Construction of chimeric 5-HT_4/5-HT_7 serotonin receptors

Thesis in pharmacology

for the degree *Candidata pharmaciae*

by

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Oslo, November 2007 Chrishanthy EliZabeth

The Lord is my Shepherd; I shall not want Psalm 23:1

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ABBREVIATIONS

Abbreviation	***Full name***
AC	adenylyl cyclase
ATP	adenosine triphosphate
β-AR	β-adrenergic receptors
bp	base pair
cAMP	adenosine-3',5'-cyclic monophosphate
cDNA	complementary DNA
CE	capillary electrophoresis
c-Myc	complementary myelocytomatosis
CNS	central nervous system
5-CT	5-carboxamidotryptamine
dATP	deoxyadenosine-5'-triphospate
ddNTP	dideoxyribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
DNA	deoxyribonucleic acid
EL	extracellular loop
EPR	EP prostaglandin receptor
ERK	extracellular signal-regulated kinase
GPCRs	G-protein-coupled receptors
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
G_s protein	stimulatory G protein
h	human
HA	hemagglutinin
HEK	human embryonic kidney
5-HT	5-hydroxytryptamine
IC	intracellular
kb	kilobase
mRNA	messenger ribonucleic acid
OD	optical density
ORF	open reading frame
pcDNA	plasmid complementary DNA
PCR	polymerase chain reaction
PKA	protein kinase A
PLC	phospholipase C
PNS	peripheral nervous system
RNA	ribonucleic acid
SCN	suprachiasmatic nucleus
SR	split receptor
TAE	Tris-acetate EDTA
TM	transmembrane
TMDs	transmembrane domains
YFP	yellow fluorescent protein
UV-light	ultraviolet light

1. SUMMARY

Unlike other G-protein-coupled receptors the human 5-HT_7 receptor reduces the signalling through other receptors when expressed together in cells (Bruheim *et al.*, 2003;Andressen *et al.*, 2006). To understand this unusual property of the 5-HT_7 receptor, we aim to determine the key amino acid sequences of the 5-HT_7 receptor which are responsible for the attenuation of signalling through other G_s-coupled receptors.

As a first step to determine the key amino acid sequences of the 5-HT_7 receptor responsible for this attenuation we constructed chimerae of the 5-HT_7 receptor and another serotonin receptor which does not display this unusual property, the 5-HT_4 receptor. Plasmids encoding the 5-HT_7 and 5-HT_4 receptors were engineered using molecular biology techniques to achieve the (recombinant) plasmids encoding the desired chimeric receptors.

A strategy involving insertion of restriction sites for the restriction endonuclease ***Bsi*WI** was employed to construct chimeric receptors of human (h) 5-$HT_{7(b)}$ and h5-$HT_{4(b)}$. The mutagenesis primers for the respective receptors were designed using the computer software SE Central designed for cloning.

The plasmids were mutated by two methods. Mutation in extracellular loop 2 was performed by method I (Gene Tailor™ Site-Directed Mutagenesis System) and mutation in extracellular loop 1 was performed by method II (Standard mutagenesis strategy). Once the plasmids containing the receptor genes were mutated, they were subsequently cut by endonuclease enzymes. The cut DNA fragments were interchanged and ligated with the opposite parts from both receptor types. The recombinant DNA was verified by both restriction digestion and sequencing.

Chimeric receptor constructs based on cutting in extracellular loop 1 (EL1) of the receptors were successfully made. The constructed chimers were 5-HT_7 up to EL1, 5-HT_4 from EL1 and 5-HT_4 up to EL1, 5-HT_7 from EL1. Chimeric receptor constructs based on cutting in extracellular loop 2 (EL2) were also made, but sequencing revealed that a string of 10 bp had been lost. The constructed chimeric receptors will subsequently be expressed in HEK293 cells and their pharmacological properties determined by binding and adenylyl cyclase assays.

2. INTRODUCTION

2.1 G-PROTEIN-COUPLED RECEPTORS

2.1.1 General structure and classification of the G-protein-coupled receptors

G-protein-coupled receptors (GPCRs) are the most numerous and versatile family of proteins that control cell-cell communications. Their endogenous ligands include hormones, neurotransmitters, growth and survival factors. Exogenous factors such as smells, tastes and photons also act as ligands for the GPCRs. (Joubert *et al.*, 2002).

All the GPCRs have an extracellular N-terminal segment, seven transmembrane domains (TMDs) which form the TM core, three extracellular loops (exoloops), three intracellular loops (cytoloops) and an intracellular C-terminal segment (Fig.1). The C-terminal segment can be palmitoylated at a cystein residue, thus forming a fourth cytoloop. Each of the seven TMDs is generally composed of 20-27 amino acids, whereas the N-terminal segments vary from 7-595 amino acids, the loops from 5-230 amino acids and the C-terminal segments from 12-359 amino acids. These variations in size specify their diverse structures and functions (Ji *et al.*, 1998).

Odd numbers of TMDs place the N- and C- terminal segments at opposite membrane surfaces. It allows glycosylation and ligand binding at the N-terminal segment, and phosphorylation and palmitoylation at the C-terminal segment for desensitization and internalization. It seems like the seven TMDs may be the minimum necessary to form six loops and therefore gives the TM core a sufficient size and versatility. The structure of GPCRs offers a prodigious number of specificities, regulatory mechanisms, and contact sites for G-protein and other signal molecules such as Jak2 kinase, phospholipase C_{γ}, GPCR-kinases, arrestin, calmodulin and/or protein kinase C.

The TM core is tightly packed by hydrogen bonds and salt bridges, leaving no room for a channel or tunnel structure (Ji *et al.*, 1998).

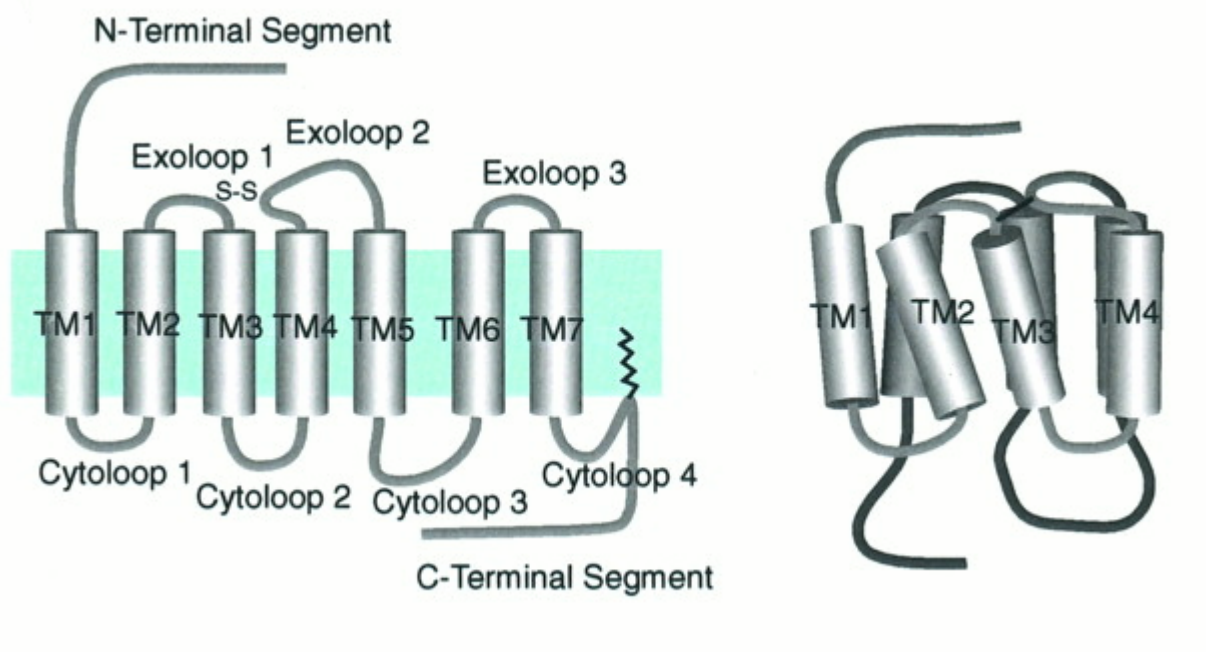


Figure 1. Schematic presentation of the general structure of G-protein coupled receptors (GPCRs). Adapted from (Ji *et al.*, 1998)

2.1.2 Ligand binding and signal generation

Individual GPCRs can signal through several G-protein subtypes and through G-protein-independent pathways, often in a ligand-specific manner. This functional plasticity can be attributed to structural flexibility of the GPCRs and the ability of the ligands to induce or stabilize ligand-specific conformations. Ligands for a given GPCR can show different efficacy profiles for coupling to distinct signalling pathways.

Recent studies suggest that agonist binding and receptor activation occur through a series of conformational intermediates (Fig.2). Transition to these intermediate states involves the disruption of non-covalent intramolecular interactions that stabilize the basal state of the receptor. Binding of structurally different agonists might entail the disruption of different combinations of these intramolecular interactions, leading to different receptor conformations and differential effects on downstream signalling proteins (Kobilka and Deupi, 2007).

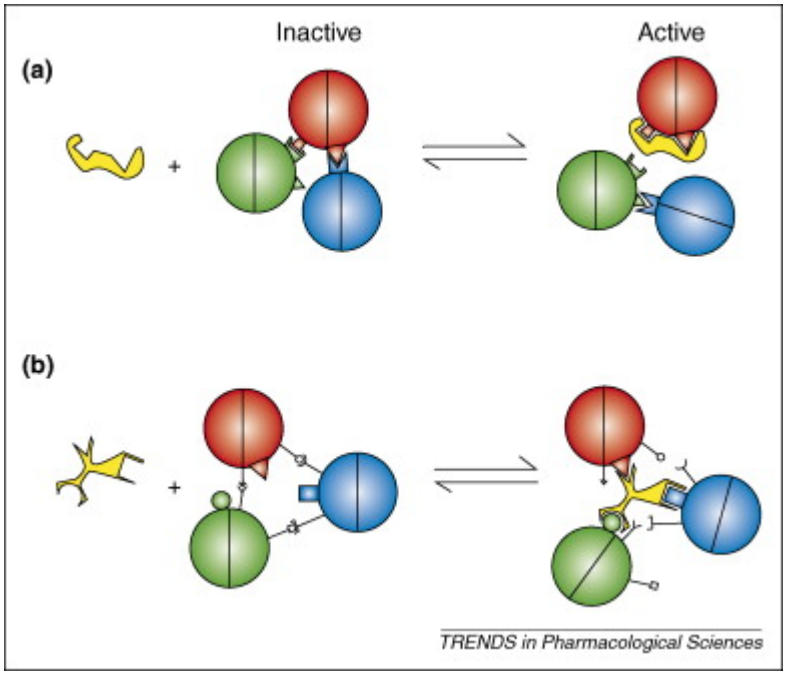


Figure 2. Possible mechanisms by which agonist binding disrupts intramolecular interactions that stabilize the inactive GPCR state. **(a)** The agonist (yellow) binds directly to amino acids involved in stabilizing the inactive receptor (red, green, blue) state. **(b)** Agonist binding stabilizes a new set of intramolecular interactions. Adapted from (Kobilka and Deupi, 2007).

2.2 HETEROTRIMERIC G PROTEINS

When an extracellular agonist binds to a GPCR, the receptor undergoes a conformational change that enables it to activate heterotrimeric GTP-binding proteins (G proteins). These G proteins reside at the cytoplasmic face of the plasma membrane, where they serve as relay molecules, functionally coupling the receptors to enzymes or ion channels in the membrane. There are various types of G proteins, each specific for a particular set of GPCRs and for a particular set of downstream target proteins in the plasma membrane.

G-proteins are composed of three protein subunits – α, β, and γ. In the unstimulated state, the α-subunit has GDP bound and the G protein is inactive. When stimulated by an activated receptor, the α-subunit releases its bound GDP, allowing GTP to bind in its place. This exchange causes the trimer to dissociate into two activated components, the α-subunit and the βγ-complex.

The dissociation of the trimeric G-protein activates its two components in different ways. GTP-binding causes a conformational change that affects the surface of the α-subunit that

associates with the βγ-complex in the trimer. This change causes the release of the βγ-complex, but it also causes the α-subunit to adopt a new shape that allows it to interact with its target proteins. The βγ-complex does not change its conformation, but the surface previously masked by the α-subunit is now available to interact with a second set of target proteins. The α-subunit is a GTPase, and once it hydrolyzes its bound GTP to GDP, it reassociates with a βγ complex to reform an inactive G protein, reversing the activation process.
Both the α-subunit and βγ-complex can regulate the activation and inhibition of different G protein-coupled effector molecules (The Cell, 4th ed. Pg 852-856).

G-proteins are divided into four subfamilies, depending on their α-subunit: 1) The 'G_s' subfamily that stimulates adenylyl cyclase (AC), 2) the '$G_{i/o}$' subfamily that inhibits AC and regulates ion channels, 3) the '$G_{q/11}$' subfamily that activates phospholipase C_β and 4) the '$G_{12/13}$' subfamily that has been shown to activate a small GTPase, Rho. Depending on the subtype(s) of the G protein that a given GPCR interacts with, a single or a combination of effectors can be activated (Wong, 2003).There are a number of subtypes of β- and γ-subunits, too. A combination of different subtypes of α-, β- and γ- subunits provides a great diversity of intracellular signalling pathways that can be regulated by GPCRs (Wong, 2003).

2.3 5-HT

Serotonin (5-hydroxytryptamine; 5-HT) is an endogenous monoamine synthesised from the amino acid tryptophan mainly in the enterochromaffin cells of the intestine (95%) and the rest in discrete areas of the brain and neuroendothelial cells lining the lung. Tryptophan is converted to 5-hydroxytryptophan by tryptophan hydroxylase. The 5-hydroxytryptophan is then decarboxylated by an amino acid decarboxylase to 5-hydroxytryptamine.

5-HT plays a crucial role in a number of physiological and pathological processes in many organs including the brain, gastrointestinal tract, heart, blood cells and vessels. It acts both as a neurotransmitter and as a local hormone in the peripheral vascular system (Rang *et al.*, 2003). The physiological effects of the 5-HT in different blood vessels or different parts of the heart depend on the 5-HT receptors involved, the intracellular signals evoked through these receptors, and their cellular localisation (Kaumann and Levy, 2006b).

The mechanisms of synthesis, storage, release and reuptake of 5-HT are very similar to that of noradrenaline, and many drugs affect both processes indiscriminately. Degradation of 5-HT occurs mainly through oxidative deamination, catalysed by monoamine oxidase, followed by oxidation to 5-hydroxyindoleacetic acid (5-HIAA), the pathway being the same as that of noradrenaline catabolism (Rang *et al.*, 2003).

5-HT has diverse effects on the cardiovascular system. The hormone displays both vasodilation and vasoconstriction depending on the vessel site mediated through different 5-HT receptor subtypes (Rang *et al.*, 2003). In the heart, 5-HT can elicit cardioexcitation, such as increased inotropic and chronotropic effects, through a direct action on the heart (Kaumann and Sanders, 1998;Brattelid *et al.*, 2004) and cardiodepression indirectly via stimulation of the central nervous system (Kaumann and Sanders, 1998).

Although 5-HT is synthesized by only a small group of neurons within the raphe nuclei of the brain stem, these cells send both ascending and descending projections to large parts of the CNS. Because of this widespread innervation, 5-HT has been implicated in numerous important physiological and pathophysiological phenomena, including sleep-wakefulness cycles and several psychiatric disorders (Hoyer *et al.*, 2002;Hedlund and Sutcliffe, 2004).

2.4 5-HT RECEPTORS

Among known neurotransmitters, 5-HT acts on the most diverse group of receptors. 5-HT exerts its effects via fourteen different receptor subtypes. All of them are G protein-coupled receptors, with the exception of 5-HT_3 receptors, which are ligand-gated cation-channels. The 5-HT receptors act primarily by means of the following second messenger systems: $5\text{-HT}_{1A,B,D,E,F}$ receptors decrease cyclic AMP (cAMP) formation; $5\text{-HT}_{2A,B,C}$ receptors increase inositol triphosphate and diacylglycerol formation; 5-HT_3 receptors increase Na^+- and Ca^{2+}-influx (Barnes and Sharp, 1999)and 5-HT_4 receptors increase cAMP formation (Kaumann, 1990).The physiological effector system for $5\text{-HT}_{5A,B}$ receptors remains elusive, although iniositol triphosphate and diacylglycerol formation have been implicated in transfected cells{Francken, 2000 3997 /id}. The $5\text{-HT}_{6,7}$ receptors are positively coupled to adenylyl cyclase and increase cAMP formation (Barnes and Sharp, 1999).

The 5-HT_7 receptors are widely expressed in different cell types in the central nervous system and in peripheral tissues. Genetic and pharmacological studies have provided evidence that 5-

HT_7 receptors may be involved in regulation of emotions, thermoregulation, circadian rhythmicity, memory processes and smooth muscle relaxation(Vanhoenacker *et al.*, 2000).

2.5 5-HT_4 RECEPTORS

5-HT_4 receptors occur in the brain, as well as in peripheral organs, such as the gastrointestinal tract, bladder and heart. One of their main physiological roles appears to be in the gastrointestinal tract, where they produce neuronal excitation and mediate the effect of 5-HT in stimulating peristalsis (Rang *et al.*, 2003). Experimental studies have shown that 5-HT through 5-HT_4 receptors exerts positive chronotropic and inotropic effects in human and porcine cardiac atria (Kaumann and Sanders, 1998).

