2006

Generating stable constructs of beta-2 adrenergic receptor by truncation

Meera Kumar
San Jose State University

GENERATING STABLE CONSTRUCTS OF BETA-2 ADRENERGIC RECEPTOR BY TRUNCATION

A Thesis

Presented to

The Faculty of the Department of Biology

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

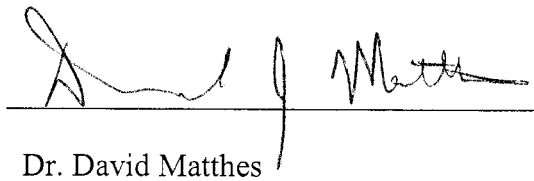
by

Meera Kumar

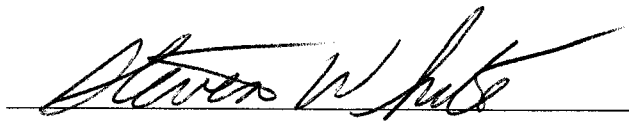
May 2006

APPROVED FOR THE DEPARTMENT OF BIOLOGY

Dr. Brian Kobilka



Dr. David Matthes



Dr. Steven White

APPROVED FOR THE UNIVERSITY

ABSTRACT

DEVELOPING STABLE CONSTRUCTS OF BETA 2-ADRENERGIC RECEPTOR BY TRUNCATION

by Meera Kumar

Constructs derived from the β2-AR were generated by deleting amino acids within the fifth loop of the receptor. These mutant receptors were expressed in insect cells, purified, and their binding affinities determined. The results showed that shortening the wild type β2-AR by removing 18 aminoacids within the fifth loop (TR-1) improved the affinity towards antagonist binding almost four fold when compared to the wild type. Further shortening the loop by removing 25 aminoacids within the fifth loop (TR-2) decreased the affinity of the receptor to its ligand when compared to the wild type. There was no change in the pharmacological properties of these mutant receptors when compared to the wild type. An increase in affinity indicates an increase in the stability of the receptor. Immunofluorescence results indicated that these truncated versions did not express as efficiently as the wild type and some of these mutant receptors were clustered intracellularly.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. David Matthes who has always been my constant source of inspiration. I want to thank him for accepting me as his student. His advice has always been very valuable to me and he has helped me a lot during my cloudy days. I still remember the first day of my genetics class. I was completely mesmerized by the depth of his knowledge. He has been one of the best teachers that I have ever had. His approach to problem solving always taught us how to get an insight into the subject. I want to thank him especially for allowing me to start working in his laboratory right from the first semester. It was this lab experience that helped me learn all the basic skills. I have absolutely no words to mention how grateful I am for all his help.

I am very thankful and grateful to Dr. Brian Kobilka who gave me an opportunity to work in his esteemed laboratory amidst a great team of scientists. Dr. Kobilka has always been very patient with me. He always took time inspite of his busy schedule to answer my questions. I feel it was a great privilege for me to work under the mentorship of someone as knowledgeable and as renowned as him. It was because of the time that I spent in his laboratory, learning all the techniques, that I landed a job at Xenoport.

The team at Stanford laboratory has been very friendly to me and I felt very comfortable working there. I would like to thank each and every person at the laboratory for being kind and helpful.

I would like to thank Dr. Zhiping Yao who provided me the truncated construct-1 and the linker for my experiments.

I would like to mention my special thanks to Dr. Carl Hurt who has been my mentor. He taught me a lot of cell biology techniques such as adenoviral infection, methods to establish titer, western blotting and the proper use of microscope. He helped me acquire the immunofluorescence images using the confocal microscopy. He is a wonderful and patient teacher. He also helped me keep going during my very bad days. His meticulous laboratory etiquette and his dedication taught me what it takes to be a good scientist.

I would like to thank Dr. Gayathri Swaminath who spent several hours teaching me the binding assays. She was very patient and helped me learn the assays and the use of all the instruments. She spent a significant amount of her time teaching me how to perform binding assays and competition assays. Her dedication and hardworking nature has always amazed me. She has been a constant source of inspiration.

I would like to thank Dr. Steven White, the amazing soul who keeps abreast of every new breakthrough in science. I enjoyed the two classes that I signed up for with him-molecular cell biology and human embryology. His enthusiasm and knowledge of the subject is unrivalled. It was because of him that I got interested in the signal transduction mechanisms. He would present the latest information in the class and would update the class with new publication in an awe inspiring way. I acquired a lot of molecular biology techniques in his class and he helped me in refining those techniques.

Thanks to my amazing and loving husband Kumar without whom none of this would have been possible. He financed my entire course. He helped me at each and every step. I still remember the days when I did not have a car and he would drive me to

the laboratory anytime I wanted to go and kept encouraging me and inspiring me every day. I do not think I could have done anything if it were not for him.

Last but not the least I want to thank my mother and father. Their encouragement kept me going. Their regular encouragement helped me complete the thesis after it had been on the shelf gathering dust for over a year. My father helped me through all my bad days by boosting confidence within me to finish this graduate work.

TABLE OF CONTENTS

Subject

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ALP	Alproterenol
AR	Adrenergic receptor
β2-AR	Beta-2 Adrenergic receptor
DHA	Dihydroalproterenol
GPCR	G Protein coupled receptor
G-Protein	Guanine nucleotide binding protein
ISO	Isoproterenol
TR1-β2AR	Truncated receptorI beta-2 adrenergic receptor
TR2-β2AR	Truncated receptorII beta-2 adrenergic receptor
WT β2-AR	Wild type Beta-2 Adrenergic receptor

INTRODUCTION

The majority of transmembrane signal transduction in response to hormones and neurotransmitters is mediated by G- protein coupled receptors (GPCRs). Based on structure, function, and ligand characteristics, nearly 2,000 GPCRs have been identified (Kolakowski, 1994). The human body uses these seven transmembrane-spanning serpentine receptors for an astounding variety of biological signaling functions. G-protein coupled receptors on the cells lining our tongue convey taste (Abe et al., 1993). GPCRs in olfactory bulbs of our nose convey information about the presence of odors and are called odorant ligands (Bargmann, 1997). A carotenoid molecule related to vitamin A is bound in the ligand position of rhodopsin in the rods and cones of our eyes where it serves to pick up photons, alter its conformation, and cause the receptor to which it is bound to release signals into the rod/cone cytoplasm that result in our perception of light (Ando et al., 1991). These serpentine receptors are of very ancient lineage. They are also seen in other species, for example, *Saccharomyces* sps communicate their sexual identity to each other by release of polypeptide mating factors that recognize these membrane-spanning serpentine receptors.

