAAAGATTTTCT
S  W  E  L  A  P  E  A  Q  E  K  I  K  S  E  C  F  S  K  R    683

ATTTTTGACCATGACAGATGTGACCAGCTCACGGATTGTTACAGCTGCACAGCCAACACC 2109
---------+---------+---------+---------+---------+---------+
TAAAAACTGGTACTGTCTACACTGGTCGAGTGCCTAACAATGTCGACGTGTCGGTTGTGG
I  F  D  H  D  R  C  D  Q  L  T  D  C  Y  S  C  T  A  N  T    703

AATGGCTGCCAGTGGTGCAATGACCATTGTGTCCCTACGAACCACAGCTGCACAGAAGGC 2169
---------+---------+---------+---------+---------+---------+
TTACCGACGGTCACCACGTTACTGGTAACACAGGGATGCTTGGTGTCGACGTGTCTTCCG
N  G  C  Q  W  C  N  D  H  C  V  P  T  N  H  S  C  T  E  G    723

CAGATCTCCATTTTCAAGTATGATCATTGCCCCAAGGATAACCCGATGTATTATTGTAAC 2229
---------+---------+---------+---------+---------+---------+
GTCTAGAGGTAAAAGTTCATACTAGTAACGGGGTTCCTATTGGGCTACATAATAACATTG
Q  I  S  I  F  K  Y  D  H  C  P  K  D  N  P  M  Y  Y  C  N    743

AAGAAGACCAGCTGCAGGAGCTGTGCCCTGGACCAGAACTGCCAGTGGGAGCCCCGGAAT 2289
---------+---------+---------+---------+---------+---------+
TTCTTCTGGTCGACGTCCTCGACACGGGACCTGGTCTTGACGGTCACCCTCGGGGCCTTA
K  K  T  S  C  R  S  C  A  L  D  Q  N  C  Q  W  E  P  R  N    763

CAGGAGTGCATCGCCCTGCCCGAAAATATCTGTGGCATTGGCTGGCATTTGGTTGGAAAC 2349
---------+---------+---------+---------+---------+---------+
GTCCTCACGTAGCGGGACGGGCTTTTATAGACACCGTAACCGACCGTAAACCAACCTTTG
Q  E  C  I  A  L  P  E  N  I  C  G  I  G  W  H  L  V  G  N    783

TCATGTTTGAAAATTACTACTGCCAGGGAGACTTATGACAATGCAAAATTGTCCTGCAGA 2409
---------+---------+---------+---------+---------+---------+
AGTACAAACTTTTAATGATGACGGTCCCTCTGAATACTGTTACGTTTTAACAGGACGTCT
S  C  L  K  I  T  T  A  R  E  T  Y  D  N  A  K  L  S  C  R    803
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---------+---------+---------+---------+---------+---------+
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N  H  N  A  F  L  A  S  L  T  T  Q  K  K  V  E  F  V  L  K    823

CAGCTGCGAATCATGCAGTCATCACAGAGCATGTCCAAGTTCACCTTAACCCCATGGGTT 2529
---------+---------+---------+---------+---------+---------+
GTCGACGCTTAGTACGTCAGTAGTGTCTCGTACAGGTTCAAGTGGAATTGGGGTACCCAA
Q  L  R  I  M  Q  S  S  Q  S  M  S  K  F  T  L  T  P  W  V    843

GGCCTTCGGAAGATCAATGTGTCCTATTGGTGCTGGGAAGATATGTCCCCGTTCACAAAT 2589
---------+---------+---------+---------+---------+---------+
CCGGAAGCCTTCTAGTTACACAGGATAACCACGACCCTTCTATACAGGGGCAAGTGTTTA
G  L  R  K  I  N  V  S  Y  W  C  W  E  D  M  S  P  F  T  N    863

AGTTCACTACAGTGGATGCCATCTGAGCCCAGTGATGCTGGATTCTGTGGGATCTTGTCA 2649
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S  S  L  Q  W  M  P  S  E  P  S  D  A  G  F  C  G  I  L  S    883