The human 5-HT_4 receptor exists in multiple splice variants, 5-$HT_{4\,(a\text{-}g)}$ and 5-$HT_{4\,(n)}$. They are identical up to Leu 358, followed by different C-terminal tails. 5-$HT_{4(hb)}$ is a splice variant which is a result of an extra exon "h" giving an insertion of 14 amino acid in the second extracellular loop (Fig.3). Exon h was only observed in combination with the 5-$HT_{4(b)}$ tail and therefore the receptor was named 5-$HT_{4(hb)}$ {Brattelid, 2004 1951 /id; Bender, 2000 2153 /id}

It seems like the 5-$HT_{4\,(b)}$ splice variant is the dominant splice variant in all human tissues examined, including the human heart tissue(Medhurst *et al.*, 2001).

The 5-HT_4 receptors are positively coupled to the G_s protein-adenylyl cyclase system which leads to increased cAMP level in the cell (Langlois and Fischmeister, 2003).

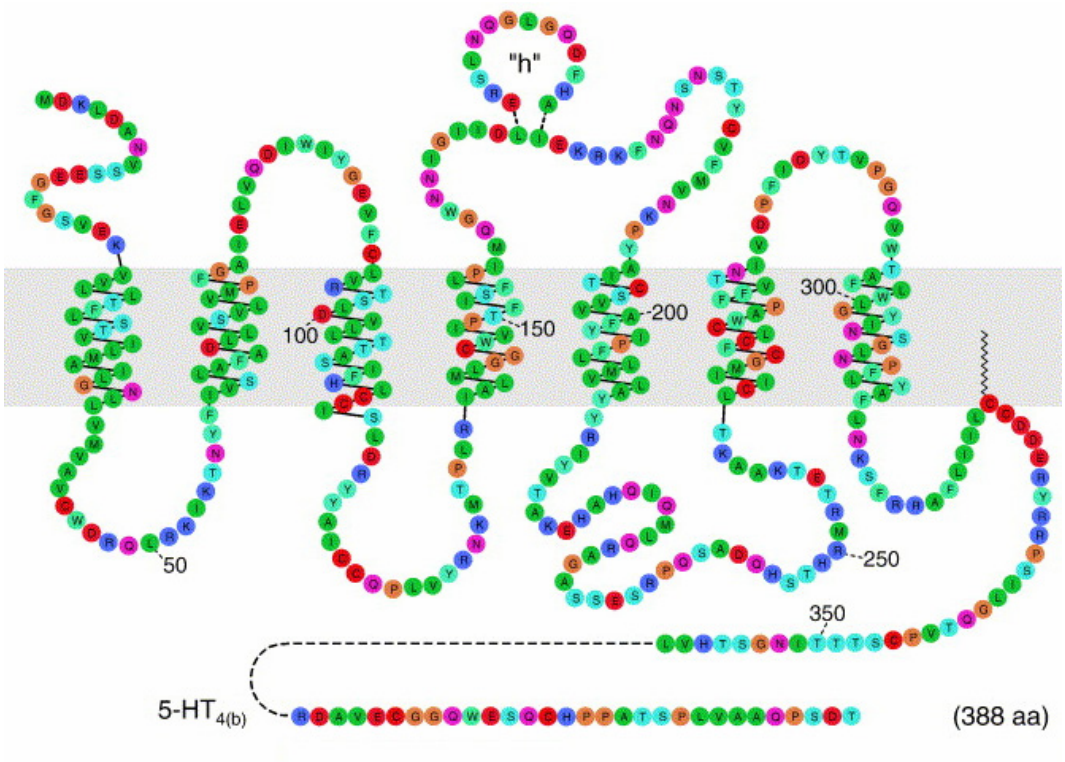


Figure 3. Structure of the human 5-$HT_{4(b)}$ receptor amino acid sequence. The 5-$HT_{4(hb)}$ splice variant has an extra 14 amino acid extracellular loop, indicated with "h". Adapted from (Kaumann and Levy, 2006a)

2.6 5-HT_7 RECEPTORS

The 5-HT_7 receptors are the most recently identified member of the 5-HT receptor family and has been characterized on the basis of structural, operational and transductional characteristics. It has been cloned from various species such as human, rat, mouse, pig, guinea pig and *Xenopus laevis*. The 5-HT_7 receptors are positively linked to adenylyl cyclase (Vanhoenacker *et al.*, 2000).

Alternative splicing of the human gene has been reported to generate three 5-HT_7 receptor isoforms (5-$HT_{7a,b,d}$), which differ in their C-termini. The amino acid sequence of the 5-$HT_{7(b)}$ receptor is shown in figure 4. To date, these isoforms show no major differences in their respective pharmacology and signal transduction properties (Heidmann *et al.*, 1998;Krobert *et al.*, 2001).

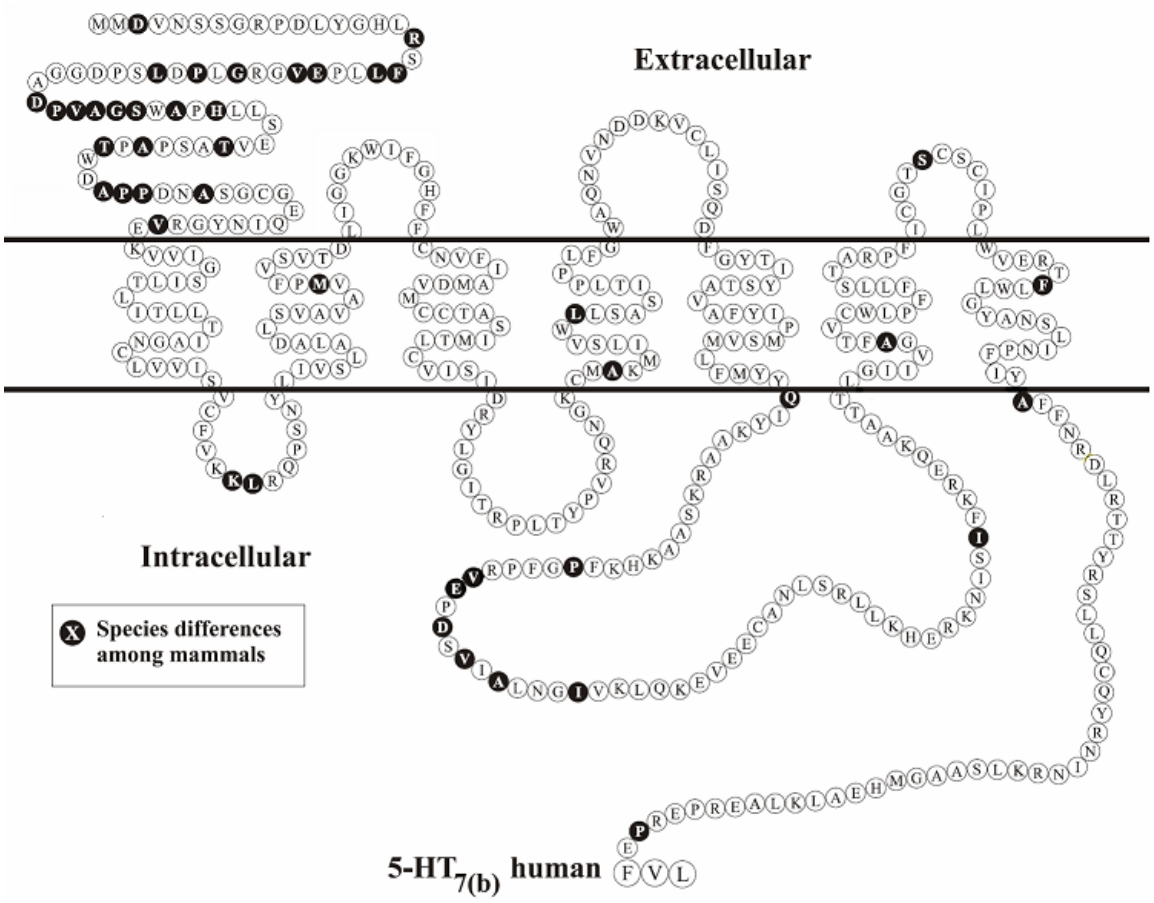


Figure 4. Structure of the human 5-$HT_{7(b)}$ receptor amino acid sequence. Adapted from (Heidmann *et al.*, 1997).

Pharmacologically, the 5-HT_7 receptor shows high affinity for 5-carboxamidotryptamine (5-CT) and 5-hydroxytryptamine (5-HT). Several antipsychotics and antidepressants have high affinity for the 5-HT_7 receptors. Several antidepressants, both tricyclic antidepressants and selective 5-HT reuptake inhibitors (SSRIs), induced *c-fos* expression in rats in a manner consistent with 5-HT_7 receptor activation within the suprachiasmatic nucleus (SCN) of the hypothalamus. The 5-HT_7 receptors are involved in thermoregulation, circadian rhythm, learning and memory, hippocampal signalling, sleep and endocrine regulation (Hedlund and Sutcliffe, 2004).

5-HT_7 receptor signalling

When expressed in cell lines, the 5-HT_7 receptors display pharmacological properties consistent with tight association with the G protein, regardless of agonist binding (Bruheim *et al.*, 2003). Activation of the G protein G_s leads to activation of adenylyl cyclase and a rapid

increase in the formation of the intracellular second messenger cAMP. This elevation of cAMP level has several intracellular effects, such as activation of cAMP-dependent protein kinase (PKA) and exchange proteins directly activated by cAMP (Epacs), guanine nucleotide exchange factors specific for Rap (Vossler *et al.*, 1997;de Rooij *et al.*, 1998;Kawasaki *et al.*, 1998).

The 5-HT_7 receptors activate the extracellular signal-regulated kinase (ERK) through a mechanism that is dependent on a Ras monomeric GTPase (Norum *et al.*, 2003). Activation of the 5-HT_7 receptors stimulate ERK in hippocampal neurons, an effect that can be of importance for hippocampal function and mood regulation (Errico *et al.*, 2001).

2.7 BACKGROUND AND PURPOSE OF THE PRESENT STUDY

Previous studies on Human Embryonic Kidney (HEK) 293 cells revealed that activation of adenylyl cyclase by other endogenous G_s-coupled receptors is attenuated by the 5-HT_7 receptors when the receptors are expressed together in the cells. Using the ecdysone-inducible expression system, which allows the titration of increasing receptor density in the same clonal cell line, the effects of 5-$HT_{4(b)}$ and 5-$HT_{7(a,b,d)}$ receptor expression on adenylyl cyclase (AC) stimulation by the endogenous G_s-coupled β-adrenergic(βAR) and prostanoid EP (EPR) receptors were compared. The conclusion of the study was that βAR and EPR-stimulated AC activity was attenuated by 5-HT_7 receptor expression in both membrane preparations and intact HEK293 cells (Fig.5). At the same time the βAR and EPR-stimulated AC activity was unaffected by expression of the G_s-coupled 5-HT_4 receptor (Andressen *et al.*, 2006). Based on these data the present study was designed.

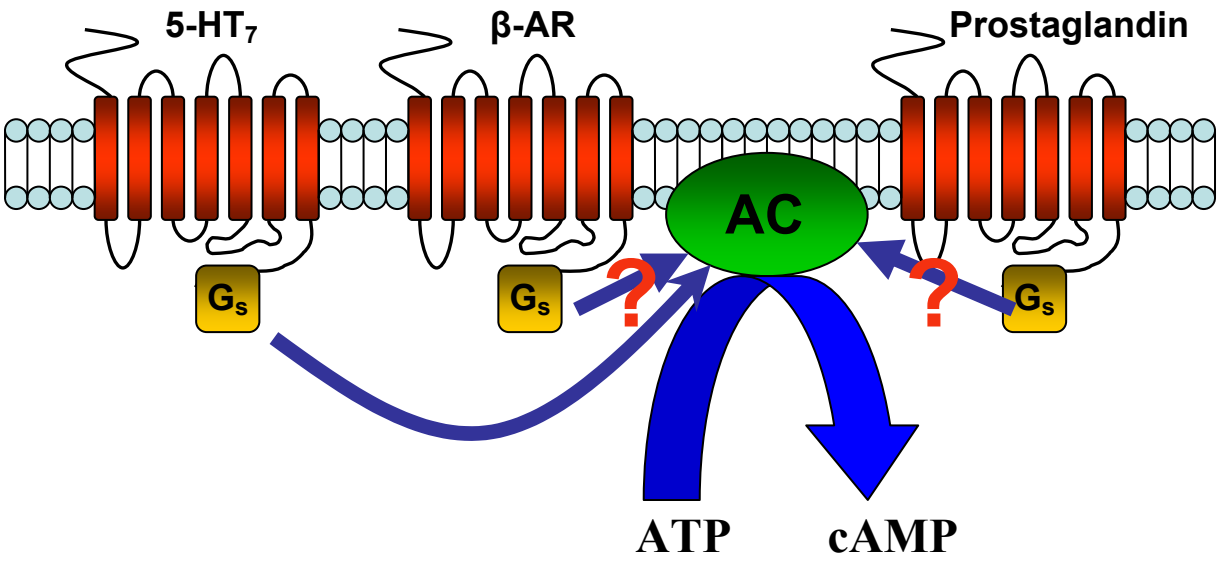


Figure 5. The figure illustrates the cellular mechanisms of the receptors 5-HT_7, βAR and EPR expressed together in HEK293 cells. (Designed by Cam Hong Thi Nguyen, 2007)

The ultimate aim of the research program of which the present study is a part is to determine the molecular properties of the 5-HT_7 receptor which leads to this unusual property of attenuation of the signalling through other endogenous G_s-coupled receptors. To achieve this goal we want to construct chimeric receptors of 5-HT_7.

Since the βAR and EPR-stimulated AC activity was unaffected by expression of the G_s-coupled 5-$HT_{4(b)}$ receptor this receptor seems to be a very good candidate in construction of the chimers of 5-HT_7. The chimers will contain parts of the 5-$HT_{4(b)}$ receptor and the 5-$HT_{7(b)}$ receptor.

The constructed chimeric receptors will subsequently be expressed in HEK293 cells and their pharmacological properties determined by binding and adenylyl cyclase assays.

3. MATERIALS AND METHODS

3.1 VECTORS UTILIZED

pcDNA 3.1 vectors

The pcDNA3.1(+) and pcDNA3.1(-) (Invitrogen®) are 5.4 kb vectors derived from pcDNA3 and designed for high-level stable and transient expression in mammalian host cells such as HEK293 cells. The vectors contain multiple cloning sites in the forward (+) and reverse (-) orientations to facilitate cloning.

The plasmid 3xHA-5-$HT_{4(b)}$ contains an N-terminally hemagglutinin-tagged 5-$HT_{4(b)}$ receptor coding sequence cloned into pcDNA3.1(+) at ***Kpn*I** (5') and ***Xho*I** (3'). The plasmid was purchased from cDNA.org.

The plasmid c-myc-5-$HT_{7(a)}$ contains an N-terminally myc-tagged 5-$HT_{7(a)}$ receptor coding sequence cloned into pcDNA3.1(+) at ***Bam*HI** (5') and ***Eco*RI** (3')(De Martelaere *et al.*, 2007) . This plasmid was kindly provided by Dr. Peter Vanhoenacker, Ghent, Belgium.

The plasmid 5-$HT_{7(b)}$ YFP contains the coding sequence of the human 5-$HT_{7(b)}$ receptor fused with a coding sequence for the C-terminal fluorescent protein called (enhanced) Yellow Fluorescent Protein, cloned into pcDNA3. When expressed in HEK293 cells and examined by fluorescence microscopy these proteins emit a yellow light showing the location and the movement of the 5-$HT_{7(b)}$ in the cells. For the construction of 5-$HT_{7(b)}$YFP, a ***Sal*I**-flanked primer with a mutated stop codon was used to generate a ***Ppu*MI/ *Sal*I** fragment covering the C-terminal end of the 5-$HT_{7(b)}$ receptor. After subcloning and sequence verification, this ***Ppu*MI/ *Sal*I** fragment was ligated to a ***Ppu*MI/ *Xba*I** fragment of ph5- $HT_{7(b)}$(De Martelaere *et al.*, 2007) and a ***Sal*I/*Xba*I** fragment of peYFPN1 (Clontech®). The resulting vector was sequence verified in both directions (Andressen et al. 2007, submitted). This plasmid was also kindly provided by Dr. Peter Vanhoenacker, Ghent, Belgium.

3.2 GENERAL EXPERIMENTAL DESIGN IN CONSTRUCTING CHIMERIC RECEPTORS

To construct chimeric receptors of h5-HT_7 and h5-HT_4 several different reactions are made along with simulation from the computer software designed for cloning. The software called SE Central enables the designer to make all kinds of gene alterations on the computer before performing the reactions in the laboratory.

First the mutagenesis is planned on the receptor genes by using the software programme in order to introduce a cutting site for a restriction endonuclease enzyme (***BsiW*I**) in the h5-HT_7 receptor and h5-HT_4 receptor. The enzyme ***BsiW*I** was chosen as the restriction enzyme since this enzyme was one of the two enzymes found which does not cut the receptors within the loops. Then the mutation primers for the respective genes are designed by the same software programme. A Polymerase Chain Reaction is run with the designed mutagenesis primers to clone the receptor genes with mutation. The mutated genes are separated by agarose gel electrophoresis, transformed into bacterial cells to be circularized and cut by restriction digestion using restriction endonuclease enzymes.

The cut DNA fragments are separated by agarose gel electrophoresis and purified by gel extraction. The DNA fragments from both receptors are interchanged and ligated with the opposite parts from both receptor types. The ligated gene products are transformed into TOP10 cells and amplified. The recombinant gene products are then sequenced to verify if the reactions proceeded as expected. The sequencing results reveal the information of the exact chimeric receptor gene sequence. The recombinant gene products are also analyzed qualitatively by restriction digestion and separation on agarose gel.