GPCRs are polytopic membrane proteins that share a common structure containing seven transmembrane segments (7-TMSs) (Probst, *et al.*, 1992). These seven transmembrane receptors can be identified by hydropathy analysis and are predicted to have α-helical structures, usually consisting of 20 to 24 amino acids each. They have an extracellular N-terminus, a cytoplasmic C-terminus, three intracellular, and three

extracellular loops (Figure 1). The extracellular portions of the seven-transmembrane spanning regions apparently form a "binding- pocket" capable of selectively binding to drugs, neurotransmitters and hormones (Figure 2). The ligand-receptor complex generates a signal that the intracellular tail and loops transmit into the cell by interacting with specialized intracellular proteins known as guanine nucleotide-binding proteins, G proteins.

The ligand binding domains of the GPCRs are very diverse and the agonists that bind to these receptors are also very different with respect to their chemical structures. GPCRs recognize a variety of ligands and respond to a broad range of stimuli (e.g., light, ions, biogenic amines, nucleosides, lipids, amino acids, and peptides). Ligands that have lower molecular weights generally bind to sites within the hydrophobic core formed by the transmembrane α helices (Kobilka, 1992). The binding sites for protein and peptide agonists include the N terminus and the extracellular hydrophilic loops that join the transmembrane domains (Strader et al., 1995). Signal transduction is accomplished by coupling via guanine nucleotide-binding proteins (G proteins) to various secondary pathways involving ion channels, adenylyl cyclases, and phospholipases. Furthermore, GPCRs may also couple to other proteins, for example those containing PDZ domains. It has recently been shown that opioid receptors interact with calmodulin at the same domain required for G protein coupling. Hence, GPCRs may have many more protein signaling partners than currently known.

Several other families share the 7-TMS architecture, most notably bacteriorhodopsin and photoreceptors, proton pumps of archebacteria, for which direct

molecular structural information is available (Henderson, *et al.*, 1990). The 7-TMS topology of the bacteriorhodopsins and GPCRs is similar (Mizobe, et al., 1996). The bacteriorhodopsin ligand, retinal, is covalently attached to a lys residue in the seventh TMS at a location identical to that for the retinal attachment site in rhodopsin, a true GPCR (Henderson, *et al.*, 1990).

The structural analysis of GPCRs has been hindered by their low natural abundance and difficulty in obtaining and purifying significant quantities of recombinant protein. The only GPCR that has been crystallized and whose structure has been determined is the bovine rhodopsin (Okada et al., 2000).

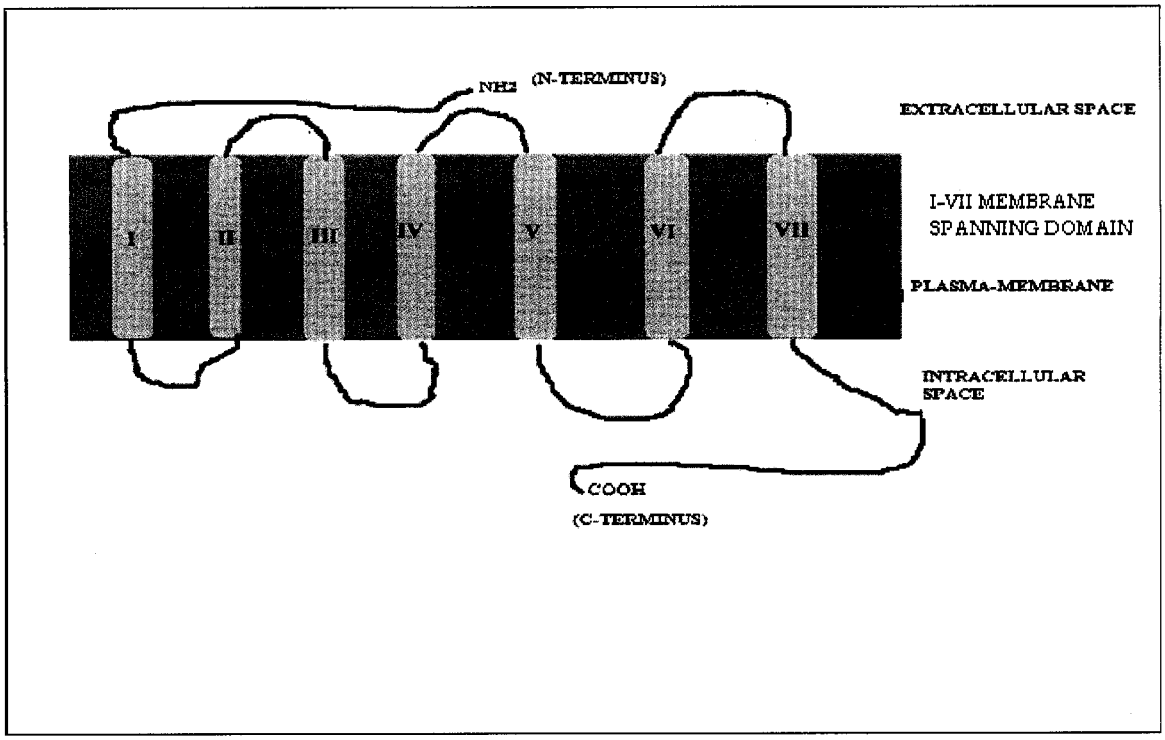


Figure 1. Schematic representation of G-protein coupled receptor showing the N-terminus, C-terminus, transmembrane domains (I-VII) and six loops.

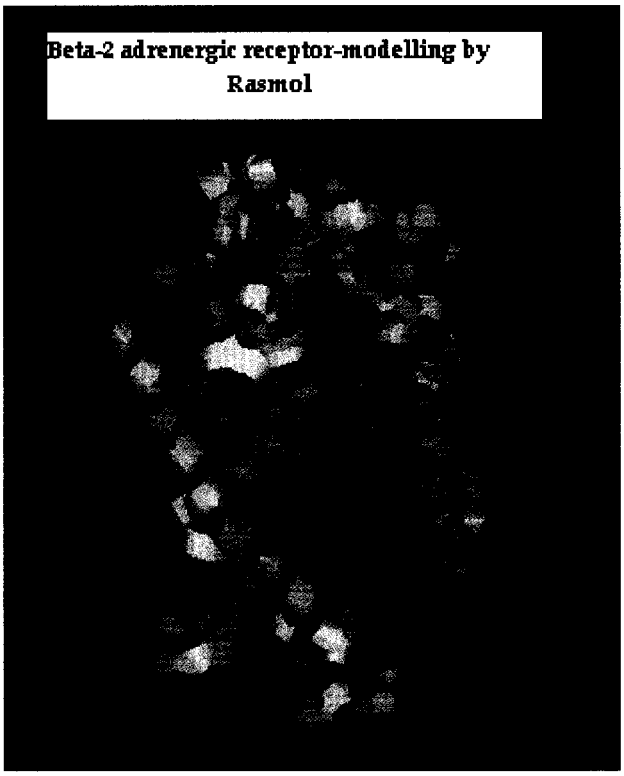


Figure 2. Model of beta-2 adrenergic receptor using Rasmol molecular modeling program.