GAACCCAGTACTCGGGGCTTAAAGGCTGCAACCTGCATCAATCCACTCAATGGTAGTGTC 2709
---------+---------+---------+---------+---------+---------+
CTTGGGTCATGAGCCCCGAATTTCCGACGTTGGACGTAGTTAGGTGAGTTACCATCACAG
E  P  S  T  R  G  L  K  A  A  T  C  I  N  P  L  N  G  S  V    903
                  MgP3
TGTGAAAGGCCTGCTAACCACAGTGCCAAGCAGTGTCGGACCCCTTGTGCCTTGCGGACA 2769
---------+---------+---------+---------+---------+---------+
ACACTTTCCGGACGATTGGTGTCACGGTTCGTCACAGCCTGGGGAACACGGAACGCCTGT
C  E  R  P  A  N  H  S  A  K  Q  C  R  T  P  C  A  L  R  T    923

GCCTGTGGGGAGTGTACCAGTGGCAGCTCGGAGTGCATGTGGTGCAGCAACATGAAGCAG 2829
---------+---------+---------+---------+---------+---------+
CGGACACCCCTCACATGGTCACCGTCGAGCCTCACGTACACCACGTCGTTGTACTTCGTC
A  C  G  E  C  T  S  G  S  S  E  C  M  W  C  S  N  M  K  Q    943

TGCGTGGACTCCAATGCCTACGTGGCCTCCTTCCCTTTTGGCCAGTGTATGGAGTGGTAT 2889
---------+---------+---------+---------+---------+---------+
ACGCACCTGAGGTTACGGATGCACCGGAGGAAGGGAAAACCGGTCACATACCTCACCATA
C  V  D  S  N  A  Y  V  A  S  F  P  F  G  Q  C  M  E  W  Y    963
   MgP8
```

```
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T  M  S  S  C  P  P  E  N  C  S  G  Y  C  T  C  S  H  C  L    983

GAGCAACCAGGCTGTGGCTGGTGCACTGATCCTAGCAATACGGGCAAAGGGAAATGCATA 3009
---------+---------+---------+---------+---------+---------+
CTCGTTGGTCCGACACCGACCACGTGACTAGGATCGTTATGCCCGTTTCCCTTTACGTAT
E  Q  P  G  C  G  W  C  T  D  P  S  N  T  G  K  G  K  C  I    1003

GAGGGCTCCTATAAAGGACCAGTGAAGATGCCTTCTCAGGGCCCTACAGGAAATTCCTAC 3069
---------+---------+---------+---------+---------+---------+
CTCCCGAGGATATTTCCTGGTCACTTCTACGGAAGAGTCCCGGGATGTCCTTTAAGGATG
E  G  S  Y  K  G  P  V  K  M  P  S  Q  G  P  T  G  N  S  Y    1023

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---------+---------+---------+---------+---------+---------+
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P  Q  P  L  L  N  S  S  M  C  L  E  D  S  R  Y  N  W  S  F    1043

ATTCATTGTCCAGCTTGCCAGTGCAATGGCCACAGCAAATGCATTAATCAGAGTATCTGT 3189
---------+---------+---------+---------+---------+---------+
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I  H  C  P  A  C  Q  C  N  G  H  S  K  C  I  N  Q  S  I  C    1063

GAGAAGTGTGAGAACCTGACCACGGGCAAGCACTGTGAGACCTGCATATCTGGCTTCTAT 3249
---------+---------+---------+---------+---------+---------+
CTCTTCACACTCTTGGACTGGTGCCCGTTCGTGACACTCTGGACGTATAGACCGAAGATA
E  K  C  E  N  L  T  T  G  K  H  C  E  T  C  I  S  G  F  Y    1083
                                                      MgP9
GGTGATCCAACCAACGGGGGAAAATGTCAACCATGCAGGTGCAATGGACATGCGTCGCTA 3309
---------+---------+---------+---------+---------+---------+
CCACTAGGTTGGTTGCCCCCTTTTACAGTTGGTACGTCCACGTTACCTGTACGCAGCGAT
G  D  P  T  N  G  G  K  C  Q  P  C  R  C  N  G  H  A  S  L    1103