The restriction cuts were made within the extracellular loops 1 and 2 of the h5-HT_7 and h5-HT_4 receptors. The mutagenesis reaction was carried out by different methods for the two extracellular loops.

3.3 MUTAGENESIS REACTION

The mutagenesis primers were first designed by the software program SE central and the designed primers were supplied by Invitrogen®. The mutagenesis reaction by method I (3.2.2) was performed according to the protocol from Invitrogen® ("Gene Tailor™ Site-Directed Mutagenesis System"). The mutagenesis reaction by method II (3.2.4) was performed according to the protocol for Standard Mutagenesis Strategy.

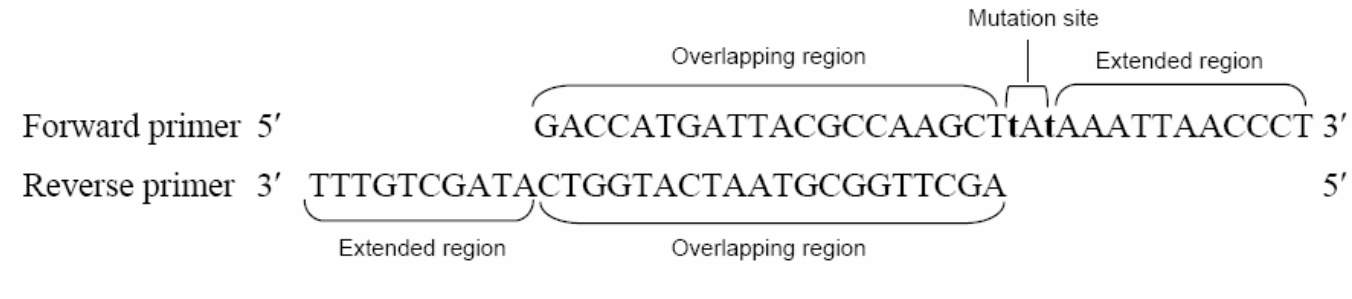
3.3.1 Primer design

Design of good mutagenesis primers is critical in order to get specific and effective amplification of the target gene as well as to avoid amplification of unwanted DNA sequences due to primer dimers or unspecific binding of the primers.

To design the mutagenesis primers for the "Gene Tailor™ Site-Directed Mutagenesis System" (3.2.2) the following specifications by Invitrogen® were followed:

- Both primers (forward and reverse) should be approximately 30 nucleotides in length, not including the mutation site on the mutagenic primer.
- Primers should have an overlapping region at the 5´ends of 15-20 nucleotides, for efficient end-joining of mutagenesis product.
- The mutation site should be located on only one of the primers, downstream from and adjacent to the overlapping region, and can be up to 21 bases (deletions, insertions, and / or any substitutions).
- On the mutagenic primer, there should be at least 10 nucleotides downstream of the mutation site for efficient annealing.

Example of a mutagenesis primer design:



The mutation can be located on either primer.

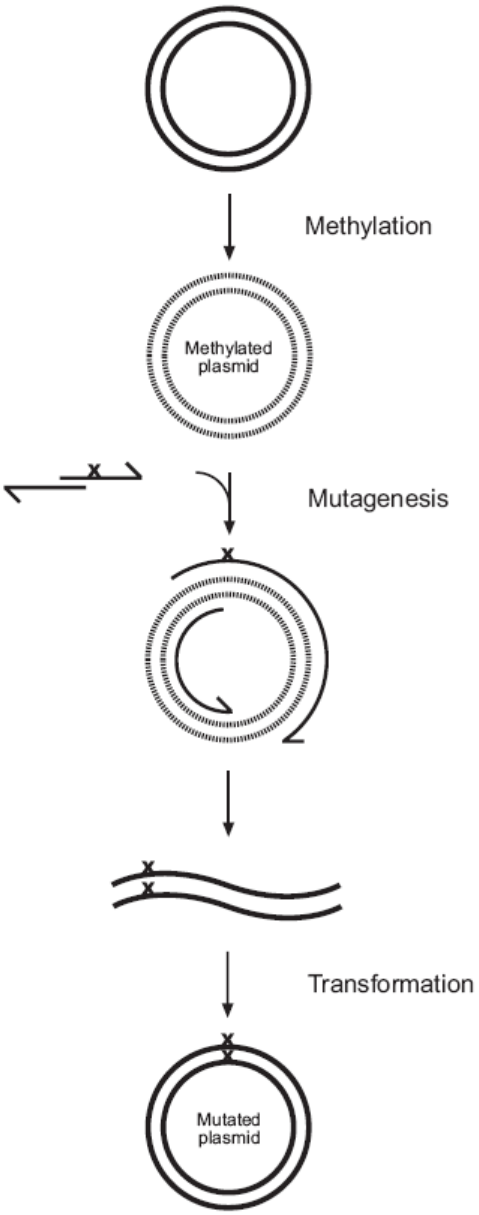
The mutagenesis primers for method II (3.2.4) were designed with mutations located on both primers (forward and reverse).

3.3.2 Method I: Gene Tailor™ Site-Directed Mutagenesis System

The "Gene Tailor™ Site-Directed Mutagenesis System" Invitrogen® is a simple and highly efficient method for standard and high-throughput *in vitro* site-directed mutagenesis. This unique system can generate base substitutions, deletions, or insertions of up to 21 nucleotides in DNA plasmids of up to 8 kb from any source, with no specialized vectors, host strains, or restriction sites required. Only one mutagenic oligonucleotide primer is required to generate a mutation site. The high efficiency (>80%) and simplified protocols of the kit allow for the generation of site-directed mutants from the same or different target genes. The mutagenesis system relies on the inherent properties of the enzymes DNA methylase and McrBC.

In this study the PCR is used to amplify the plasmid in a mutagenesis reaction with two overlapping primers, one of which contains the target mutation. The PCR product obtained is a linear double stranded DNA containing the mutation.

Figure 6 is a workflow diagram showing the different processes involved in this mutagenesis system. In the PCR the mutagenesis primers anneal to the methylated plasmid and amplify the plasmid with a mutation. The product which is a linear double-stranded DNA containing mutation is further transformed into *E. coli* cells to be circularized.



1. Methylate plasmid DNA (isolated from any source) with DNA methylase at 37°C for 1 hour.
2. Amplify the plasmid in a mutagenesis reaction with two overlapping primers, one of which contains the target mutation. The product is linear, double-stranded DNA containing the mutation.
3. Transform the mutagenesis mixture into wild type *E. coli*. The host cell circularizes the linear mutated DNA, and *Mcr*BC endonuclease in the host cell digests the methylated template DNA, leaving only unmethylated, mutated product.

Figure 6. Mutagenesis workflow diagram (Adapted from Invitrogen® "Gene Tailor™ Site-Directed Mutagenesis System" user manual).

Protocol for methylation reaction

Reagent	**Single reaction**
Plasmid DNA	100 ng
Methylation buffer	1.6 µl
10x S-adenosylmethionine(SAM)	1.6 µl
DNA methylase (4U/ µl)	1.0 µl
Sterile, distilled water	to 16 µl

1. Combine reagents and incubate at 37º C for 1 hour.

2. After methylation, proceed to Mutagenesis reaction by PCR.

The protocol of the mutagenesis reaction is integrated in a worksheet as the example below.

Example of a worksheet used for the PCR reaction:

	Stock conc.	Conc. in assay						
PCR Buffer (X)	10	1				Date of experiment	15.2.07	
$MgSO_4$ (mM)	50	1						
dNTP's (mM)	10	0.3	ID					
Polymerase (U/µl)	5	1.5	Polymerase	Plat.Taq High fidelity		Reaction volume (µl)	50	
Primer I (µM)	10	1.5	Primer I	444				
Primer II (µM)	10	1.5	Primer II	445		Template volume (µl)	2	
Primer III (µM)	10	0	Primer III	#				Extra
Primer IV (µM)	10	0	Primer IV	#		Number of samples	3	1

	µl pr. reaction	µl to mix	Added		Cycling parameters				
Nuclease-free H_2O	37.2	148.8	[ ]	94	°C Preheat	120	sec.		
PCR buffer	5	20	[ ]	95	°C Melting	30	sec.		
$MgSO_4$	1	4	[ ]	55	°C Anealing	30	sec.	No. of cycles	25
dNTP's	1.5	6	[ ]	68	°C Amplifying	7.5	min.		
Polymerase	0.3	1.2	[ ]	68	°C Extension	1	min.		
Primer I	1.5	6	[ ]	4	°C Storage	100	hrs.		
Primer II	1.5	6	[ ]						
Primer III	0	0	[ ]						
Primer IV	0	0	[ ]						
Template	2	To sample tubes only							
Total volume	48	192		→		Add	48	µl PCR mix to each sample tube	

Comments:
PCR to mutate $5HT_{7b}$ YFP with primers 444 and 445. Control reaction from Gene Tailor Site-Directed Mutagenesis system kit is carried out simultaneously (control plasmids + control primers). The control primers were declared 100 ng/µl each from the manufacturer. Therefore 1.5 µl of primer mix and 1.5 µl of nuclease-free H_2O were added.

Unmethylated plasmid $5HT_{7b}$ YFP 6 ng/µl, methylated plasmid $5HT_{7b}$ YFP and methylated control plasmid from 17.01.07 were used.

Order of addition:
1:Nuclease-free H_2O 2:dNTP 3:$MgSO_4$ 4:PCR buffer 5:polymerase 6:Primers 7:templat (outside the RNA-lab)

4) 45 µl reaction mix + 1.5 µl ctrl. primer mix + 1.5 µl nuclease-free H_2O + 2 µl control Plasmid (outside the RNA-lab)
5) 48 µl reaction mix with primers 444+445 + 2 µl of methylated $5HT_{7b}$ YFP
6) 48 µl reaction mix with primers 444+445 + 2 µl of unmethylated $5HT_{7b}$ YFP

3.3.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a powerful and sensitive method to amplify a selected DNA sequence *in vitro* by purified, thermostable DNA polymerases. These polymerases synthesize new polynucleotides complementary to an existing DNA or RNA template.

In a mix of the four deoxynucleotides (dATP, dTTP, dCTP and dGTP), primers, correct buffer and salt concentration, a thermostable DNA polymerase can replicate a DNA template in repeated rounds of replication. The polymerase is guided to the sequence to be copied by short synthetically produced oligonucleotide primers that anneal to the DNA at both flanks of the desired DNA sequence. These primers induce replication of each strand of the original DNA.

PCR reaction is a two or three step reaction which is controlled by temperature regulation. First, the reaction mixture is heated to 94ºC. At this temperature the hydrogen bonds that hold together the polynucleotides of the double helix are broken, so the target DNA becomes denatured into single-stranded molecules. In the second step, the temperature is reduced to 50-60ºC which allows the primers to anneal to their corresponding DNA sequence. Finally, the temperature is increased to the optimum of the polymerase, usually between 68-72ºC, where the DNA sequence of interest is replicated in both directions. In a two-step PCR reaction the annealing and extension occur at the same temperature.

The reaction steps are repeated for 30-40 cycles by an automated temperature-cycler. Theoretically, in a 100 % efficient reaction, every cycle doubles the amount of DNA synthesized in the previous cycle, and within a few cycles the predominant product is a single species of DNA fragment whose length corresponds to the distance between the two primers.

Usually, the products are analyzed by agarose gel electrophoresis, which will reveal a single band if the PCR proceeded as expected and has amplified a single segment of the target DNA.

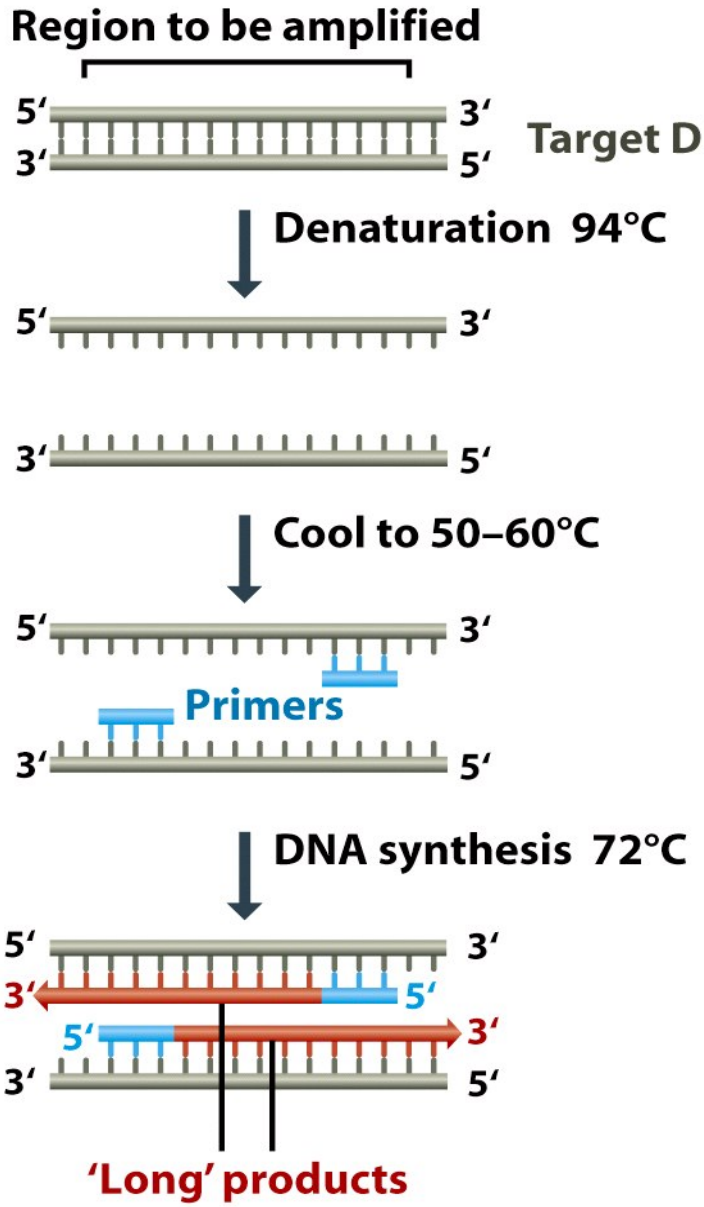


Figure 7. The polymerase chain reaction (Adapted from *Genomes 3*, © Garland Science 2007).

3.3.4 Method II: Standard mutagenesis strategy

For this mutagenesis strategy both the forward and reverse primers were designed with the mutation. The mutagenesis primers were designed for introducing a cutting site for the endonuclease ***Bsi*WI** (…CGTACG…) in extracellular loop 1 in the receptors. The mutagenesis primers were supplied by the same manufacturer (Invitrogen®).

In this method two sets of PCR were run. In the first set of PCR (PCR-I), two separate reactions were carried out for each plasmid. The wild type 5-HT receptor plasmids were used as templates along with one of the mutagenesis primers and a flanking primer in each reaction, comprising two reactions for each plasmid. The PCR products obtained were separated on agarose gel and purified.
In the second PCR (PCR-II), the PCR products from PCR-I serve as templates and the flanking primers from PCR-I were used to initiate the DNA synthesis. The following flanking primers and mutagenesis primers were used to perform the PCR reactions.

h5-HT$_{4(b)}$ receptor:

465: h5HT4b EL1 U 1252C 1255A 1257G
GCC CTT TGG TGC CAT TGA GCT cGT aCg AGA CAT C
135: Flanking primer (reverse)
TCA GTC GGC AGA CCA GC
478: h5HT4b EL1 L 1252C 1255A 1257G
CTC CCC ATA AAT CCA GAT GTC TcG TaC gAG CTC AAT
150: Flanking primer (forward)
TAA TAC GAC TCA CTA TAG GG

h5-HT$_{7(b)}$ receptor:

468: h5HT7b EL1 U 466T 467A 468C
GTC AGC GTC ACC GAC CTC ATC Gta cGC AAG TGG ATC TTT GG
119: Flanking primer (reverse)
GAA CAG AAA GCA GCC ACC
481: h5HT7b EL1 L 466T 467A 468C
TGT CCA AAG ATC CAC TTG CGt acG ATG AGG TC
115: Flanking primer (forward)
CGC GAT GAT GGA CGT TAA CAG C

Protocol:

In a microfuge tube kept on ice mixed the following with one mutagenesis primer and one flanking primer per reaction for each plasmid and run the PCR for all the four reactions.

PCR-I

Ingredients	**µl per reaction**	**Final Conc.**
Nuclease free water	18	
Termopol buffer (10x)	3	1 x
dNTP (10 µM)	3	1 µM
DMSO	2	6.7 %
Vent ® Polymerase 2U/µl	1	0.06 U
Forward Mutagenesis primer (10 µM)	1	0.3 µM
Reverse Flanking primer (10 µM)	1	0.3 µM
Template (Plasmid DNA)	1	
Total reaction mix volume	30	

The four products from PCR – I reaction were separated on an agarose gel and purified. These products served as templates for the PCR-II reaction.

PCR-II

Ingredients	**µl per reaction**	**Final Conc.**
Nuclease free water	23	
Termopol buffer (10x)	5	1 x
dNTP (10 µM)	5	1 µM
DMSO	2	4.0 %
Vent ® Polymerase 2U/µl	1	0.04 U
Forward Flanking primer (10 µM)	2	0.4 µM
Reverse Flanking primer (10 µM)	2	0.4 µM
Template 1 (PCR product I)	5	
Template 2 (PCR product I)	5	
Total reaction mix volume	50	

The following figure (figure 8) summarizes the two sets of PCRs giving the final mutated DNA products. These mutated products are the DNA coding regions for the two receptors 5-

$HT_{4(b)}$ and 5-$HT_{7(b)}$. These mutated DNA products and their respective plasmids are cut by endonucleases and their DNA fragments ligated together.