G-protein Mediated Signal Transduction Pathway

Guanine nucleotide-binding proteins (G proteins) are specialized signaling molecules that directly interact with specific intracellular regions of the GPCR. G-proteins can be categorized into many families, G_i, G_s, G_q, G_t, and G_{12} etc., whose members are important in regulating distinct cellular processes. G proteins are comprised of three structurally different polypeptide subunits: alpha, beta, and gamma (Wall, 1995). In its inactive state, the G protein's three subunits are linked together, forming a heterotrimer (Figure 3). The inactive heterotrimer's alpha subunit has two important functions: it binds to a molecule of guanosine diphosphate (GDP) and serves as a major contact point between the G protein and receptor's third intracellular loop (Cerione et al., 1985) (Figure 3). The beta and gamma subunits in the inactive G protein enhance the interaction between the receptor and the alpha subunit (Hamm et al., 1998; Coleman et al., 1994).

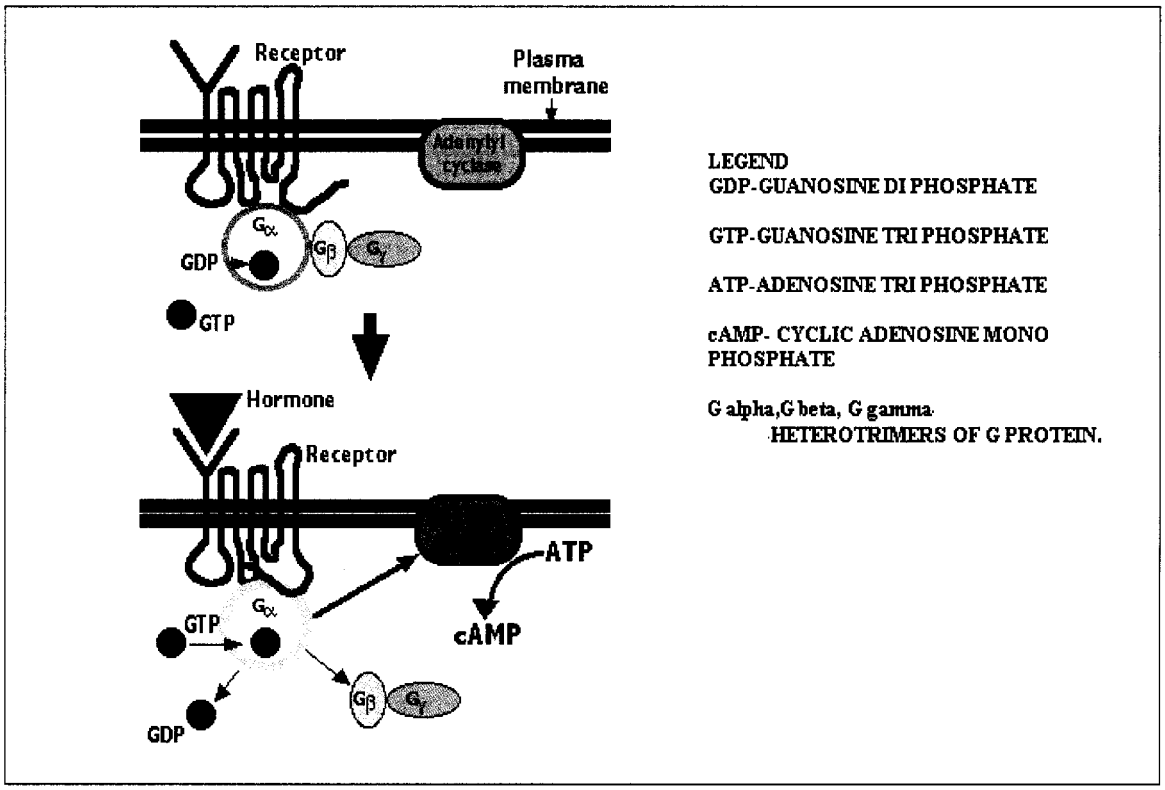


Figure 3. Schematic illustration depicting the G-protein interaction in beta adrenergic receptors.

Receptor-Ligand Interaction

Receptor activation by an agonist molecule induces a conformational change in the receptor's three-dimensional structure. It is this structural change that is responsible for activating the G protein. G protein activation is characterized by an exchange of GDP for GTP on the alpha-subunit and subsequent dissociation of the G protein from the receptor (Strader et al., 1994). As the G protein disengages from the receptor, it splits into two separate components: a free alpha subunit-GTP complex and a beta-gamma dimer. The alpha subunit, $G\alpha_s$, binds to and activates a specialized membrane protein, adenylyl cyclase. Adenylyl cyclase is a membrane enzyme that produces the cyclic nucleotide, cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). The production of cAMP is a critical component of this signaling pathway, for it regulates the activity of a second enzyme, protein kinase A (PKA). PKA is a multi-subunit enzyme complex composed of regulatory and catalytic subunits. Cyclic AMP activates PKA by binding to the regulatory subunit, causing it to disengage from the catalytic subunit. The released catalytic subunit of PKA phosphorylates serine and threonine residues on different intracellular proteins (Bourne, 1997). Protein phosphorylation is an important mechanism by which cells regulate the activity of intracellular proteins. As a result of protein phosphorylation by PKA, a number of diverse physiologic processes such as heart rate, glucose, fat metabolism, platelet adhesion, blood flow, and bronchoconstriction are affected. Phosphodiesterases that hydrolyze cAMP to adenosine monophosphate (AMP) terminate the intracellular signal

carried by cAMP. The alpha subunit and beta-gamma dimer can each interact with a variety of intracellular proteins, leading to either an increase or decrease in their activity. The G protein's cycle is completed once the GTP is hydrolyzed by the alpha subunit, and the three subunits recombine to reconstitute the G protein (Strader et al., 1995; Bourne, 1997).