TGCAATACCAACACGGGCAAGTGCTTCTGCACCACCAAGGGCGTCAAGGGGGATGAATGT 3369
---------+---------+---------+---------+---------+---------+
ACGTTATGGTTGTGCCCGTTCACGAAGACGTGGTGGTTCCCGCAGTTCCCCCTACTTACA
C  N  T  N  T  G  K  C  F  C  T  T  K  G  V  K  G  D  E  C    1123
```

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---------+---------+---------+---------+---------+---------+
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Q  L  C  E  V  E  N  R  Y  Q  G  N  P  L  K  G  T  C  Y  Y    1143

ACTCTTCTTATTGACTATCAGTTCACCTTTAGCCTATCCCAGGAAGATGACCGCTATTAC 3489
---------+---------+---------+---------+---------+---------+
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T  L  L  I  D  Y  Q  F  T  F  S  L  S  Q  E  D  D  R  Y  Y    1163

ACAGCCATCAATTTTGTGGCGACACCTGATGAACAAAACAGGGATTTGGACATGTTCATC 3549
---------+---------+---------+---------+---------+---------+
TGTCGGTAGTTAAAACACCGCTGTGGACTACTTGTTTTGTCCCTAAACCTGTACAAGTAG
T  A  I  N  F  V  A  T  P  D  E  Q  N  R  D  L  D  M  F  I    1183
                                        MgP4

AATGCCTCCAAAAACTTCAACCTCAACATCACCTGGGCTGCCAGCTTCTCAGCTGGAACC 3609
---------+---------+---------+---------+---------+---------+
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N  A  S  K  N  F  N  L  N  I  T  W  A  A  S  F  S  A  G  T    1203

CAAGCTGGAGAAGAGATGCCTGTTGTTTCAAAAACCAACATTAAGGAGTACAAAGATAGT 3669
---------+---------+---------+---------+---------+---------+
GTTCGACCTCTTCTCTACGGACAACAAAGTTTTTGGTTGTAATTCCTCATGTTTCTATCA
Q  A  G  E  E  M  P  V  V  S  K  T  N  I  K  E  Y  K  D  S    1223

TTCTCTAATGAGAAGTTTGATTTTCGCAACCACCCAAATATCACTTTCTTTGTTTATGTC 3729
---------+---------+---------+---------+---------+---------+
AAGAGATTACTCTTCAAACTAAAAGCGTTGGTGGGTTTATAGTGAAAGAAACAAATACAG
F  S  N  E  K  F  D  F  R  N  H  P  N  I  T  F  F  V  Y  V    1243

AGTAATTTCACCTGGCCCATCAAAATTCAGATTGCATTCTCCCAGCACAGCAATTTTATG 3789
---------+---------+---------+---------+---------+---------+
TCATTAAAGTGGACCGGGTAGTTTTAAGTCTAACGTAAGAGGGTCGTGTCGTTAAAATAC
S  N  F  T  W  P  I  K  I  Q  I  A  F  S  Q  H  S  N  F  M    1263

GACCTAGTACAGTTCTTCGTGACCTTCTTCAGTTGTTTCCTCTCTCTGCTCCTGGTGGCT 3849
---------+---------+---------+---------+---------+---------+
CTGGATCATGTCAAGAAGCACTGGAAGAAGTCAACAAAGGAGAGAGACGAGGACCACCGA
D  L  V  Q  F  F  V  T  F  F  S  C  F  L  S  L  L  L  V  A    1283
```

```
GCTGTGGTTTGGAAGATCAAACAAAGTTGTTGGGCTTCCAGACGTAGAGAGCAACTTCTT 3909
---------+---------+---------+---------+---------+---------+
CGACACCAAACCTTCTAGTTTGTTTCAACAACCCGAAGGTCTGCATCTCTCGTTGAAGAA
A  V  V  W  K  I  K  Q  S  C  W  A  S  R  R  R  E  Q  L  L    1303

CGAGAGATGCAGCAGATGGCCAGTCGTCCCTTTGCCTCTGTAAATGTCGCCTTGGAAACA 3969
---------+---------+---------+---------+---------+---------+
GCTCTCTACGTCGTCTACCGGTCAGCAGGGAAACGGAGACATTTACAGCGGAACCTTTGT
R  E  M  Q  Q  M  A  S  R  P  F  A  S  V  N  V  A  L  E  T    1323

GATGAAGAGCCTCCTGATCTTATTGGGGGGAGTATAAAGACTGTTCCCAAGCCCATTGCC 4029
---------+---------+---------+---------+---------+---------+