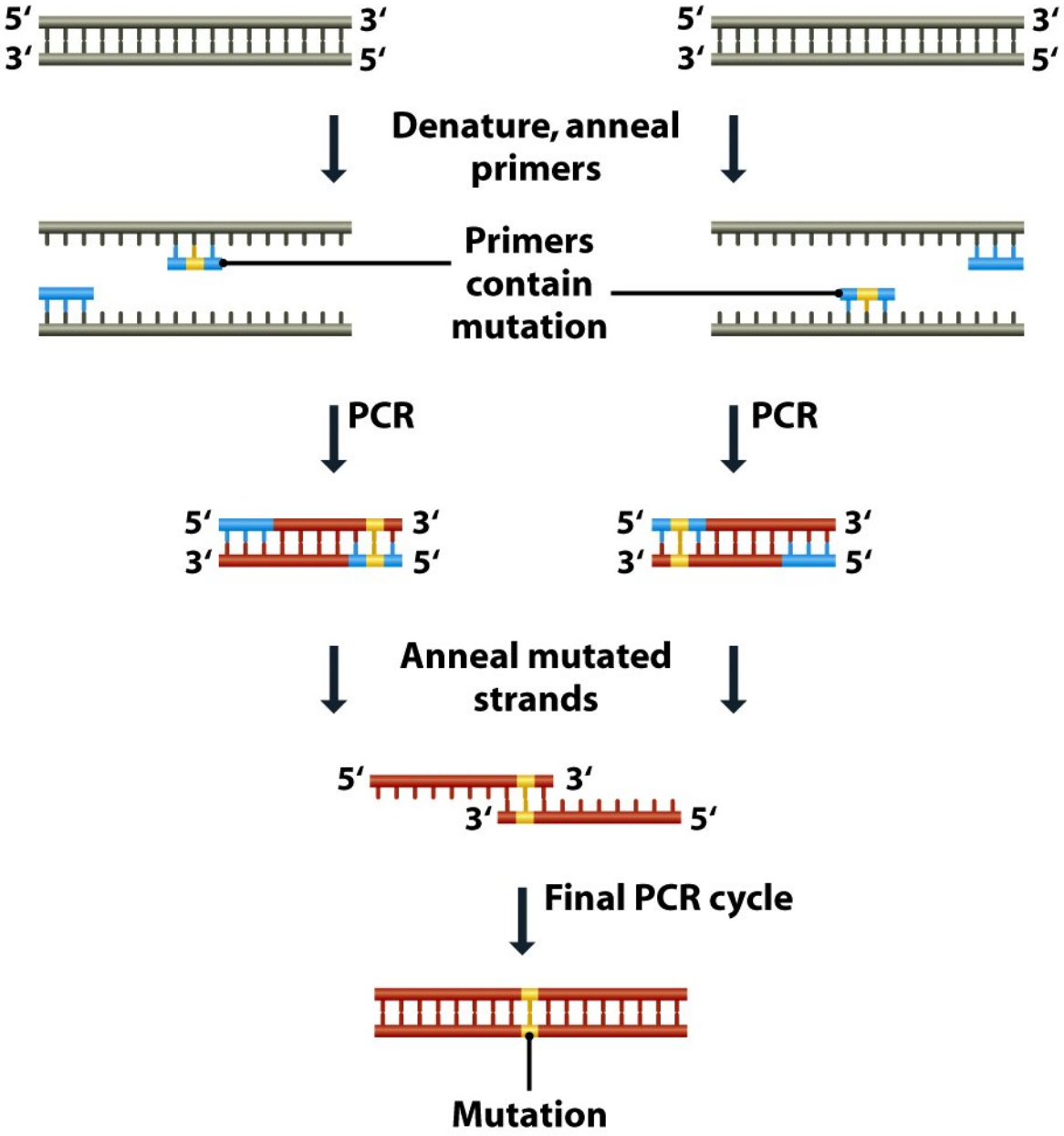


Figure 8. Standard Mutagenesis Strategy (Adapted from Technical Note 5.2, *Genomes 3*, © Garland Science 2007).

The plasmid 3x HA 5-$HT_{4(b)}$ and the mutated DNA product coding the DNA of 5-$HT_{4(b)}$ are cut by endonucleases ***Nhe*I** and ***Cla*I** and their DNA fragments ligated together. The plasmid 5-$HT_{7(b)}$ YFP and the mutated DNA product coding the DNA of 5-$HT_{7(b)}$ are cut by endonucleases ***Blp*I** and ***Xho*I** their DNA fragments ligated together. The ligated products

obtained are the plasmids containing the cutting site for the endonuclease ***Bsi*WI** in the extracellular loop 1.

3.4 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is performed to identify and separate the DNA fragments. Agarose dissolves upon heating to the boiling point and forms a gel when cooled. Agarose gel is a fine network of linear polysaccharide molecules which are suitable for separating DNA fragments from 200 bp to 50 kb.

At neutral pH, DNA is negatively charged and in an electrical field it will migrate towards the positive electrode (cathode). The migration rate of DNA in the gel is dependent on the conformation and size of DNA, agarose concentration, buffer concentration and voltage. To determine the sizes of the separated DNA molecules, the samples are compared to the DNA standard (DNA Ladder) which is run on the same gel. The DNA Ladder consists of DNA fragments of known sizes which serve as good markers in the agarose gel electrophoresis. The gel is preloaded with the UV-fluorophore ethidium bromide (EtBr) which intercalates between the basepairs of the DNA double helix, and allows the DNA fragments to be visualized under the exposure of UV-light.

In this study agarose gel electrophoresis was used for two purposes, to identify and isolate the PCR products for transformation into DH5α^{TM}- T1^{R} and TOP10 *E. coli* cells, and to separate and purify the restriction digestion products for ligation reaction.

Protocol:

1. For a 0.8 % gel, dissolve 0.8 g agarose in 100 ml 1x TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA).
2. Heat the solution in a microwave until the agarose dissolves completely.
3. Let the solution cool to about 50-60 ºC and add 20 µl of 2.5 µg/µl ethidium bromide. Pour the warm solution into a gel mould fitted with the appropriate comb. Remove the air bubbles and allow the gel to set at room temperature.
4. Transfer the gel to an electrophoresis chamber, cover it with 1x TAE buffer and remove the comb.

5. Add 6x Loading buffer to the samples (PCR products) and DNA standards (100 bp Ladder and 1 kb Ladder; Invitrogen®). The volume of loading buffer is adjusted to achieve 1x loading buffer in the final sample.

6. Load the DNA standards and samples into separate gel wells and run the electrophoresis at 100 V (Power Pac 200, Bio-Rad Laboratories Inc.) for 1-2 hours.

7. Visualize the DNA in the gel under UV-light. Excise the agarose gel slice containing the desired DNA fragment and extract the DNA from the gel (3.4).

3.5 EXTRACTION OF DNA FROM AGAROSE GELS

Isolation and purification of DNA fragments from agarose gel was performed using QIAquick Gel Extraction Kit (QIAGEN). The principle of the procedure is to solubilise the agarose gel in the presence of a chaotropic salt ($NaClO_4$) which breaks the hydrogen bonds in the agarose polymer. The solution is then filtered through a column of silica particles which bind DNA at a high salt concentration. Binding of DNA to the silica particles is optimal at pH 7.5. QG buffer which contains a pH indicator indicates optimal pH by turning the solution yellow at this pH range. The DNA is washed with an ethanol containing solution and eluted from the column by adding nuclease-free water or a buffer at low salt concentration.

Protocol:

1. Add 3 volumes of solubilisation buffer QG to 1 volume of excised gel slice. Incubate at 50 ºC for 10 minutes or until the gel slice has completely dissolved. Vortex the tubes every 2-3 minutes during the incubation.

2. Add 1 gel volume of isopropanol to the sample and mix. Addition of isopropanol increases yield of DNA fragments of <500 bp and >4 kb.

3. Place a QIAquick spin column in a provided 2 ml collection tube. Apply the sample to the QIAquick column and centrifuge at 13,000 rpm in a tabletop microcentrifuge for 1 minute. Discard the flow-through and re-insert the column into the collection tube.

4. To wash, add 0.75 ml of Buffer PE to QIAquick column. Let stand for 5 minutes and centrifuge for 1 minute. Discard the flow-through and centrifuge for an additional minute.

5. Place the QIAquick column into a new sterile 1.5 ml microcentrifuge tube. Elute the DNA by adding 50 µl of nuclease-free water onto the centre of the spin column. Centrifuge the column at maximum speed for 1 minute. Store at -20 ºC.

The linear mutated plasmid is now ready to be transformed into the bacterial cells.

3.6 PLASMID PROPAGATION

When a linear plasmid is transformed into a bacterial cell, the host cell circularizes the linear plasmid. The plasmids are also replicated independently of chromosomal DNA and thus transformation can also be used to produce high numbers of copies of plasmid constructs. The bacterial cells used for transformation are strains of *Escherichia coli* which are pre-treated with various chemicals to become competent for DNA transformation. In this study the linear mutagenesis plasmids were transformed into both DH5α^{TM}-T1^{R} *E. coli* and TOP10 Chemically Competent *E. coli* cells, both from Invitrogen®.

The transformed bacteria were grown on selective medium (LB medium with agar containing antibiotic ampicillin 100µg/ml). The pcDNA 3.1 vector contains a gene coding for ampicillin resistance, therefore only the bacteria that are transformed with this vector will survive in this medium. The plasmid DNA is isolated using either small- or large-scale plasmid preparations. In this study Wizard® Plus SV Miniprep Kit (Promega) was used for small-scale and the HiSpeed Plasmid Maxi Kit (Qiagen) was used for large-scale plasmid preparations.

3.6.1 Transformation

Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Transformation should be started immediately after thawing the cells on ice. Mixing should be done by swirling or tapping the tube gently, not by pipetting. Heat shock treatment of the cells was used to increase DNA uptake.

Protocol:

1. Pipet 5 µl plasmid DNA from each mutagenesis reaction mixture directly into each vial (50 µl) of cells and mix gently.

2. Incubate on ice for 15 minutes.

3. Heat shock the cells at 42 ºC for 30 seconds without shaking.

4. Incubate on ice for 1 minute.

5. Add 250 µl of pre-warmed SOC medium to each vial and mix gently.

6. Incubate the cells in a horizontal shaker at 37 ºC, 200 rpm for 30 minutes.

7. Label and warm the LB agar plates containing 100 µg/ml ampicillin for about 30 minutes in a 37 ºC incubator.

8. Aliquot 50, 100 and 150 µl from each transformation vial onto the labelled LB agar plates and spread it on the plate.

9. Invert the plates and incubate overnight at 37 ºC.

10. Pick 9 isolated colonies and inoculate each colony in 10 ml LB medium containing 100 µl/ml ampicillin.

11. Grow overnight at 37 ºC in a shaker incubator for small-scale plasmid preparation (3.5.2)

3.6.2 Small-scale plasmid preparation

For small-scale isolation and purification of plasmids from transfected *E. coli* cells, the Wizard® Plus SV Miniprep kit from Promega® was used. This system provides a simple and rapid isolation of plasmid DNA.

The bacterial cells are lysed with a solution containing NaOH and SDS. By neutralising and increasing the salt concentration, the chromosomal DNA, cell membrane components and denatured proteins will precipitate, while plasmid DNA remains intact in the solution.

An alkaline protease solution is added to inactivate endonucleases and to degrade proteins non-specifically. These steps prevent contamination of chromosomal DNA breakdown products or plasmid digestion products by endonucleases, and it also decreases the overall contamination of proteins in the final product. The plasmid-containing lysate is run through a silica-based membrane which binds the plasmid DNA. To elute the contaminants, the membrane is washed with ethanol containing high salt concentration. Finally, the plasmid DNA is eluted in Nuclease-free Water.

Protocol:

1. Harvest 10 ml of overnight bacterial culture by centrifugation for 5 minutes at 10,000 x *g* in a tabletop centrifuge. Discard the supernatant.

2. Add 250 µl of Cell Resuspension Solution and completely resuspend the cell pellet by vortexing.

3. Add 250 µl of Cell Lysis Solution and mix by inverting the tube 4 times.

4. Add 10 µl of Alkaline Protease solution and mix as above. Incubate for 5 minutes at room temperature.

5. Add 350 µl of Neutralisation Solution and mix as above.

6. Centrifuge the bacterial lysate at 14,000 x *g* in a microcentrifuge for 10 minutes at room temperature.

7. Decant the cleared lysate into Miniprep Spin Column inserted into a 2 ml collection tube. Centrifuge at 14,000 x *g* for 1 minute at room temperature. Discard the flow-through.

8. Add 750 µl Column Wash Solution, previously diluted with 95 % ethanol, to the spin column. Centrifuge as above and discard the flow-through.

9. Repeat the wash procedure using 250 µl of Column Wash Solution. Centrifuge at 14,000 x *g* for 2 minutes and discard the flow-through.

10. Transfer the column to a sterile 1.5 ml microcentrifuge tube and elute the plasmid DNA by adding 100 µl nuclease-free water to the spin column. Centrifuge at 14,000 x *g* for 1 minute at room temperature. Store at -20 ºC.

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and qualitative analysis on an agarose gel.

3.7 QUANTIFICATION OF DNA

Quantification of DNA was performed by UV spectrophotometric analysis. Nucleotide spectra are complicated to analyse quantitatively because they have many non-bonded electrons with indistinct transitions giving a multitude of absorptions between 200 nm and 300 nm. All nucleotides do however have a λ_{max} near 260 nm which is fairly specific for the purine and pyrimidine bases. This wavelength can thus be used to estimate the nucleic acid concentration in a sample. DNA also absorbs light at 230 nm and 280 nm, but to a lesser extent. Pure DNA samples should have the following absorption ratios:

OD_{260} / OD_{280} ratio ~ 1.8-1.9

OD_{260} / OD_{230} ratio ~ 1.8-2.2

The amount of DNA was calculated by using the following formula:

DNA (µg/µl) = OD_{260} x dilution/20

UV spectrophotometer: Ultrospec 2100 pro UV/Visible spectrophotometer, GE Healthcare.

3.8 SUB-CLONING

In this context, sub-cloning means moving an insert from one vector to another. The method involves preparation of inserts and vectors by digesting with restriction enzymes, separation and isolation of the DNA fragments by agarose gel electrophoresis and subsequent ligation.
The method was used to move the desired coding region of h5-HT_7 receptor into the specific coding region of h5-HT_4 receptor and vice versa.

3.8.1 Restriction digestion

Sequence specific DNA endonucleases (restriction enzymes) were used to prepare vectors and inserts for subcloning. These enzymes degrade DNA molecules by breaking the phosphodiester bonds that link one nucleotide to the next. The enzymes used were all type II restriction endonucleases. These enzymes recognise specific sequences of four to eight nucleotides and cut double stranded DNA with either "sticky" (with 5' or 3' overhang) or "blunt" ends (no overhang). The choice of buffer is crucial for cutting the DNA with specificity and accuracy. The optimal temperature is usually 37 ºC. One unit (1 U) of a restriction enzyme is defined as the amount of enzyme that cuts 1 µg of λ-phage DNA in one hour. For digestion of circular DNA, 4-5 U per µg of DNA is required.

Protocol:

1. Mix the following solution in a microcentrifuge tube kept on ice:
~ 1 µg/µl plasmid DNA, adjust volume to contain totally 5 µg plasmid DNA
5 µl 10x assay buffer
2 µl of each restriction digestion enzyme (10 U/ µl)
Nuclease-free H_2O ad 50 µl
2. Incubate at 37 ºC for 1 hour.
3. Perform agarose gel electrophoresis to separate digestion products.

The endonuclease enzyme ***Bsi*WI** (New England Biolabs®) cuts the gene coding the serotonin receptors 3xHA-5HT_4 and 5HT_{7b} YFP in the extracellular loop 1 or 2 according to where the cutting site is introduced by mutation. Endonuclease enzyme ***Xba*I** (New England Biolabs®) cuts the plasmid after the coding region of the serotonin receptor genes.

Agarose gel electrophoresis was used to identify and separate the restriction cut DNA fragments of interest. If the two bands of the expected size (the size of the insert and cut vector) were visible upon UV-irradiation of the gel, they were excised and purified by gel extraction. The DNA fragments of the two receptors were interchanged and ligated to give a recombinant DNA which codes for the chimeric receptors of interest.

3.8.2 Ligation of the restriction digestion products

The vectors and inserts that were made under restriction digestion reaction and separated by agarose gel electrophoresis were ligated by T4 DNA ligase. T4 DNA ligase catalyzes the formation of a phosphodiester bond between the 3´-hydroxyl and the 5´-phosphate groups in nicked DNA. This reaction requires presence of NAD^+ and special buffer conditions. Stochiometrical parameters may also affect ligation specificity and efficacy.
T4 DNA ligase has optimal activity at 37 ºC, but at this temperature the hydrogen bonded joint between the sticky ends is unstable. A compromise between rate of enzyme action and association of the sticky ends has to be made, and has been established to be in the range of 4-16 ºC.

Protocol:

1. Mix 60 fmol insert, 20 fmol vector (3:1 molar ratio) and nuclease-free H_2O to a total volume of 8.5 µl in a microcentrifuge tube kept on ice.
2. Add 1 µl of 10x ligase buffer (Fermentas Inc.)
3. Add 0.5 µl of T4 DNA ligase (1U/µl). Incubate at 16 ºC for 12 hours.

The ligated products can be used immediately for transformation to produce small- or large-scale plasmid preparations, or stored at -20 ºC until use.