Adrenergic Receptors

Adrenergic receptors (ARs) form a distinct family within the G-protein coupled receptor superfamily (Strader et al., 1995) that controls a wide variety of functions in the autonomous nervous system by regulating physiological activities. The adrenergic receptor is a kind of G-protein coupled receptor that binds to the ligand epinephrine, also known as adrenaline, which is released by the adrenal glands above the kidneys in response to stressful stimuli. Once released, epinephrine flows through the blood stream and adsorbs to the adrenergic receptors on the surfaces of cells in various tissues throughout the body. The result is the establishment of the primitive mammalian fight or flight reaction. This reaction increases heart rate, decreases blood flow to the gut, increases blood flow to skeletal muscles, and increases blood glucose by causing liver and muscle cells to break down glycogen and release the resulting glucose into the circulation. The epinephrine ligand is not internalized but binds to its receptor for a short time causing the receptor to release biochemical signals into the cell cytoplasm due to changes in the 3-dimensional conformation of the receptor-ligand complex.

There are three subfamilies of AR: $\alpha 1$, $\alpha 2$, and β. Each of these families has three

members. The tissue specific expression patterns and their biological responses are summarized in Table 1.

The α1-ARs are involved in inositol phosphate release, which activates phospholipase C by interacting with $G_q\alpha$ subunit of the G-protein (Wu et al., 1992). The α2-ARs inhibit adenylate cyclase by interacting with the Gi subunit of the G-proteins (Kurose et al., 1991).

Table 1. Subtypes of receptors showing tissue specific expression and biological response

Alpha 1	Contraction of vascular smooth muscle
	Contraction of gastro uterine smooth muscle
	Glycogenolysis and glucogenesis of liver
	Relaxation of intestinal smooth muscle
	Proarrhythmia of heart
Alpha 2	Aggregation of platelets
	Decreased norepinephrine release from nerve endings
	Contraction of vascular smooth muscle
	Decreased insulin secretion from pancreatic islets
Beta 1	Increased rate and force of myocardial contraction
	Increased AV nodal conduction velocity
Beta 2	Relaxation of smooth and skeletal muscle
Beta 3	Lipolysis of fat

Beta Adrenergic Receptor

Beta-1 receptors are the predominant subtype in cardiac tissue where they mediate positive inotropic and chronotropic effects and in the kidney where they enhance renin release (Frielle et al., 1987).

Beta-2 receptors mediate relaxation of smooth muscle including vascular beds, bronchus, intestine and uterus. They mediate glycogenolysis and glucogenesis in the liver and regulate cell metabolism in skeletal muscle. They inhibit the activity of leukocytes and other blood cells. They are also expressed in the heart. The receptors are located presynaptically in nerves, where they facilitate neurotransmitter release, and in the brain, where they regulate a variety of physiological processes.

Beta-3 receptors have limited distribution. They are found in low levels in adipose tissue and the gastrointestinal tract, where they stimulate lipolysis and increased gut motility (Emorine et al., 1998). They appear to coexist with beta-2 receptors in skeletal muscle (Strosberg, 1997).

Beta 2 Adrenergic Receptor

The beta 2-adrenergic receptor (β2-AR) is an integral membrane glycoprotein of apparent molecular weight approximately equal to 64,000 Dalton. (Kobilka et al., 1987) The amino acid sequence deduced from the β2-AR gene reveals homology with the visual pigment rhodopsin of retinal rod outer segments (Dixon et al., 1986). The 38 kDa amino-terminal domain contains the ligand binding site (as revealed by photoaffinity labeling) and the sites of glycosylation (as revealed by its sensitivity to endoglycosidase F), whereas the 26 kDa carboxyl-terminal domain contains all of the phosphorylated

sites. Of the four canonical sites for N-linked glycosylation, two exist near the amino and two near the carboxyl terminus (Kobilka et al., 1987) and only those in the amino-terminal domain (Asn6 and Asn15) are sensitive to endoglycosidase F. Carboxypeptidase Y treatment of reconstituted native beta-adrenergic receptor generates a truncated (approximately 57 kDa) glycopeptide that has lost most of the sites phosphorylated by beta-AR kinase, and one of the sites phosphorylated by protein-kinase A. The various features delineated, including the length of the carboxypeptidase Y-sensitive region, the extracellular location of the trypsin-sensitive site, the location of the sites of phosphorylation and glycosylation all constrain the receptor to a rhodopsin like structure with multiple membrane spanning segments (Okada et al., 2001).

Structure of Beta-2 Adrenergic Receptor versus Rhodopsin Structure

Bovine rhodopsin is the only GPCR that has been crystallized (Okada et al., 2000). Recently, low-resolution structures of bovine rhodopsin, frog rhodopsin and squid rhodopsin have become available. The complex seven transmembrane structure of GPCRs makes them very difficult to crystallize. The β2-AR exhibits a high degree of structural similarity to bovine rhodopsin. Table 2 summarizes the comparison between bovine rhodopsin and β2-AR.

Table 2. Comparison of characteristics between beta-2 adrenergic receptor and bovine rhodopsin.

Characteristics	**Beta-2 adrenergic receptor**	**Bovine rhodopsin**
1st messenger	Norepinephrine	Retinal
G-protein coupled receptor	Beta-2 adrenergic receptor	Opsin
G-protein	G_s protein	Transducin
Activated enzyme	Adenylyl cyclase	Phosphodiesterase
2nd messenger	Cyclic AMP	Cyclic GMP
Ion channel is permeable to	K+	Na+,K+,Ca2+

The multiple sequence alignment of β2-AR and bovine rhodopsin in appendix B shows the similarities between the two proteins. A comparison of the secondary structures of the two proteins (Figure 4) shows that the β2-AR has a longer intracellular loop and a longer C-terminus than that of rhodopsin.