CTACTTCTCGGAGGACTAGAATAACCCCCCTCATATTTCTGACAAGGGTTCGGGTAACGG
D  E  E  P  P  D  L  I  G  G  S  I  K  T  V  P  K  P  I  A    1343

CTGGAGCCGTGTTTTGGCAACAAAGCTGCTGTCCTCTCTGTGTTTGTGAGGCTCCCTCGA 4089
---------+---------+---------+---------+---------+---------+
GACCTCGGCACAAAACCGTTGTTTCGACGACAGGAGAGACACAAACACTCCGAGGGAGCT
L  E  P  C  F  G  N  K  A  A  V  L  S  V  F  V  R  L  P  R    1363

GGCCTCGGTGGAATCCCTCCTCCTGGGCAGTCCGGGCTCGCAGTGGCCAGTGCCCTGGTG 4149
---------+---------+---------+---------+---------+---------+
CCGGAGCCACCTTAGGGAGGAGGACCCGTCAGGCCCGAGCGTCACCGGTCACGGGACCAC
G  L  G  G  I  P  P  P  G  Q  S  G  L  A  V  A  S  A  L  V    1383

GACATTTCTCAGCAGATGCCAGTCGTCTACAAAGAGAAGTCAGGAGCAGTGAGAAACCGA 4209
---------+---------+---------+---------+---------+---------+
CTGTAAAGAGTCGTCTACGGTCAGCAGATGTTTCTCTTCAGTCCTCGTCACTCTTTGGCT
D  I  S  Q  Q  M  P  V  V  Y  K  E  K  S  G  A  V  R  N  R    1403

AAACAGCAGCCCCCTGCGCAGCCTGGGACCTGCATTTGAATGCTGGGGCCGGGACTCCCTG 4269
---------+---------+---------+---------+---------+---------+
TTTGTCGTCGGGGGACGCGTCGGACCCTGGACGTAAACTACGACCCCGGCCCTGAGGGAC
K  Q  Q  P  P  A  Q  P  G  T  C  I  *                        1415

TGAGCAAGGAGTGGCGTGCCAGAGCCGCCTGCCAGGAGGGAGCAGGCAGGGAGAAGATGC 4329
---------+---------+---------+---------+---------+---------+
ACTCGTTCCTCACCGCACGGTCTCGGCGGACGGTCCTCCCTCGTCCGTCCCTCTTCTACG

TATGCGGGGCTGAAGACTGGAAACCCTCGAATCATCCACCTCCTCTGCATGTTCACAAGC 4389
---------+---------+---------+---------+---------+---------+
ATACGCCCCGACTTCTGACCTTTGGGAGCTTAGTAGGTGGAGGAGACGTACAAGTGTTCG
```

```
TTTCTTTGACAGTTTCTCCCATCTGTACTCCAGCATCTAACCTTTTACTTCTG 4442
---------+---------+---------+---------+---------+---
AAAGAAACTGTCAAAGAGGGTAGACATGAGGTCGTAGATTGGAAAATGAAGAC
                              MgPend
```

Figure 35: Coding cDNA and deduced amino acid sequence of bovine attractin gene

Start and stop codons are shown in a boxes. Primers are shown in grey, MgR2 primer overlapping with the Mgex3F1 primer is shown in a box. 5' end of the RACE clone is the beginning of the Mgex1F1 primer (position 234), BAC clone was sequenced using Mgex1R8 primer until the end of the shown sequence, location of a boundary between cDNA and genomic sequence at the 5' end is unknown.

Curriculum vitae

Surname	Graphodatskaya
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1981-1991	Secondary school N 130, Novosibirsk
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1992-1997	Biological Department of the Novosibirsk State University
1997	Diploma work at the Laboratory of Biochemical Genetics, Institute of Cytology and Genetics, Novosibirsk
1998	Scolarship in the group of Prof. G. Stranzinger at the Institute of Animal Sciences, Swiss Federal Institute of Technology (ETH)
1999-2002	Ph D studies and assistant in the group of Prof. G. Stranzinger at the Institute of Animal Sciences, Swiss Federal Institute of Technology (ETH)

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