In this study the ligated products were transformed to produce a high copy number of plasmids, isolated by large-scale plasmid preparation (3.8).

3.9 LARGE-SCALE PLASMID PREPARATION

To produce larger amounts of purified plasmid DNA, large-scale plasmid preparation was set up using HiSpeed Plasmid Maxi kit (QIAGEN®). The lysis principle is the same as for small-

scale plasmid preparations. RNase A is added at the beginning of the procedure to digest the liberated RNA as to minimise RNA contamination. The lysate is run through an anion-exchange column consisting of silica beads with high density of diethylaminoethyl (DEAE) groups. In acidic environment the DEAE groups become positively charged and interact with negatively charged molecules, such as phosphate groups of the RNA and DNA backbone. Impurities such as RNA, protein, carbohydrates and other small molecules are washed off with medium-salt buffer, while plasmid DNA is eluted with salt buffer. The plasmid eluate is concentrated and desalted by isopropanol precipitation.

Protocol:

1. Prepare a starter culture of 2-5 ml LB medium containing 100 µg/ml ampicillin and incubate overnight at 37 ºC in a shaker incubator.

2. Dilute the starter culture 1/500 in LB medium by adding 1 ml of the starter culture in 500 ml LB medium containing 100 µg/ml ampicillin. Incubate overnight (12-16 hours) at 37 ºC in a shaker incubator.

3. Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 minutes at 4 ºC. Decant the supernatant.

4. Resuspend the bacterial pellet in 10 ml buffer P1.

5. Add 10 ml of buffer P2 and mix thoroughly by inverting the tube 4-6 times. Incubate at room temperature for 5 minutes.

6. Add 10 ml of chilled P3 buffer and mix immediately by inverting the tube 4-6 times. Pour the lysate into the capped barrel of a QIAfilter Cartridge and incubate for 10 minutes at room temperature.

7. Equilibrate a HiSpeed Maxi Tip by applying 10 ml buffer QBT.

8. Remove the cap from the QIAfilter outlet nozzle, gently insert the plunger and filter the cell lysate into the HiSpeed Maxi Tip.

9. Wash the HiSpeed Maxi Tip with 60 ml buffer QC.

10. Elute DNA with 15 ml buffer QF.

11. Precipitate DNA by adding 10.5 ml isopropanol. Mix and incubate at room temperature for 5 minutes.

12. Filter the eluate / isopropanol mixture through a QIAprecipitator Maxi Module (membrane filter) attached to a 30 ml syringe. Discard the flow-through.

13. Wash the DNA with 2 ml 70 % ethanol. Dry the membrane by pressing air through the QIAprecipitator quickly and forcefully. Repeat this step.
14. Attach the QIAprecipitator to a 5 ml syringe and elute DNA into a 1.5 ml sterile microcentrifuge tube by adding 700 µl buffer TE. Transfer the eluate back to the syringe and elute for a second time into the same 1.5 ml tube to ensure that maximum amount of DNA in the QIAprecipitator is solubilised and recovered. Store at -20 ºC.

3.10 DNA SEQUENCING

DNA of the recombinant plasmids were sequenced to verify whether the inserts and vectors were correctly ligated and whether the sequence was intact. The sequencing was performed by the staff at the molecular microbiology laboratory of Institute of Microbiology. The instrument used for DNA sequencing is ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Four different nucleotides with their respective fluorescent probe are used. Each probe is a dideoxynucleotide (ddNTP) and a linear amplification reaction will incorporate the probes and unmarked deoxynucleotides (dNTP) to the growing sequence of DNA polymerisation. Since ddNTP lack a free 3'-hydroxyl group, the synthesis is terminated when ddNTP is incorporated instead of dNTP. The sample is separated by capillary electrophoresis (CE) and driven through a capillary filled with a polymer (POP6) by high voltage. The polymer will slow migration of molecules according to increasing length so that small molecules will be least retarded and elute first. The fluorescence is recorded by a laser beam at the end of the capillary. Each probe has its own colour coding, so that the sequence can be read manually on the output of the ABI prism program (Applied Biosystems).

The following primers were used for the sequencing reaction of the recombinant DNA of the two chimeric receptors.

Chimeric receptor: 5-HT_7 up to EL1, 5-$HT_{(4b)}$ from EL1
Forward primers:
115: **5' CGC GAT GAT GGA CGT TAA CAG C 3'**
150 (T7 primer): **5'TAA TAC GAC TCA CTA TAG GG 3'**
Reverse primers

128: **5' TTA CGC CAT CTG CTG CCA 3'**

135: **5'TCA GTC GGC AGA CCA GC 3'**

204 (pcDNA3.1 RSP*): **5' TAG AAG GCA CAG TCG AGG 3'**

Chimeric receptor: 3xHA-5-HT_4 up to EL1, 5-$HT_{7(b)}$ YFP from EL1

Forward primers:

150 (T7 primer): **5'TAA TAC GAC TCA CTA TAG GG 3'**

155: **5' GAT GGT GGC TGT GTG CTG 3'**

Reverse primers:

119: **5' GAA CAG AAA GCA GCC ACC 3'**

120: **5' TAT GCC TTC TTC AAC CGG 3'**

204 (pcDNA3.1 RSP*): **5' TAG AAG GCA CAG TCG AGG 3'**

The recombinant plasmids were also analyzed qualitatively by restriction digestion and separation on agarose gel. The expected fragments from restriction digestion will give an indication of the construction of the plasmid.

Both DNA sequencing and agarose gel analysis confirms the plasmid construction.

4. RESULTS

This thesis is based on previous studies and findings of cellular mechanisms of the different receptors co-expressed in HEK293 cells (in the Department of Pharmacology and Center for Heart Failure Research, University of Oslo, Norway), which revealed that the serotonin receptor 5-HT_7, when expressed in HEK293 cells, attenuated the signalling through endogenous G_s-coupled receptors such as β-AR and EPR (Andressen *et al.*, 2006). The ultimate aim of this line of research is to identify the molecular determinants for this property of the 5-HT_7 receptor by molecular biology techniques. As an initial guide to identify the key amino acids of the 5-HT_7 receptor responsible for the attenuation of signalling through other G_s-coupled receptors, chimeric receptors were constructed using plasmids encoding the 5-$HT_{7(b)}$ receptor and 5-$HT_{4(b)}$ receptor. By comparison of the 5-$HT_{4(b)}$ and 5-$HT_{7(b)}$ receptor sequences, DNA sequences were identified corresponding to the first, second and third extracellular loops of the receptors, where a cutting site for the restriction endonuclease enzyme ***Bsi*WI** could be introduced at corresponding sites in the two receptors. Different mutagenesis strategies were then employed to introduce the cutting sites for the endonuclease enzyme ***Bsi*WI** on the plasmids encoding the 5-$HT_{4(b)}$ and 5-$HT_{7(b)}$ receptors. First, mutagenesis of extracellular loop 2 was carried out by mutagenesis method I ("Gene Tailor™ Site- Directed Mutagenesis System", Invitrogen®). Second, mutagenesis of extracellular loop 1 was carried out by mutagenesis method II ("standard method"). Due to time constraints, mutagenesis of extracellular loop 3 was not carried out. The mutated 5-HT receptor plasmids were cut in the cutting site introduced in the extracellular loop by ***Bsi*WI** and after the coding regions of the receptors by ***Xba*I**. The cut fragments were interchanged and ligated to yield plasmids encoding the chimeric receptors.

4.1 DESIGNED MUTAGENESIS PRIMERS

Mutagenesis primers were designed for all the three extracellular loops of both the 5-$HT_{4(b)}$ and 5-$HT_{7(b)}$ receptor plasmids. The following primers were designed for the mutagenesis reaction to introduce a cutting site for the restriction endonuclease enzyme ***Bsi*WI** (…CGTACG…) in the extracellular loops 1, 2 and 3 of the plasmids encoding each 5-HT receptor.

The mutagenesis primers of h5-$HT_{4(b)}$ receptor :

465: h5-$HT_{4(b)}$ EL1 U 1252C 1255A 1257G

5' GCC CTT TGG TGC CAT TGA GCT cGT aCg AGA CAT C 3'

478: h5-$HT_{4(b)}$ EL1 L 1252C 1255A 1257G

5' CTC CCC ATA AAT CCA GAT GTC TcG TaC gAG CTC AAT 3'

450: h5-$HT_{4(b)}$ EL2 – mut- L45

5' CGT AGA GTT AGA GTT CTG GTc GtA CgT CCT CTT TTC TAT CAA ATC 3'

451: h5-$HT_{4(b)}$ EL2 – nomut-U41

5' ACC AGA ACT CTA ACT CTA CGT ACT GTG TCT TCA TGG TCA AC 3'

479: h5-$HT_{4(b)}$ EL3 U 1862G 1865C 1866G

5' CAC CAA TAT TGT GGA TCC TTT CgT Acg CTA CAC TG 3'

480: h5-$HT_{4(b)}$EL3 L 1862G 1865C 1866G

5' GGG ACA GTG TAG CgT Acg AAA GGA TCC ACA ATA 3'

The mutagenesis primers of h5-$HT_{7(b)}$ receptor :

468: h5-$HT_{7(b)}$EL1 U 466T 467A 468C

5' GTC AGC GTC ACC GAC CTC ATC Gta cGC AAG TGG ATC TTT GG 3'

481: h5-$HT_{7(b)}$EL1 L 466T 467A 468C

5' TGT CCA AAG ATC CAC TTG CGt acG ATG AGG TC 3'

448: h5-$HT_{7(b)}$ EL2 – nomut- L33

5' GCC CAT CCA AAG AGT GGA GGT AAG GTG ATG GAG 3'

449: h5-$HT_{7(b)}$ EL2 –mut-U34

5' CCT CCA CTC TTT GGA TGG GCT CcG tAc GTA AAT G 3'

482: h5-$HT_{7(b)}$ EL3 U 1095C 1097T 1100G

5' CAG ACC CTT CAT CTG TcG tAC gTC CTG CAG CTG CAT C 3'

483: h5-$HT_{7(b)}$ EL3 L 1095C 1097T 1100G

5' GCT GCA GGA cGt ACg ACA GAT GAA GGG TCT GG 3'

484: h5-$HT_{7(b)}$YFP 1516 L25

5' AAC TTC AGG GTC AGC TTG CCG TAG G 3'

4.2 MUTAGENESIS IN EXTRACELLULAR LOOP 2 (MUTAGENESIS METHOD I)

The mutation in the plasmid encoding the extracellular loop 2 of the 5-$HT_{4(b)}$ and 5-$HT_{7(b)}$ receptors was made by the mutagenesis primers in a polymerase chain reaction using method I ("Gene Tailor™ Site- Directed Mutagenesis System", Invitrogen®). According to the manufacturer's primer design specifications only one of the primer pair contains the mutation. The plasmids which were used for the mutagenesis reaction are 5-$HT_{7(b)}$ YFP and 3xHA-5-$HT_{4(b)}$.

Figures 9 and 10 show the mutations made in the plasmids to introduce a cutting site for the endonuclease ***Bsi*WI**. The mutations led to changes in the amino acid sequences in the extracellular loop 2 of the 5-HT receptors.

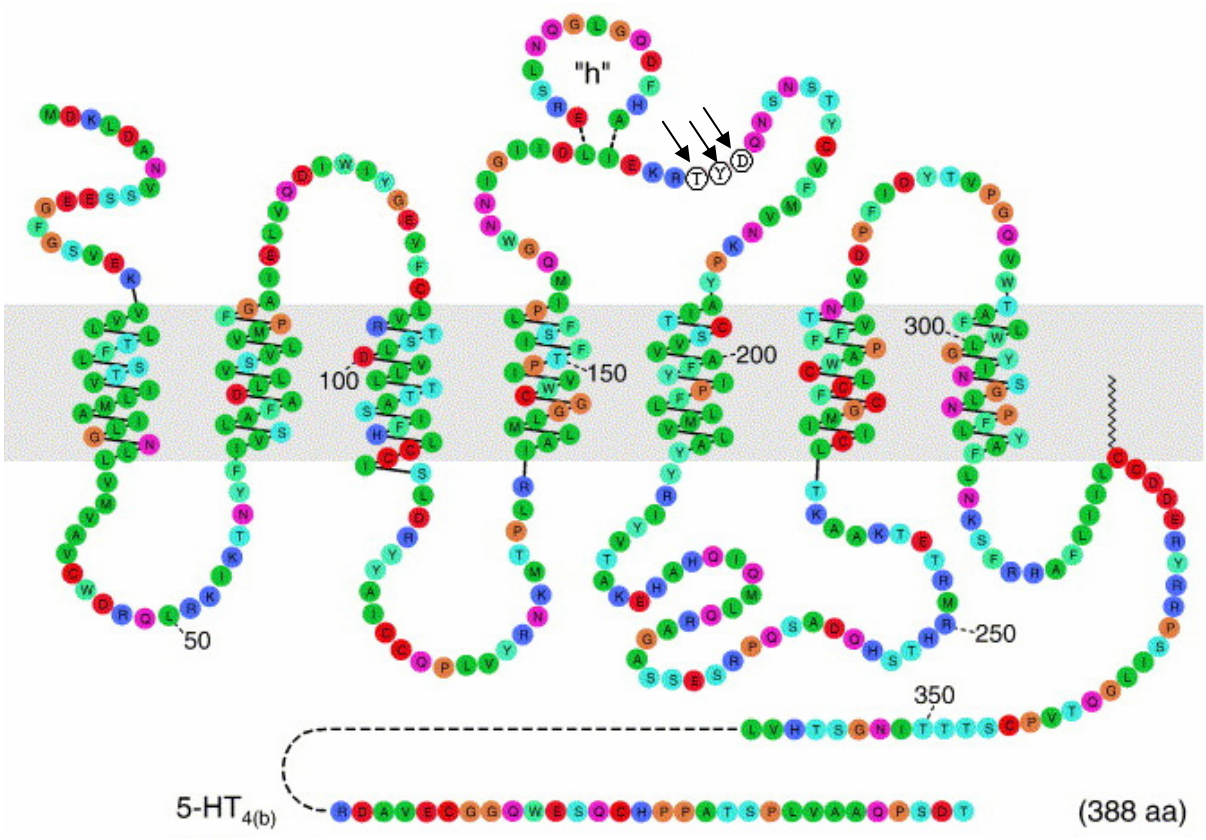


Figure 9. The figure shows the exact location in the 5-$HT_{4(b)}$ receptor where a restriction site for the endonuclease enzyme ***Bsi*WI** was introduced and later used for cutting and religating the 5-$HT_{7(b)}$ receptor. The arrows in the figure show the amino acids K(Lysine), F(Phenylalanine) and N(Asparagine) replaced by amino acids T(Threonine),Y(Tyrosine) and D(Aspartate) due to mutations. Adapted from (Kaumann and Levy, 2006a) with modification.

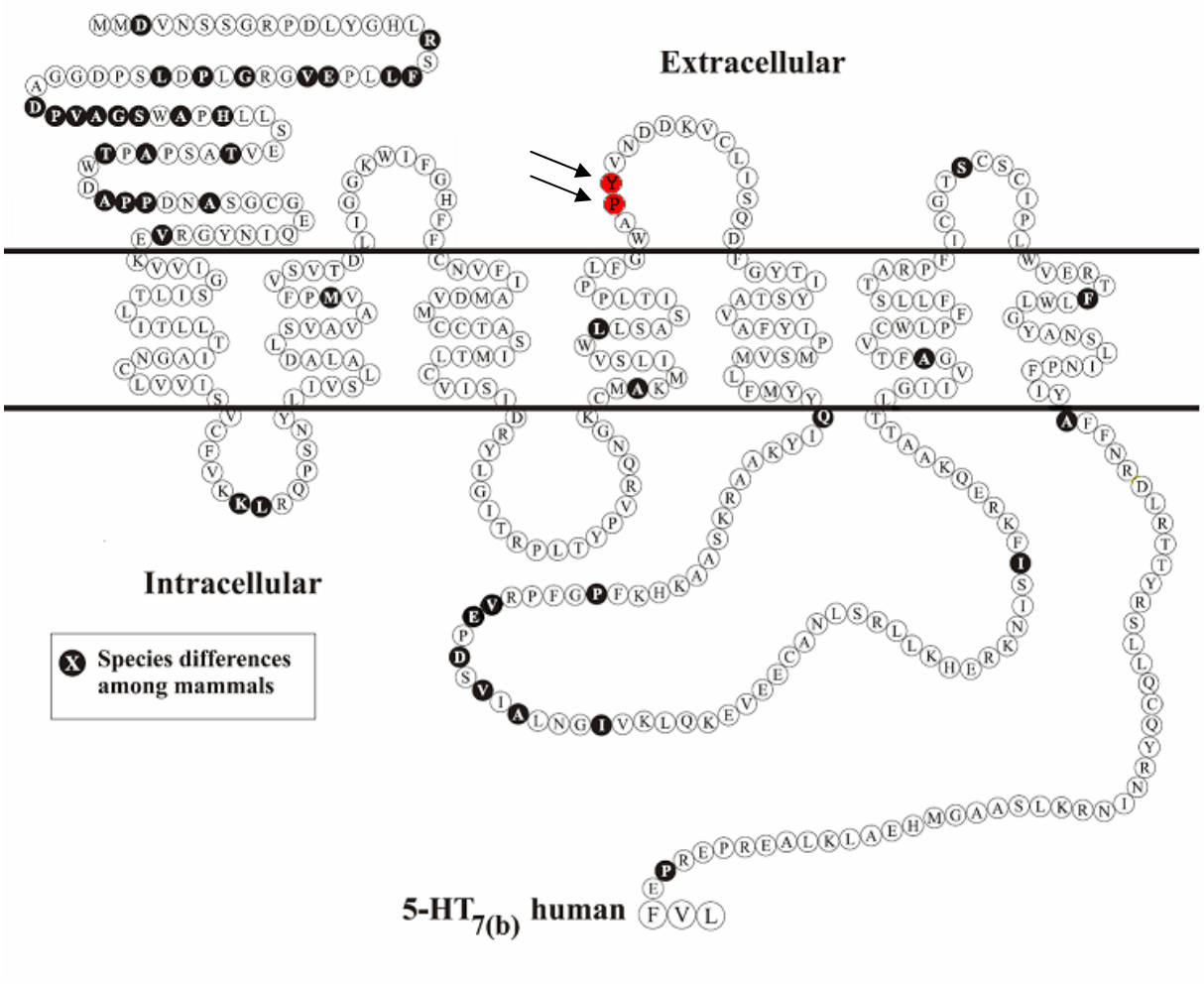


Figure 10. The figure shows the exact location in the 5-$HT_{7(b)}$ receptor where a restriction site for the endonuclease enzyme ***Bsi*WI** was introduced and later used for cutting and religating the 5-$HT_{4(b)}$ receptor. The arrows in the figure show the amino acids Q(Glutamine) and N(Asparagine) replaced by amino acids P(Proline) and Y(Tyrosine) due to mutations. Adapted from (Heidmann *et al.*, 1997) with modification.