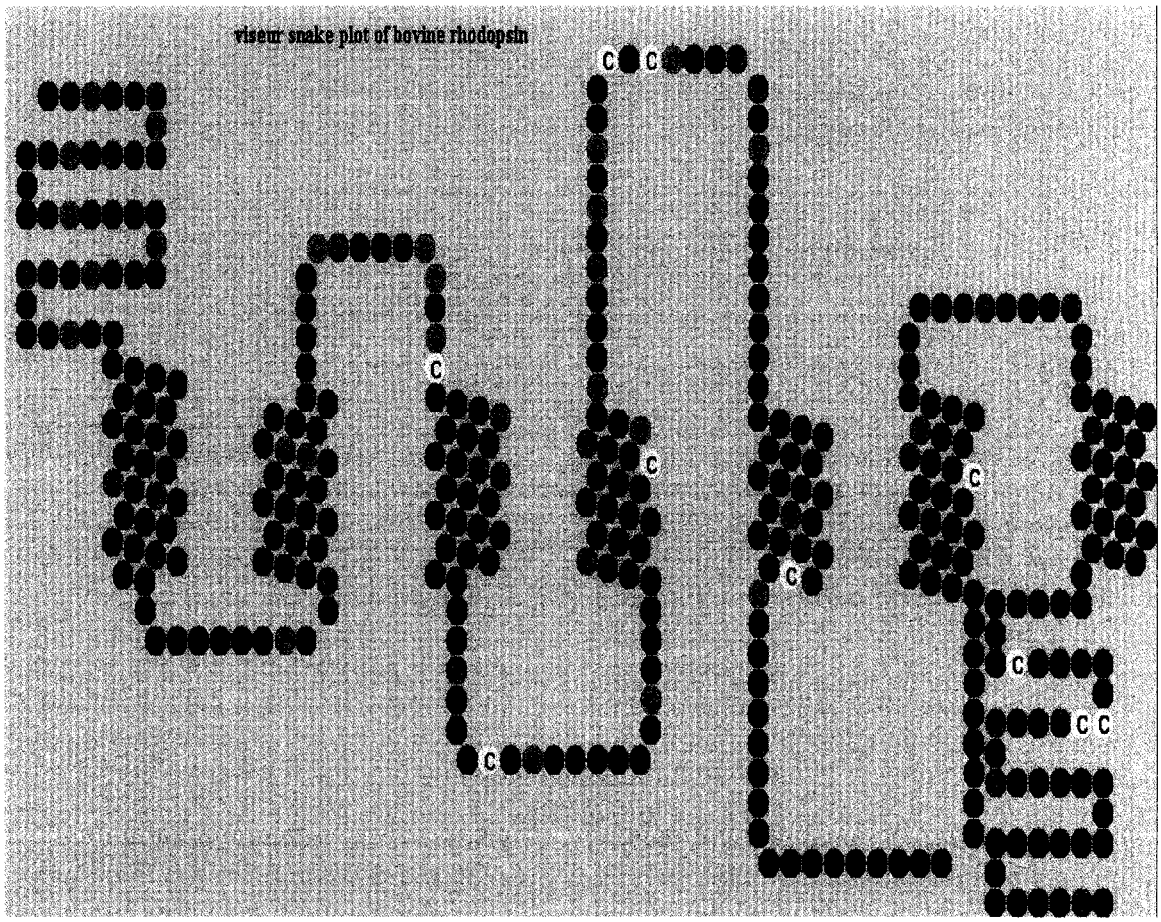


Figure 4. Two-dimensional structure of bovine rhodopsin.

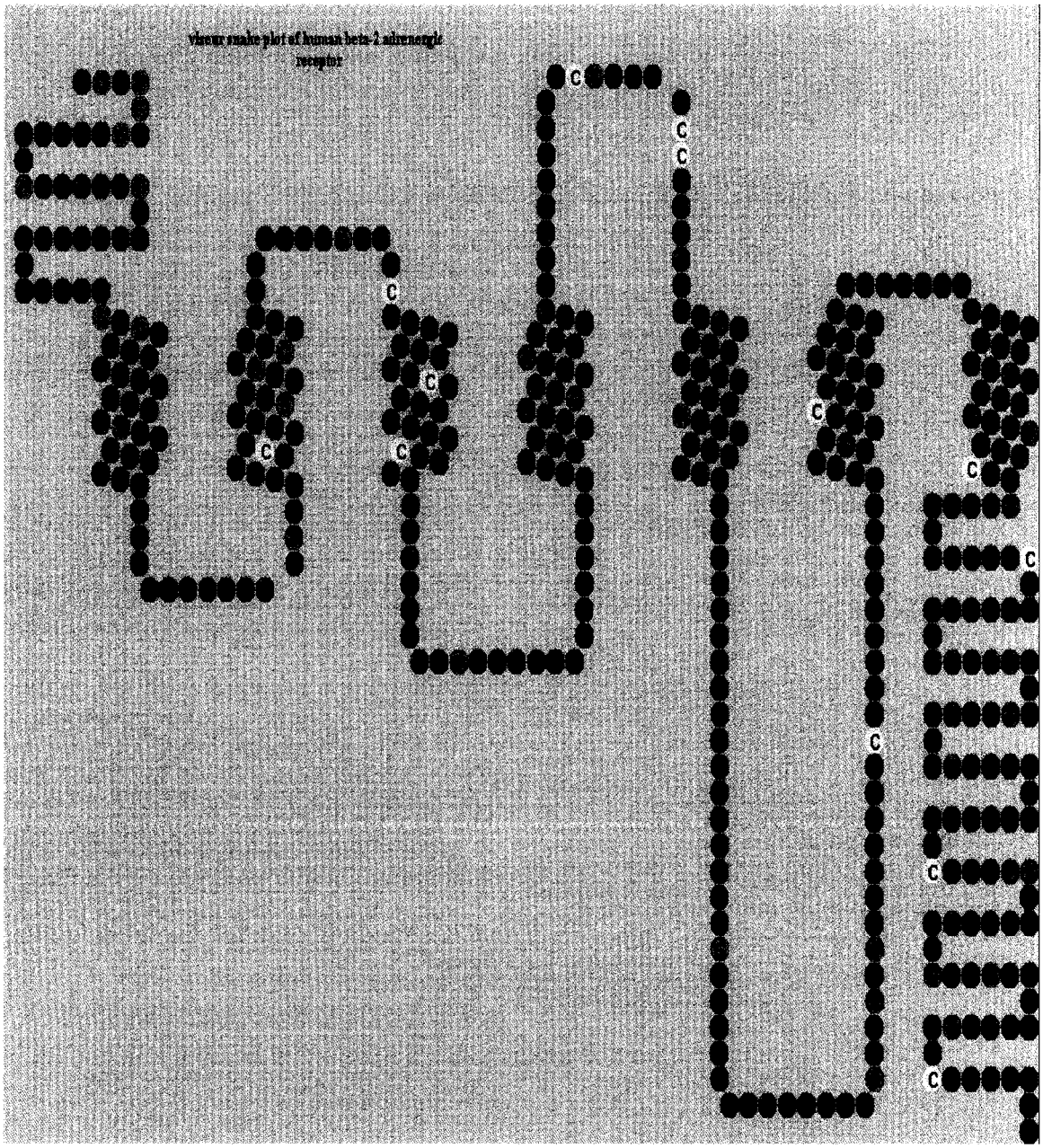


Figure 5. Two-dimensional structure of beta 2-adrenergic receptor. Beta2-AR has a longer fifth loop and a longer C-terminus compared to bovine rhodopsin.

Molecular Mechanisms in Beta 2-Adrenergic Receptor Activation

The specific amino acid residues that are involved in the binding of agonists and antagonists to the β_2-adrenergic receptor have been identified in TM 3, 5, 6, and 7. It has been found from spectroscopy studies that the binding pocket is buried in the receptor molecule (Tota et al., 1990). It was observed that these receptors have a basal activity and could stimulate the G proteins even in the absence of any ligand. Certain mutations in the receptor were able to turn this basal level into a constitutively activated agonist-independent activity (Samama et al., 1993). These mutants were found to be structurally unstable and have an enhanced conformational flexibility that allows them to undergo swift transition between inactive and active states (Gether et al., 1997). Mutation of the conserved aspartic acid residue in TM3 caused a dramatic activation of the receptor. This supported the protonation hypothesis that charge neutralizing mutations such as this one not only activated the receptor but also affected the conformation of the receptor by tilting the TM6 (Rasmussen et al., 1999). The first direct approach used to study conformational changes was carried out using fluorescence spectroscopic studies (Gether et al., 1995). Studies conducted on mutants that were created with cysteines available for labeling suggested movements of TM3 and TM6 (Gether et al., 1997). This data suggests that movement in TM3 and TM 6 is very important in generating an active state in the receptor. Once activated, the receptor may undergo dimerization by interacting between the transmembrane segments (Hebert et al., 1996). The spectroscopic analyses suggest

that agonist induced conformational changes occur slowly while the reversal of these changes occurs very quickly (Gether et al., 1998).

Background on Baculoviral System

The Baculovirus Expression Vector System (BEVS) is a convenient and versatile eukaryotic system for heterologous gene expression. Baculovirus expression provides correct folding of recombinant protein as well as disulfide bond formation, oligomerization, and other important post-translational modifications. Consequently the overexpressed protein exhibits the proper biological activity and function. The Baculovirus Expression Vector System is based on the introduction of a foreign gene into a nonessential region of the viral genome via homologous recombination with a transfer vector containing the cloned gene, an event that occurs in the co-transfected insect cells. The production of foreign protein is then achieved by infection of additional Sf9 insect cell cultures with the resultant recombinant virus for amplification. The Baculovirus Expression Vector System from BD Biosciences Pharmingen employs a modified *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome, BD BaculoGold™ DNA, and an appropriate transfer vector. The baculovirus expression system offers the following advantages over prokaryotic and other eukaryotic systems:

- **High Level of Protein Expression**. Yields of up to 100 mg of protein per 10^9 cells.
- **Post-Translational Modifications**. Including disulfide bond formation, phosphorylation, glycosylation, oligomerization and proper folding.

- **Relevant Cellular Compartmentalization of Proteins**. Secreted, membrane-bound, cytoplasmic or nuclear.
- **Capacity of Large cDNA Inserts.** Accommodates genes up to 15 kb.

PVL1392 Vector

pVL1392 is a baculovirus transfer vector that contains the complete polyhedrin gene locus of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) cloned into a pUC8 vector, but lacks part of polyhedrin gene coding region. A multiple cloning site (MCS) has been inserted 37 nucleotides downstream of the polyhedrin ATG start site that was changed into ATT. This means that the insert beta-2AR inside the vector must have its own starting ATG signal at the 5' end of the gene. The distance between the cloning site and the start ATG site of the insert should not exceed 100 nucleotides to ensure high expression of the protein. This vector was supplied with the baculogold transfection kit from PharMingen. This was the vector that was used to clone the truncated receptors. A diagram of the vector map is shown in Appendix C.

Life Cycle of Baculovirus

The life cycle of the baculovirus is depicted in Figure 6.

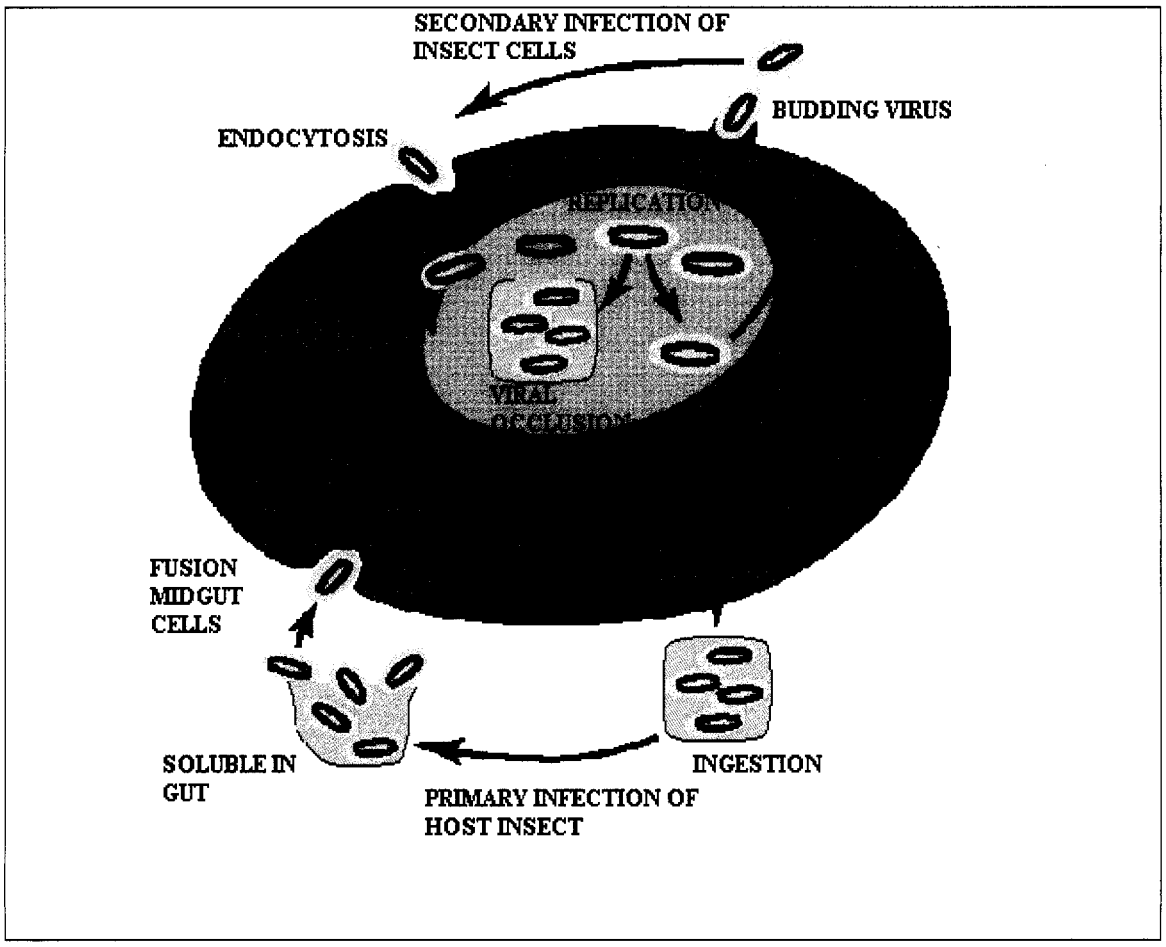


Figure 6. Life cycle of baculovirus inside an insect cell showing replication, budding, infection, and amplification.