The polymerase chain reaction gave linear double-stranded DNA containing mutations. The linear double-stranded DNA was transformed into "TOP10" *E.coli* bacteria where it was circularized. Nine colonies of each plasmid were grown in culture tubes for small scale plasmid preparation. Only seven of the culture tubes containing 5-$HT_{7(b)}$YFP and five culture tubes containing 3xHA-5-$HT_{4(b)}$ had dense growth and were chosen for the small-scale isolation and purification.

4.2.1 Restriction digestion in extracellular loop 2

The purified mutated plasmids were cut in the DNA encoding the extracellular loop 2 by the endonuclease enzyme ***Bsi*WI** and after the coding region of the 5-HT receptor coding sequences by ***Xba*I**. The sizes of the DNA fragments were 653 bp (insert) and 5967 bp (cut vector) for the 3xHA-5-$HT_{4(b)}$ and 1409 bp (insert) and 6084 bp (cut vector) for the 5-$HT_{7(b)}$YFP (Fig.11).

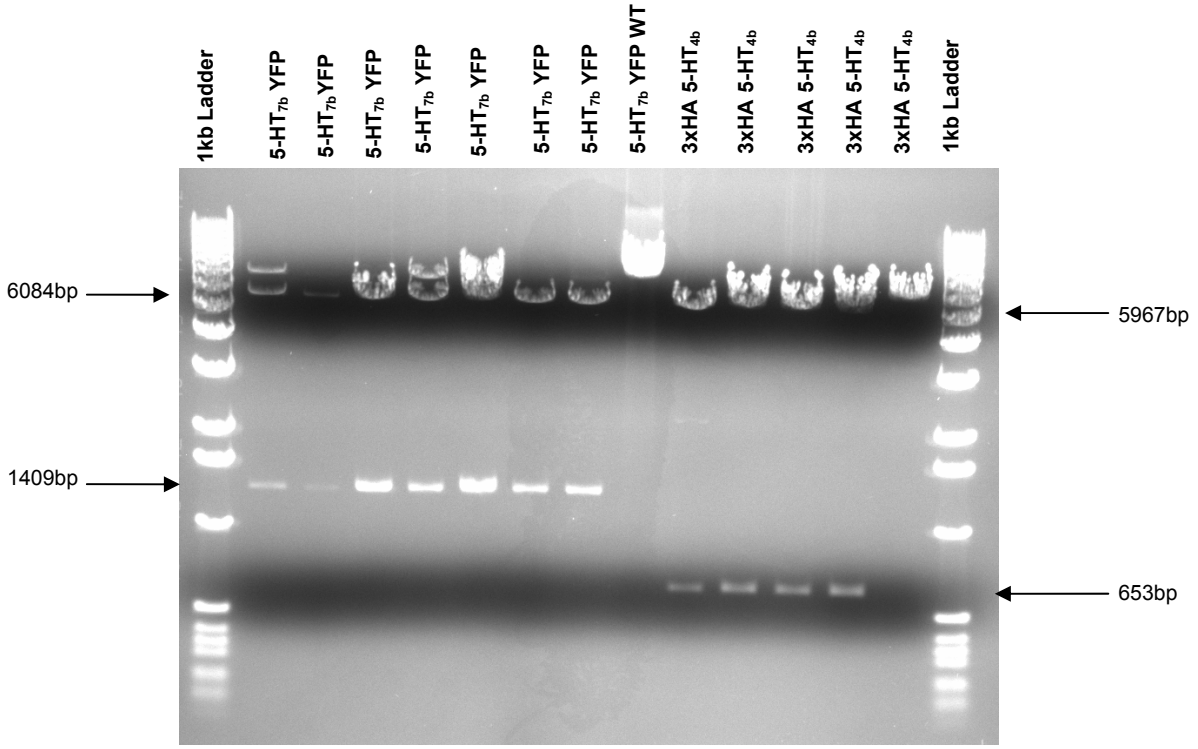


Figure 11. The restriction digestion products of 3xHA-5-$HT_{4(b)}$ and 5-$HT_{7(b)}$YFP cut with BsiWI /XbaI were separated on agarose gel (0.8 %). The samples in lanes 2-8 are restriction digestion products of 5-$HT_{7(b)}$YFP and in lanes 10-14 are the restriction digestion products of 3xHA-5-$HT_{4(b)}$. Lane 9 is an uncut 5-$HT_{7(b)}$YFP. 1 kb DNA Ladder as markers are run in lane 1 and 15.

The samples which gave the best separation on the gel of the DNA fragments from each plasmid were chosen for purification and ligation. The insert (653 bp) from 3xHA-5-$HT_{4(b)}$ was ligated into the cut vector (6084 bp) of 5-$HT_{7(b)}$YFP and the insert (1409 bp) from 5-$HT_{7(b)}$YFP was ligated into the cut vector (5967 bp) of 3xHA-5-$HT_{4(b)}$. The ligated products were transformed into "TOP10" *E.coli* bacteria and the recombinant DNA was amplified.

The constructed chimeric receptor DNA was sequenced to verify if the DNA fragments were correctly inserted. The sequencing result revealed a deletion of ~10bp in the beginning of the 5-$HT_{7(b)}$YFP coding region in both the mutated 5-$HT_{7(b)}$YFP plasmid and in the derived chimeric construct. For unknown reasons, the opposite chimeric construct did not reveal any interpretable sequence and neither of the chimers had the correct, intact sequences either.

Since this mutagenesis method did not give a satisfactory result we decided to use the standard mutagenesis strategy to make mutations in extracellular loop 1.

4.3 MUTAGENESIS IN EXTRACELLULAR LOOP 1 (MUTAGENESIS METHOD II)

The mutation in the extracellular loop 1 by method II (standard mutagenesis strategy) was made by the mutagenesis primers and flanking primers in two sets of polymerase chain reaction.According to standard mutagenesis strategy the mutation was made in both the forward and reverse primers. The plasmids used were 5-$HT_{7(b)}$YFP and 3xHA-5-$HT_{4(b)}$.

Figures 12 and 13 show the mutations made in the plasmid DNA to introduce a cutting site for the endonuclease ***Bsi*WI**. The mutation led to changes in the amino acid sequences in the extracellular loop 1 of the 5-HT receptors.

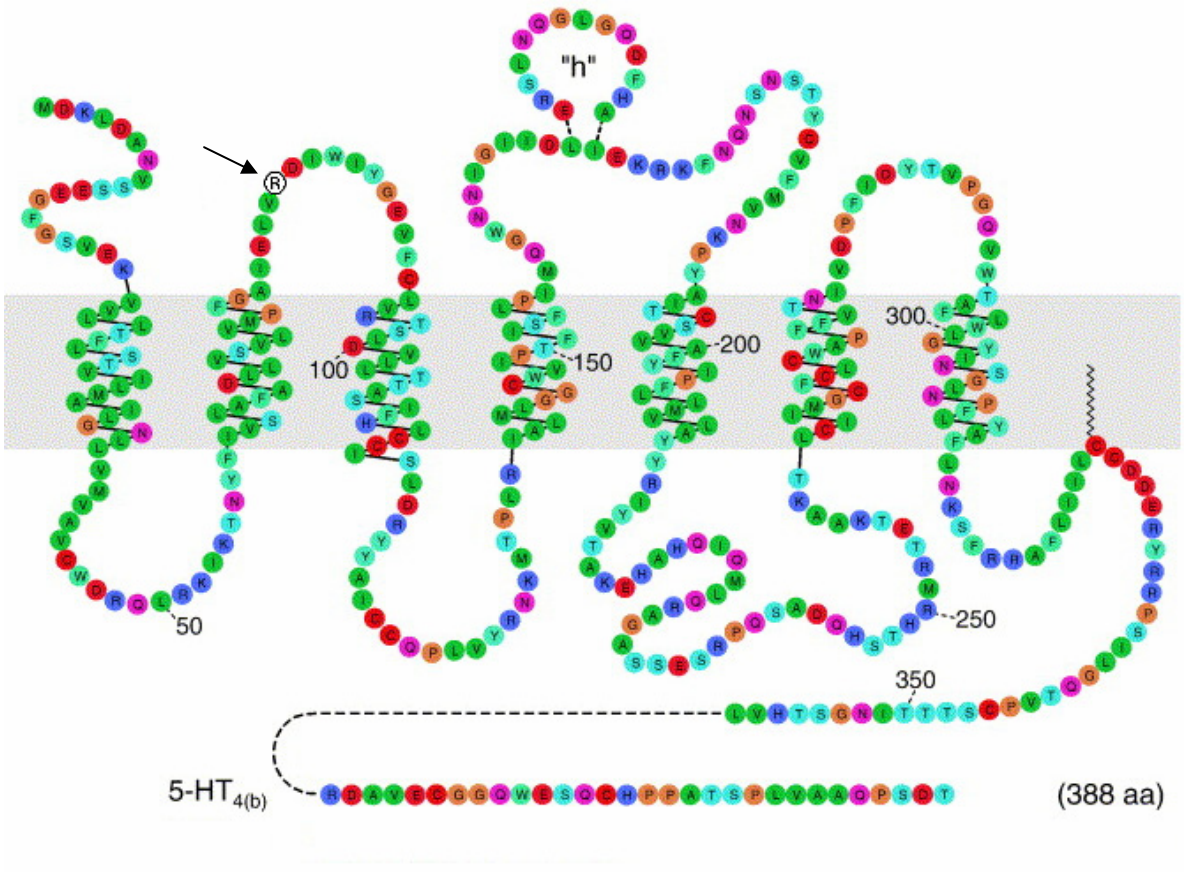


Figure 12. The figure shows the exact location in the 5-$HT_{4(b)}$ receptor where a restriction site for the endonuclease enzyme ***Bsi*WI** was introduced and later used for cutting and religating the 5-$HT_{7(b)}$ receptor. The arrow in the figure shows the amino acid Q (Glutamine) replaced by amino acid R (Arginine) due to mutations.

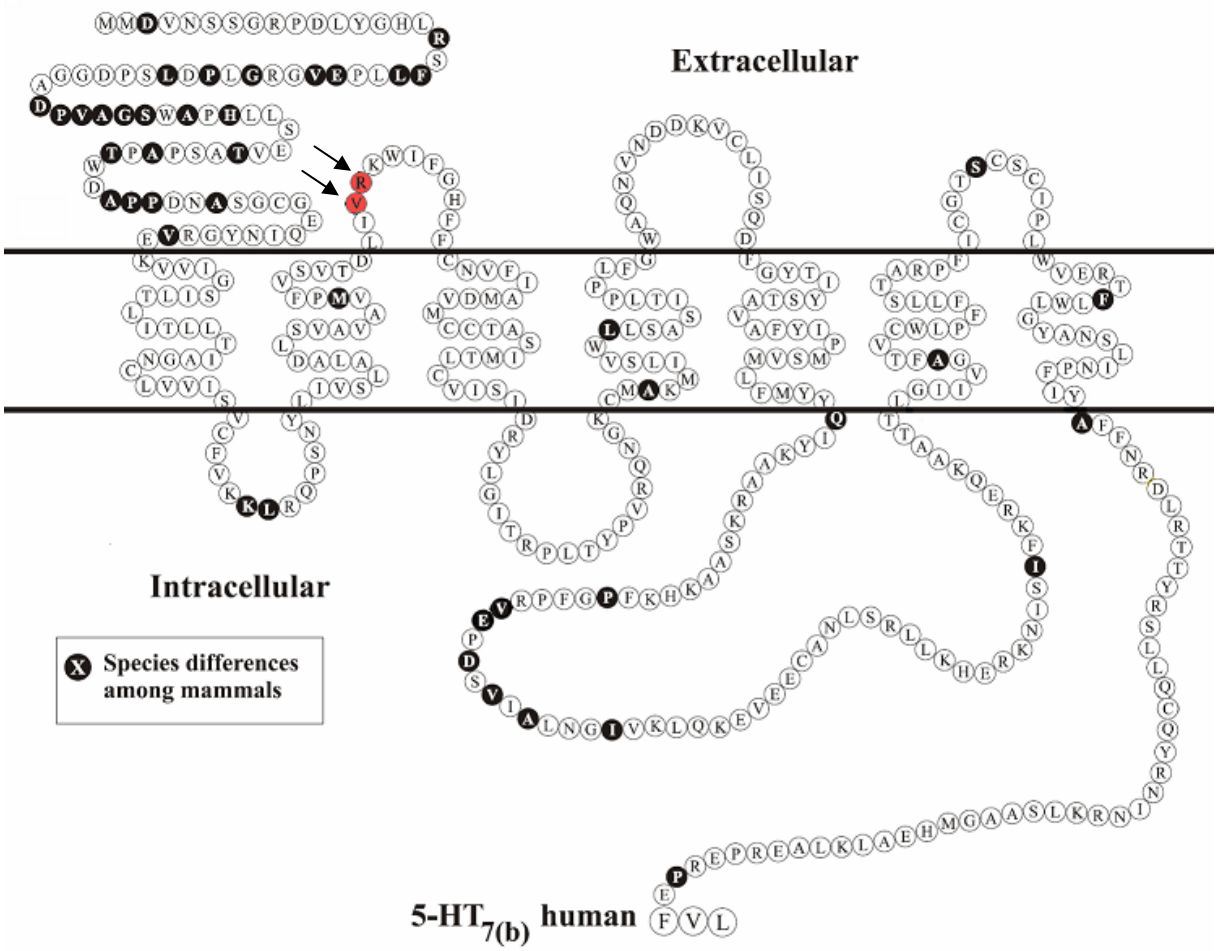


Figure 13. . The figure shows the exact location in the 5-$HT_{7(b)}$ receptor where a restriction site for the endonuclease enzyme ***Bsi*WI** was introduced and later used for cutting and religating the 5-$HT_{4(b)}$ receptor. The arrows show the amino acids G (Glycine), G (Glycine) are replaced by V (Valine) and R (Arginine) due to mutations. Adapted from (Heidmann *et al.*, 1997) with modification.

4.3.1 Restriction digestion in extracellular loop 1

The mutated plasmids are cut in the DNA encoding the extracellular loop 1 by the restriction endonuclease ***Bsi*WI**, and after the coding region of the 5-HT receptor coding sequences by ***Xba*I**. The sizes of the DNA fragments were 931 bp (insert) and 5689 bp (cut vector) for the 3xHA-5-$HT_{4(b)}$ and 1645 bp (insert) and 5848 bp (cut vector) for the 5-$HT_{7(b)}$YFP, respectively (Fig.14).