Transfection into baculovirus is done by cotransfecing the gene of interest in one of the transfer vectors that has the polyhedrin promoter and the baculo gold or baculo platinum DNA into the Sf9 cells. Homologous recombination occurs and recombinant viral particles bud out from Sf9 cells. The recombinant protein can be produced by

infection. When the viral particles bud out they could be used to cause secondary infection of cells (Figure 7).

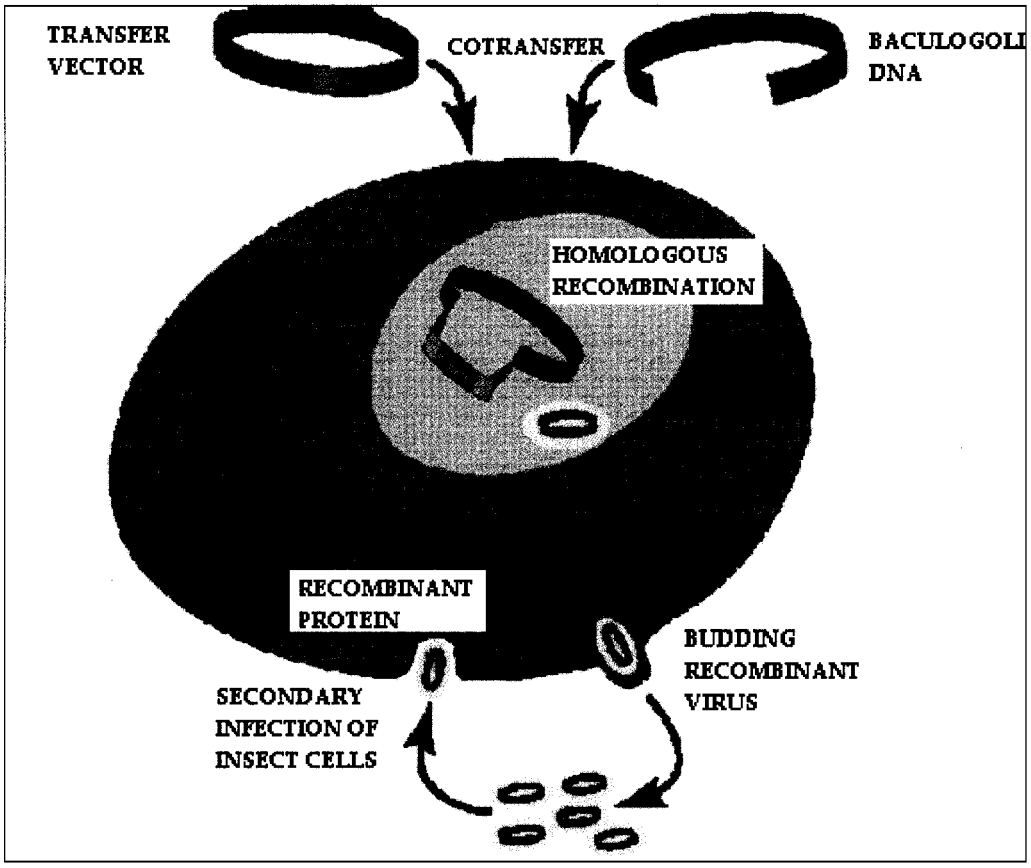


Figure 7. Representation of cotransfection of the gene of PvL vector and the baculo-gold DNA into insect cell.

Background on Membrane Binding Experiments

a) Saturation Binding Experiments

Saturation binding assays are done to quantitate the number of receptors in a tissue, and the affinity of those receptors for the radioligand. The term "saturation" is used to describe the fact that with increasing ligand concentration, the receptors are eventually saturated with ligand. "Affinity" refers to the strength with which the receptor binds to ligand; high affinity means that the ligand binds very tightly to the receptor and low affinity means that it is loosely bound (Motulsky et al., 1992).

In saturation binding experiments one incubates the tissue with various concentrations of radioligand until equilibrium is reached. The total binding at each concentration is determined. The nonspecific binding at each radioligand concentration is also determined by incubating the tissue with excess cold ligand. Specific binding is then obtained by subtracting total from nonspecific binding. The nonspecific binding is linear with increasing ligand concentration, meaning the nonspecific binding sites are in excess and have lower affinity and that makes it difficult to saturate. The specific binding on the other hand gradually saturates (Motulsky et al., 1992).

The saturation binding experiment gives two valuable pieces of information-

"B_{max}"-B max refers to maximal binding which in turn can be used to calculate the total number of receptors.

"K_D"- K_D is the equilibrium dissociation constant of receptors for the radioligand which gives the amount of radioligand that is needed to bind to half of the receptors. The reciprocal of K_D gives the affinity of radioligand, i.e a low K_D means the radioligand has

a higher affinity for the receptor, and a higher K_D means that the radioligand has a lower affinity for the receptor (Motulsky et al., 1992).

b) Competition Binding Experiments

Competition binding experiments test the ability of drugs/hormones to compete with the radioligand for the receptor-binding site. These experiments are done by incubating the radioligand at one particular concentration with different concentrations of unlabelled compound, until equilibrium is reached. The amount of radioligand that binds to the receptor at each of the various concentration of unlabelled compound is then determined. The radiolabelled and the unlabelled compound compete with each other for the receptor binding. As the concentration of unlabelled compound increases, less radioligand binds to the receptor.

The concentration of the competitor that is needed to block 50% of the specific radioligand binding is called the IC50, 50% inhibitory concentration. We can also determine the dissociation constant K_i of the inhibitory agent for the receptor (Motulsky et al., 1992).