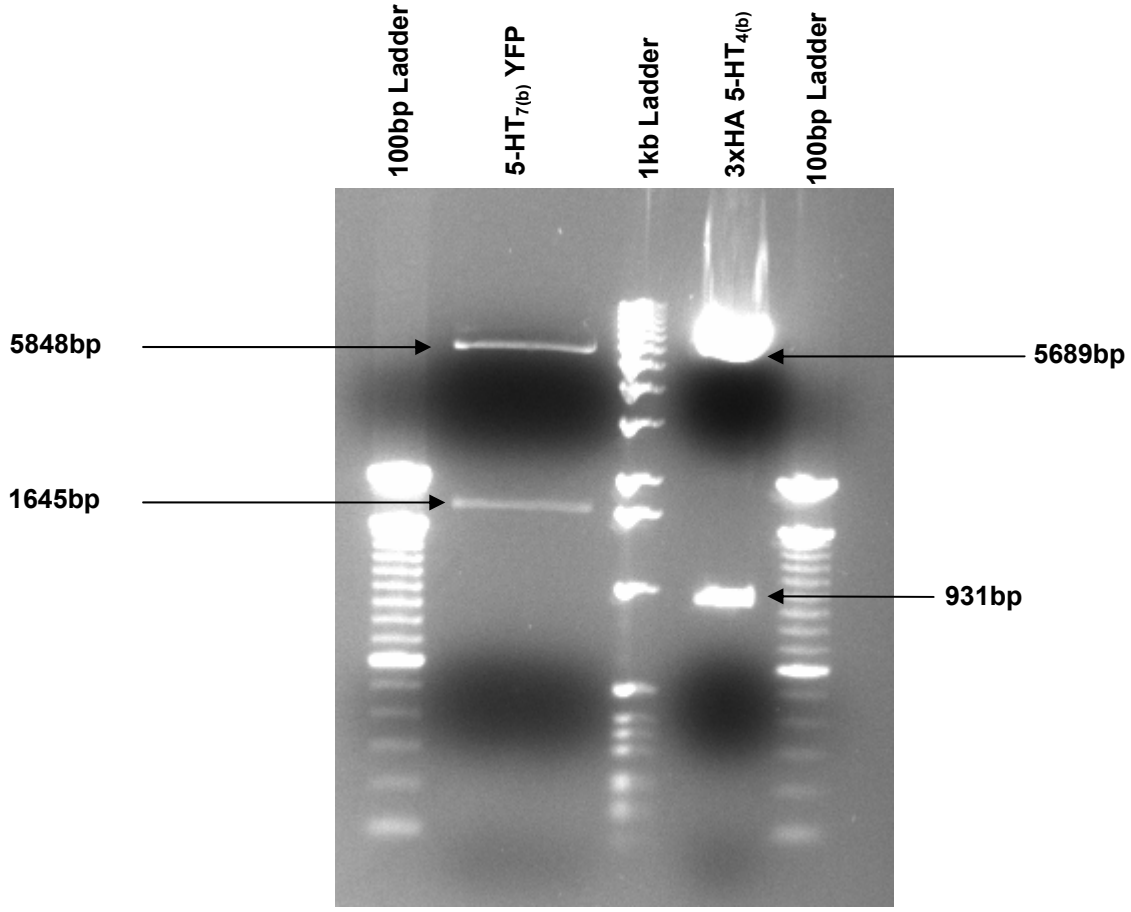


Figure 14. The restriction digestion products of 3xHA-5-$HT_{4(b)}$ and 5-$HT_{7(b)}$YFP cut with ***BsiWI /XbaI*** were separated on agarose gel (1.0 %). The sample in lane 2 are the restriction digestion products of 5-$HT_{7(b)}$YFP. The sample in lane 4 are the restriction digestion products of 3xHA-5-$HT_{4(b)}$. In lane 1 & 5 are the 100 bp DNA Ladder and in lane 3 the 1 kb DNA Ladder.

The ***Bsi*WI/*Xba*I**- fragment (931 bp) of 3xHA-5-$HT_{4(b)}$ was ligated into the ***Bsi*WI/*Xba*I** cut vector (5848 bp) of 5-$HT_{7(b)}$YFP, whereas the ***Bsi*WI/*Xba*I**-fragment (1645 bp) of 5-$HT_{7(b)}$YFP was ligated into the ***Bsi*WI/*Xba*I** cut vector (5689 bp) 3xHA-5-$HT_{4(b)}$, respectively. The ligated products were subsequently transformed into "TOP10" *E.coli* bacteria and the recombinant DNA was amplified.

The chimeric receptor DNAs were sequenced to verify if the DNA fragments were correctly inserted. The sequencing result revealed that the chimers had correct and intact sequences. Fig.15 illustrates the chimers made.

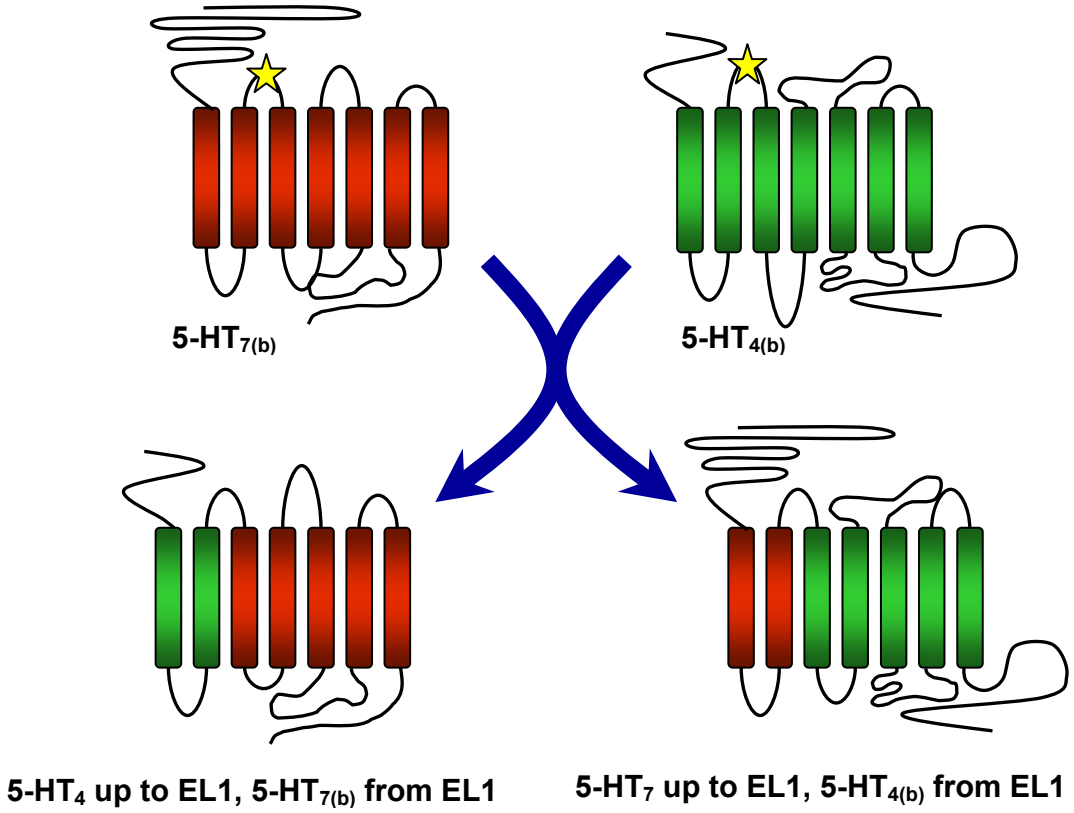


Figure 15. The figure shows the chimeric receptors 5-HT_4 up to EL1, 5-$HT_{7(b)}$ from EL1 and 5-HT_7 up to EL1 and 5-$HT_{4(b)}$ from EL1. (Designed by Cam Hong Thi Nguyen, 2007).

Figure 15 is an illustration of the chimeric receptors made at the molecular level. Only the plasmids containing the recombinant DNA for the chimers were constructed. These plasmids containing the constructed chimeric receptor DNA will subsequently be expressed in HEK293 cells to produce the chimeric receptor proteins of interest.

The following graphic maps show the plasmids containing the recombinant gene for the chimers made.

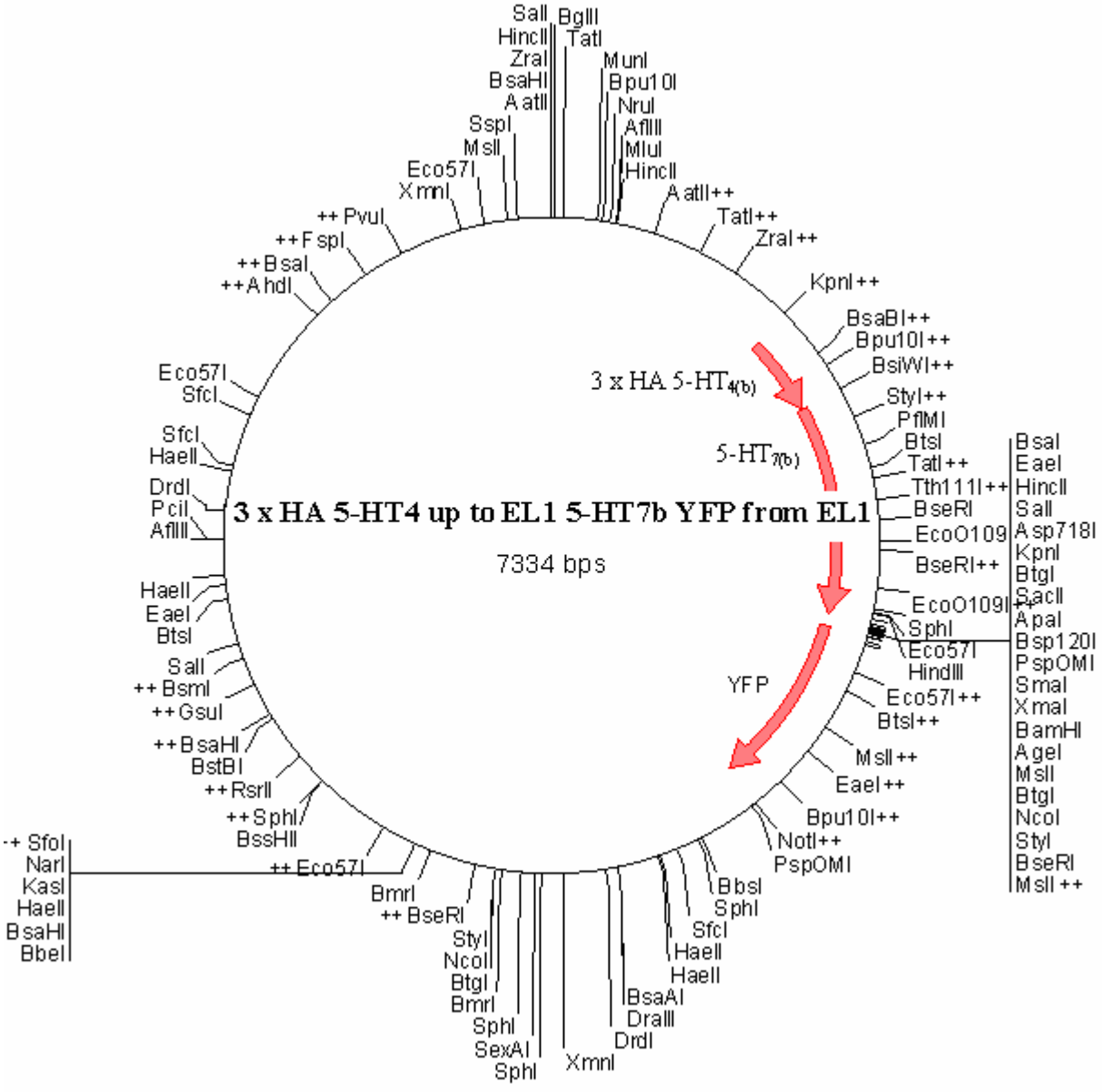


Figure 16. Graphic map of the plasmid containing the chimeric receptor gene 3xHA-5-HT$_4$ up to EL1, 5-HT$_{7(b)}$ YFP from EL1.

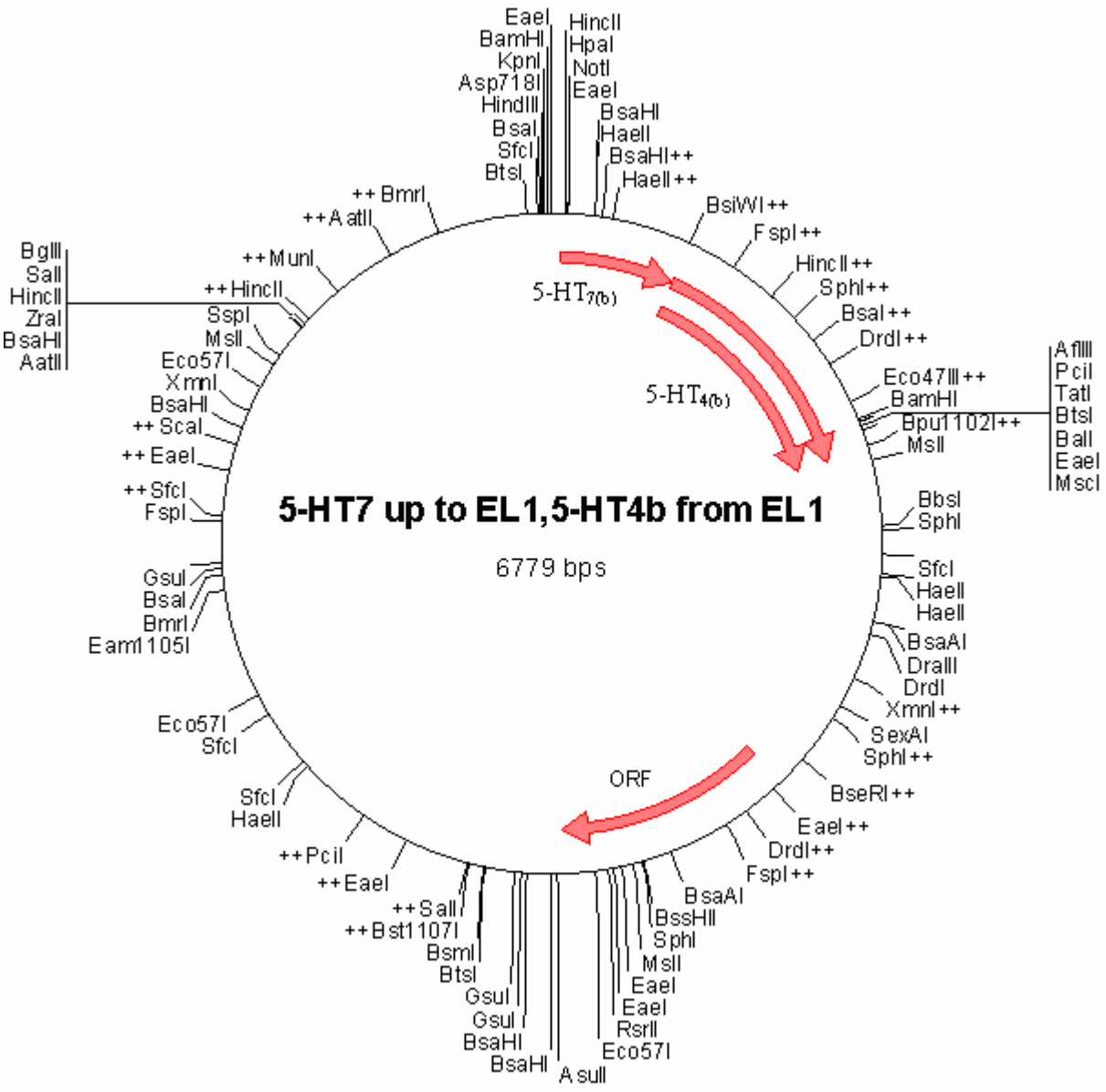


Figure 17. Graphic map of the plasmid containing the chimeric receptor gene 5-HT_7 up to EL1, 5-$HT_{4(b)}$ from EL1.

5. DISCUSSION

5.1 PURPOSE OF 5-$HT_{7(b)}$ RECEPTOR MUTAGENESIS AND CONSTRUCTION OF CHIMERIC RECEPTORS

The 5-HT_7 receptor is the most recently identified member of the 5-HT receptor family. Much is yet unknown about this receptor subtype. So far a couple of studies have revealed the existence of three isoforms of the human 5-HT_7 receptor (5-$HT_{7a,b,d}$), which differ in their C-termini. To date, these isoforms show no major differences in their respective pharmacology and signal transduction properties (Heidmann *et al.*, 1998;Krobert *et al.*, 2001).

The present study of constructing chimers was designed based on data from previous studies revealing that the 5-HT_7 receptor when co-expressed with other G_s-coupled receptors in HEK293 cells attenuate the signalling through the other receptors (Bruheim *et al.*, 2003;Andressen *et al.*, 2006). The purpose of constructing chimeric 5-HT_7/5-HT_4 receptors is to elucidate the structural basis for this attenuating property of the 5-HT_7 receptor. Since the 5-$HT_{4(b)}$ receptor does not posses this kind of attenuating property we decided to use this receptor to construct different chimers with the 5- $HT_{7(b)}$ receptor.

The mutagenesis reactions to introduce cutting sites for the endonuclease enzyme ***Bsi*WI** on the plasmids encoding the 5-HT receptors were carried out by two different methods. Method I ("Gene Tailor™ Site-Directed Mutagenesis System") was initially chosen and was tried with several different modifications, but eventually resulted in a product lacking 10 bp. Method II (standard mutagensis strategy) was therefore chosen for the second mutagenesis and was successful, enabling construction of the desired chimeric receptor constructs.

5.2 MUTAGENESIS REACTION IN EXTRACELLULAR LOOP 2 BY METHOD I

The mutation in extracellular loop 2 was performed using the "Gene Tailor™ Site-Directed Mutagenesis System", according to the protocol from Invitrogen®. It was very difficult to perform the mutagenesis reaction initially. Several PCR attempts were made and different PCR parameters were changed in order to optimize the conditions for a successful mutation of the plasmids.

According to the manufacturer's protocol the plasmids should first be methylated and then amplified in a polymerase chain reaction with the mutagenesis primers. The procedure worked well for the plasmid 3xHA-5-$HT_{4(b)}$ (6620 bp) but it was ineffective for the plasmid c-myc-5-$HT_{7(a)}$ (6795 bp).

The plasmid c-myc-5-$HT_{7(a)}$ was the one we initially used for the mutagenesis reaction. Many attempts were made to mutate the plasmid c-myc-5-$HT_{7(a)}$ with different PCR enzymes (Accuprime™ Pfx, Platinum® Taq and Platinum® Taq HiFi) and with different cycling conditions such as amplifying time, number of cycles etc. None of the PCR reactions gave any product, so we decided to use the plasmid 5-$HT_{7(b)}$-YFP (7493 bp) instead of plasmid c-myc-5-$HT_{7(a)}$.

The mutagenesis reaction was made both on methylated and unmethylated 5-$HT_{7(b)}$-YFP plasmid. The analysis of the mutagenesis products on agarose gel showed a single band in the sample containing the unmethylated plasmid 5-$HT_{7(b)}$-YFP. A few attempts were made to mutate the methylated 5-$HT_{7(b)}$-YFP plasmid with Vent® DNA polymerase and Platinum® Taq HiFi . Analysis on agarose gel showed no band in the sample containing methylated plasmid. We therefore decided to use the unmethylated mutated 5-$HT_{7(b)}$-YFP plasmid further for the restriction digestion reaction.

5.2.1 The purpose of methylation reaction

The plasmid DNA is methylated in a methylation reaction prior to the mutagenesis reaction by PCR. The enzyme DNA methylase methylates cytosine residues within a specific sequence throughout the double-stranded DNA of the original plasmid (template). When the PCR product is transformed into *E. coli* bacteria, the bacteria digest the methylated template DNA with an endonuclease enzyme called ***Mcr***BC leaving only unmethylated, mutated product. The purpose of this procedure is to increase the signal-to-noise ratio, i.e. the relative frequency of mutated plasmids. However, for us it resulted instead in loss of efficiency and we were not able to obtain any product from the methylated plasmids. Apparently, this step is not necessary, since we were successful in obtaining a product from non-methylated plasmid, although, unfortunately, this plasmid was lacking a stretch of 10 bp.

5.2.2 Mutagenesis primers

Design of good mutagenesis primers is very critical for the PCR. Only good primers will yield specific and effective amplification of the target gene, and at the same time avoid amplification of any unwanted DNA sequences due to primer dimers or unspecific binding of the primer. We carried out a couple of control reactions to test the mutagenesis primers by running a PCR with the known primers from the Levy laboratory (which gives a PCR product of 300-500 bp) along with the designed primers and on both the tagged plasmids and the wild type receptor plasmids. All the designed mutagenesis primers functioned well.

5.2.3 Magnesium ion concentration in the PCR

The significance of the chemistry of the Mg^{2+} ion to the enzymes lies in the unusual reaction chemistry of the ion to fulfil a range of functions. Mg^{2+} interacts with substrates, enzymes and occasionally both (Mg^{2+} may form part of the active site). Some enzymes do not need any additional components to show full activity but DNA polymerase requires magnesium ion as a co-factor to function efficiently. DNA polymerase is an enzyme that catalyzes the polymerization of deoxyribonucleotides in DNA synthesis and the magnesium ion either alters the conformation of the enzyme or takes part in the chemistry of the catalytic reaction. In the absence of the magnesium ion or when the magnesium ion concentration is very low the DNA polymerase does not function well at all. The manufacturer's protocol denotes that the methylation reaction buffer also contains magnesium ions, and that it is therefore sufficient to add 1 µl of 50 mM $MgSO_4$ per 50 µl mutagenesis reaction mixture for the methylated plasmid, as opposed to 2 µl for the unmethylated plasmid.

Though many attempts were made to mutate the plasmids and perform the restriction digestion with subsequent ligation the sequencing results revealed that the sequence of the mutated 5-$HT_{7(b)}$-YFP were not intact. Ten bases were deleted in the final mutated 5-$HT_{7(b)}$-YFP plasmid.

There are several different possible reasons for the deletion of 10 bases. One of the reasons could be that *Taq* DNA polymerase could have put the wrong bases in that region during DNA replication and the proof reader just removed those bases. Alternatively the magnesium concentration in the PCR mix was too low for the polymerase to function efficiently. Another

reason could probably be the exposure of the DNA in the agarose gel to UV-light while examining the gel. Ultraviolet radiation is an ionising radiation and depending upon its intensity it exerts its effects on DNA by point, insertion and/or deletion (nicking) mutations. UV radiation of 260 nm induces dimerisation of adjacent pyrimidine bases, especially if these are both thymines, resulting in a cyclobutyl dimer. Other pyrimidine combinations also form dimers. UV-induced dimerisation usually results in a deletion mutation when the modified strand is copied (Genomes, 2ndedition, T.A. Brown).

Longer exposure of the plasmid DNA to alkaline protease during the DNA purification (Wizard® Plus SV Miniprep Kit, Promega) might lead to some nicking of the plasmid DNA. Alkaline protease (an enzyme that degrades proteins) inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA. If the bacterial culture is incubated for more than 5 minutes with alkaline protease then nicking of the plasmid may occur.

One possible explanation why only one of the two plasmids (3xHA-5-HT$_{4(b)}$) was able to be methylated and the other (5-HT$_{7(b)}$-YFP) not is may be because the two plasmids had different origin and therefore the differences in the construction of the plasmids might have an impact for the methylation reaction. The plasmid 5-HT$_{7(b)}$-YFP (7493 bp) is also bigger in size compared to the 3xHA-5-HT$_{4(b)}$ (6620 bp).

Since the mutagenesis reaction according to the protocol from Invitrogen® resulted in a final product with a deletion, we decided to use method II (standard mutagenesis strategy) for the next chimer construct.

5.3 MUTAGENESIS REACTION IN EXTRACELLULAR LOOP 1 BY METHOD II

This mutagenesis strategy is a commonly used strategy to make mutations in the DNA. Using this strategy we were able to introduce a cutting site for the enzyme ***Bsi*WI** in the extracellular loop 1, followed by restriction digestion and ligation.

In this PCR protocol we used Vent® polymerase with termopol buffer. Vent® polymerase is a high-fidelity thermophilic DNA polymerase with an intergral 3'→5' proofreading

exonuclease activity. The polymerase also tolerates a wide range of cosolvents and in this protocol DMSO was used as a cosolvent.

DMSO is a polar aprotic solvent that dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water. This solvent is used in the PCR to reduce secondary structures in the DNA template or the primers. At the same time this solvent also seems to increase the mutation rate in PCR.

Though two sets of PCR were run to mutate the plasmids this method gave the correct and intact chimeric receptor sequences and proved to be an easier way to induce mutation for larger plasmids. Since this mutagenesis method seems to be more reliable than method I perhaps the other chimers could be made using this method.

5.4 PREVIOUS FINDINGS ON THE ATTENUATING PROPERTY OF THE 5-HT$_7$ RECEPTORS

Many studies have indicated that the amino acid sequences of IC loop 3 and the C-terminus are the main sites for coupling to G proteins in a variety of GPCRs, and thus these receptor domains are the most critical domains for the signal transduction. However, it is completely unknown which parts of the 5-HT$_7$ receptor are causing the attenuation of signalling by other G_s-coupled receptors. We have some indications based on unpublished data from our group (in collaboration with Evelien Gellynck and Peter Vanhoenacker from Ghent, Belgium) that when the IC loop 3 and the C-terminus of the 5-HT$_7$ receptors were removed the receptors did not couple efficiently to adenylyl cyclase but the receptor was still able to attenuate signalling through other G_s-coupled receptors (β-adrenergic receptors and prostaglandin receptors). Thus, these receptor domains do not seem to affect the preassociation of the 5-HT$_7$ receptors with G_s, presuming this preassociation is related to the attenuation of signalling through other G_s-coupled receptors. It is also believed that other domains of the 5-HT$_7$ receptors could be involved in the preassociation with G_s.

Constructing and expressing a series of chimeric receptors could reveal the key amino acid sequences which are involved in the preassociation of the 5-HT$_7$ receptors with G_s.

5.5 CHIMERIC RECEPTORS

Many studies have been done in the recent years using chimeric receptors to determine the structural basis for the receptor function in agonist binding and selectivity of G protein activation. In this study we are interested in determining the molecular basis for the attenuating property of the 5-HT_7 receptor by constructing and testing a series of chimers of the 5-HT_7 and 5-HT_4 receptors.

We decided to construct the chimers by introducing restriction sites to allow "cutting and pasting" in the extracellular domains. To date, there is not much data available concerning the property of the 5-HT_7 receptor which we are interested in (attenuation of signalling by other G_s-coupled receptors), and therefore it was not obvious where in the receptors to "cut and paste". One reason to start with the extracellular domains was to leave the intracellular loops intact, since it seems likely that they are somehow involved in the attenuation, either alone or in combination. So far only the chimers fused in extracellular loop 1 have been successfully made. The attempt of constructing the chimers fused in extracellular loop 2 was unsuccessful, due to the 10-bp deletion as described above. The chimers fused in extracellular loop 3 will be constructed in the future and tested along with the chimers made in this study.

In order to construct these chimers we had to mutate one or two amino acids to introduce the cutting site for the endonuclease enzyme. If these amino acids are the key amino acids for the normal functioning of the 5-HT_7 receptor, this change of amino acids will have an impact on the receptor behaviour (either a loss of function or an unusual behaviour) when expressed in mammalian cells (for example HEK293). It would be relevant to test the properties of the 5-HT_7 and 5-HT_4 receptors mutated in the extracellular domains in addition to testing the chimeric receptors, to check the importance of introducing these amino acid changes. Alternatively, the chimeric receptors could be "corrected" by mutagenesis to change the sequence around the fusion site back to the original amino acids (in other words make the mutation on other amino acids away from the one which was initially mutated).

Only when the attenuating property of the various chimeric receptors are correlated with the 5-HT_4 and 5-HT_7 amino acid sequences of these chimeric receptors, it is possible to assign the functional properties to specific structural domains of the receptors. For a more detailed resolution of the precise amino acid sequences involved in the attenuating property one could

insert a smaller segment of the 5-HT_7 receptor sequence into the 5-HT_4 receptor or by substituting single amino acids.

5.6 CHIMERIC RECEPTORS VS OTHER STRATEGIES

To use chimeric receptors to understand the structural basis for the receptor function is one approach. Among a few other strategies to study the receptor function, the split receptor strategy gives a clue of the importance of certain receptor domains. Split receptor has been studied to explore the role of different hydrophobic domains of the GPCR receptors. Split receptors are mainly made by inserting a termination codon after an amino acid that encodes a hydrophobic domain or in the intracellular / extracellular loop. Split receptors could also be made by deleting different loop regions of the receptor.

In a study made on β_2-AR by B.K. Kobilka et al. (Kobilka *et al.*, 1988) the receptor was expressed as two separate peptides, one encoding amino acid 1- 262, containing hydrophobic domains 1 to 5, SR (1-5), and the other containing hydrophobic domains 6 to 7, SR (6-7). It was possible to express SR (1-5) and SR (6-7) together in *Xenopus* oocytes and obtain a functional receptor, but injecting mRNA for SR (6-7) alone did not lead to the expression of the protein in the oocyte membranes.

The mutant SR (1-5) did not bind ligands or activate adenylyl cyclase, but the mutant SR (6-7) was able to activate the adenylyl cyclase although the activation was only ~ 25 % as efficient as the wild-type β_2-AR. The conclusion of the study was that although the hydrophobic domain 7 (or 6 and 7) seemed to be the major determinant(s) of β_2-AR in activating adenylyl cyclase, this region of the molecule alone was inefficient to bind β_2-AR ligands or activate adenylyl cyclase.

A majority of studies also used deletion mutation to understand the structural basis of a receptor function. The premise in these studies was that there is a single motif within GPCRs which is largely responsible for G protein activation and selectivity and therefore the deletion of this domain would uncouple GPCR from G protein. This assumption, while used successfully to identify functional domains for many single membrane-spanning biomolecules such as kinase superfamily, must be taken with caution for the GPCRs. Because GPCRs have seven-membrane-spanning architecture which allows the intracellular loops to interact with

each other in forming a functional domain(s) to interact with G protein, deletion of a single domain can not conclude about the receptor activity or function. The interpretation of the data from deletion studies will therefore lead to some uncertainty.

6. SUMMARY AND CONCLUSIONS

The ultimate aim of the present series of studies is to determine the key amino acid sequences of the 5- HT_7 receptor which are responsible for the attenuation of signalling through other G_s-coupled receptors.

Many studies on chimeric receptors have given valuable data on the structural basis for the receptor function of several other GPCRs. Chimeric receptors seem to be a more convincing strategy than for example deletion studies to gain knowledge about the structural determinants which are crucial for a receptor function and thus to understand the cellular mechanisms. Deletion mutation studies has many disadvantages such as loss of function of the receptor, change in the overall folding of the receptor protein at the intracellular regions of the receptor which are important for the preassociation of G_s etc. Chimeric receptors may not have these disadvantages since the functional response of the chimers will be linked with both the receptors (5-$HT_{4(b)}$ and 5-$HT_{7(b)}$).

In further studies of chimeric 5-$HT_{4(b)}$/5-$HT_{7(b)}$ receptors the results will be interpreted on the basis that of the two receptors only one (5-$HT_{7(b)}$) possesses the attenuating property while the other receptor (5-$HT_{4(b)}$) does not.

The function of specific structural domains of the 5-HT_7 receptor will only be determined when the chimers are expressed in HEK293 cells and their pharmacological properties determined by ligand binding and adenylyl cyclase assays.

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8. APPENDIX

8.1 GENE TAILOR™ SITE-DIRECTED MUTAGENESIS SYSTEM-KIT CONTENTS

Components
DNA methylase (4 units/µl)
Methylation buffer
200x SAM
dNTP mix (10 mM each)
Control Plasmid (100 ng/ µl)
Control Primers (10 µM each)
Sterile, distilled water
One-Shot® MAX Efficiency® DH5α™ -T1^{R}

* Patent pending

8.2 BUFFERS AND SOLUTION

Buffer	Ingredients
Buffer P1	50 mM Tris-HCl, pH 8.0 10 mM EDTA 100 µg/ml RNase A
Buffer P2	200 mM NaOH 1 % SDS
Buffer P3	3.0 M potassium acetate pH 5.5
Buffer QBT	750 mM NaCl 50 mM MOPS pH 7.0 15 % isopropanol 0.15 % Triton®-X-100
Buffer QC	1.0 M NaCl 50 mM MOPS pH 7.0 1.5 % isopropanol
Buffer QF	1.25 M NaCl 50 mM Tris-HCl pH 8.5 15 % isopropanol

dNTP Mix, 10 mM	10 mM of dATP, dGTP, dCTP, dTTP
LB-medium	10 g/l tryptone 5 g/l yeast extract 10 g/l (170mM) NaCl adjusted to pH 7
LB- plates	LB- medium 15 g/l Bacto-agar
Loading buffer 6X	0.25 % bromophenol blue 0.25 % xylene cyanol FF 15 % Ficoll in dH_2O
NE buffer 3 10x	100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 1 mM Dithiothreitol pH 7.9 at 25°C
NE buffer 4 10x	50 mM potassium acetate 20 mM Tris-acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol pH 7.9 at 25°C
SOC	20 g/l tryptone 5 g/l yeast extract 10 mM NaCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 2.5 mM KCl 20 mM glucose
TAE	0.04 M Tris- acetate 0.001 M EDTA
TE (membrane)buffer	50 mM Tris-HCl, pH 7,5 at 20°C 1 mM EDTA

Thermopol buffer 10x	10 mM KCl 10 mM $(NH_4)_2$ SO_4 20 mM Tris-HCl (pH 8.8 at 25ºC) 2 mM $MgSO_4$ 0.1 % Triton X-100
Washing buffer	50 mM Tris-HCl, pH 7.0 at 20℃ 2 mM $MgCl_2$.
Wizard Plus SV Minipreps Cell Resuspension Solution	50 mM Tris-HCl pH 7.5 10 mM EDTA 100 μg/ml RNase A
Wizard Plus SV Minipreps Cell Lysis Solution	0.2 M NaOH 1 % SDS
Wizard Plus SV Minipreps Neutralization Solution	4.09 M guanidine hydrochloride 0.759 M potassium acetate 2.12 M glacial acetic acid
Wizard Plus SV Minipreps Column Wash Solution	60 % ethanol(v/v) 162.8 mM potassium acetate 27.1 mM Tris-HCl, pH 7.5

8.3 MANUFACTURERS: CHEMICALS AND CLONING MATERIALS

Chemical/cloning materials	**Abbreviation**	**Manufacturer**
100bp DNA ladder		Invitrogen
1kb DNA ladder		Invitrogen
AccuPrime Pfx DNA Polymerase		Invitrogen
Agar		AppliChem
Agarose for gel electrophoresis		Camber
*Blp*I		NE Biolabs
*Bsi*WI		NE Biolabs
*Cla*I		NE Biolabs
DH5α™ -T1^{R} *E.coli*		Invitrogen
Dimethylsulfoxide	DMSO	Sigma
Ethidium bromide	EtBr	Sigma
Ethylendiaminetetraacetic acid, Disodium salt, Dihydrate, approx. 99%	EDTA	Sigma
Gene Tailor™ Site-Directed Mutagenesis System		Invitrogen
HiSpeed plasmid maxi kit		Qiagen
Isopropanol		Arcus
*Kpn*I		Invitrogen
Magnesium sulphate	$MgSO_4$	Invitrogen
Mutagenesis primers		Invitrogen
*Nhe*I		NE Biolabs
QIAquick Gel Extraction Kit		Qiagen
Platinum Taq DNA polymerase		Invitrogen
Platinum Taq HiFi		Invitrogen
QIAquick GEL Extraction KIT		QIAGEN
SOC- medium		Invitrogen
Sodium chloride solution, 0.9%	NaCl, 0.9%	Braun
Sodium hydroxid	NaOH	Applichem
T4 DNA ligase		Fermentas Inc.
10X buffer for T4 DNA ligase		Fermentas Inc.
Thermopol Reaction buffer		NE Biolabs
TOP-10 Chemically competent E.coli cells		Invitrogen
TOPO TA Cloning		Invitrogen
Tryptone		AppliChem
Vent® polymerase		NE Biolabs
Wizard®Plus SV Miniprep Kit		Promega
*Xba*I		NE Biolabs
*Xho*I		NE Biolabs
Yeast Extract		AppliChem

8.4 INSTRUMENTS

Instruments	Manufacturer
ABI Prism 3730 DNA Analyzer	Applied Biosystems
Power Pac 200	Bio-Rad Laboratories Inc.
PTC-100 Programmable Thermal Controller	MJ Research, Inc.
Ultrospec 2100 pro UV/ Visible spectrophotometer	GE Healthcare