The aim of my project was to create truncated constructs of β2-AR that would be more stable than the wild type receptor, and could therefore be used for crystallizing the protein. When compared to bovine rhodopsin, which has been crystallized, the β2-AR has a longer third intracellular loop (V loop). It was proposed that the β2-AR could be stabilized by reducing the size of the fifth loop to mimic the structure of rhodopsin. This loop was considered for truncation for the following reasons-

- This loop is long in β2-AR but short in bovine rhodopsin. Truncating this loop would bring the two portions of the receptor (I-V) and (VI to VII) closer to one another, presumably increasing the stability of the structures. This region has not been shown to be involved in the binding of the ligand and therefore any truncation in this region would not hinder the binding properties of the receptor.
- The long C-terminus of the receptor was also truncated; it was shown to have caused problems during previous crystallization attempts. Also bovine rhodopsin does not possess a very long C-terminus.

To overexpress the truncated receptors, the constructs were expressed in the baculovirus system and the beta adrenergic receptors were purified from the cell membrane of the Sf9 cells. These membranes were then isolated and tested for their binding properties using saturation binding curves, and their pharmaceutical properties using competition assays.

METHODS

Wild Type Beta 2-Adrenergic Receptor

The parental β2-adrenergic receptor used in this study has a tobacco etch virus (TEV) protease site introduced in the N terminus. This β2-adrenergic receptor was constructed by incorporating the TEV site with the amino acid sequence ENLYFQG (Figure 8). Treatment of the protein with TEV protease allows the removal of two glycosylation sites in the N terminus; this process has been shown to increase the stability of the protein. TEV protease cleaves the amino acid sequence ENLYFQG between QG with high specificity (Parks et al., 1994).

This construct also has an N terminal FLAG epitope (IBI, New Haven, CT) that is recognized by the FLAG antibody. The FLAG tag consists of the 8 amino acid sequence DYKDDDDK. The addition of the FLAG tag to the N-terminus of the β2-AR protein allows the receptor to retain complete biological activity, while making it easier to detect (Kobilka, 1995). A simple staining process with the FLAG antibody allows to qualitatively determine the transfection efficiency of the receptor. The cells that are transfected with β2-AR can be detected with the fluorescent anti-FLAG antibody. This parental wild type construct was already available in the lab and was used as a positive control for all my experiments.

Truncation-1-Beta 2-Adrenergic Receptor (TR-1-ß2AR)

The first β2-AR construct referred to as truncation-1- β2-AR, or TR1 was made by removing 18 aminoacids within the third intracellular loop (V loop), and truncating

the C-terminus. The proposed two-dimensional structure of this construct is shown in Figure 9. This construct was kindly provided to me by Yao Zhiping.

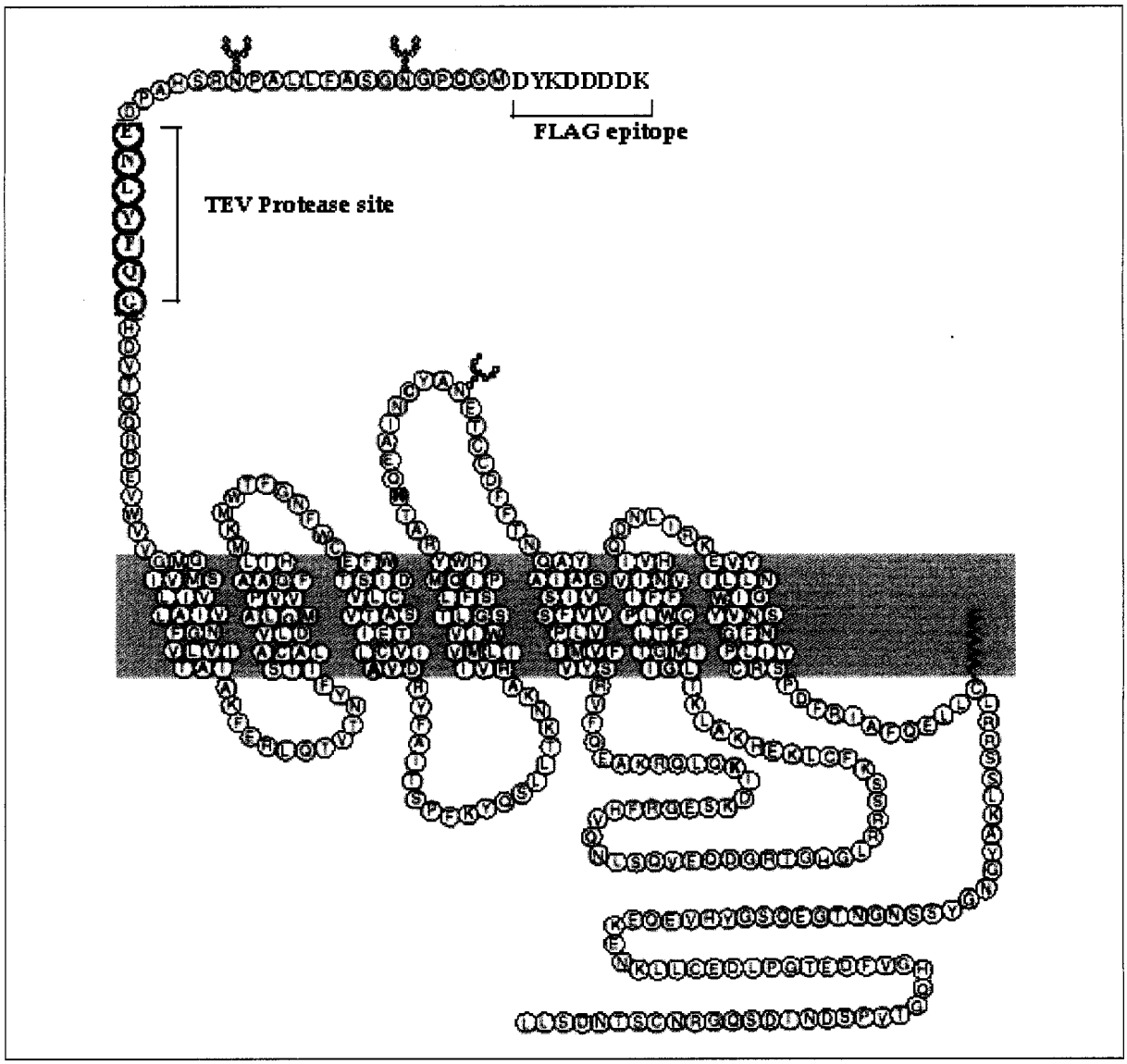


Figure 8. Two-dimensional structure of the parental β2 adrenergic receptor, showing the FLAG epitope at the N-terminus and the TEV site inserted near the N-terminus. The FLAG epitope "DYKDDDDK" is indicated in red color. The TEV protease site "ENLYFQG" is indicated by red color inside black circles near N terminus.

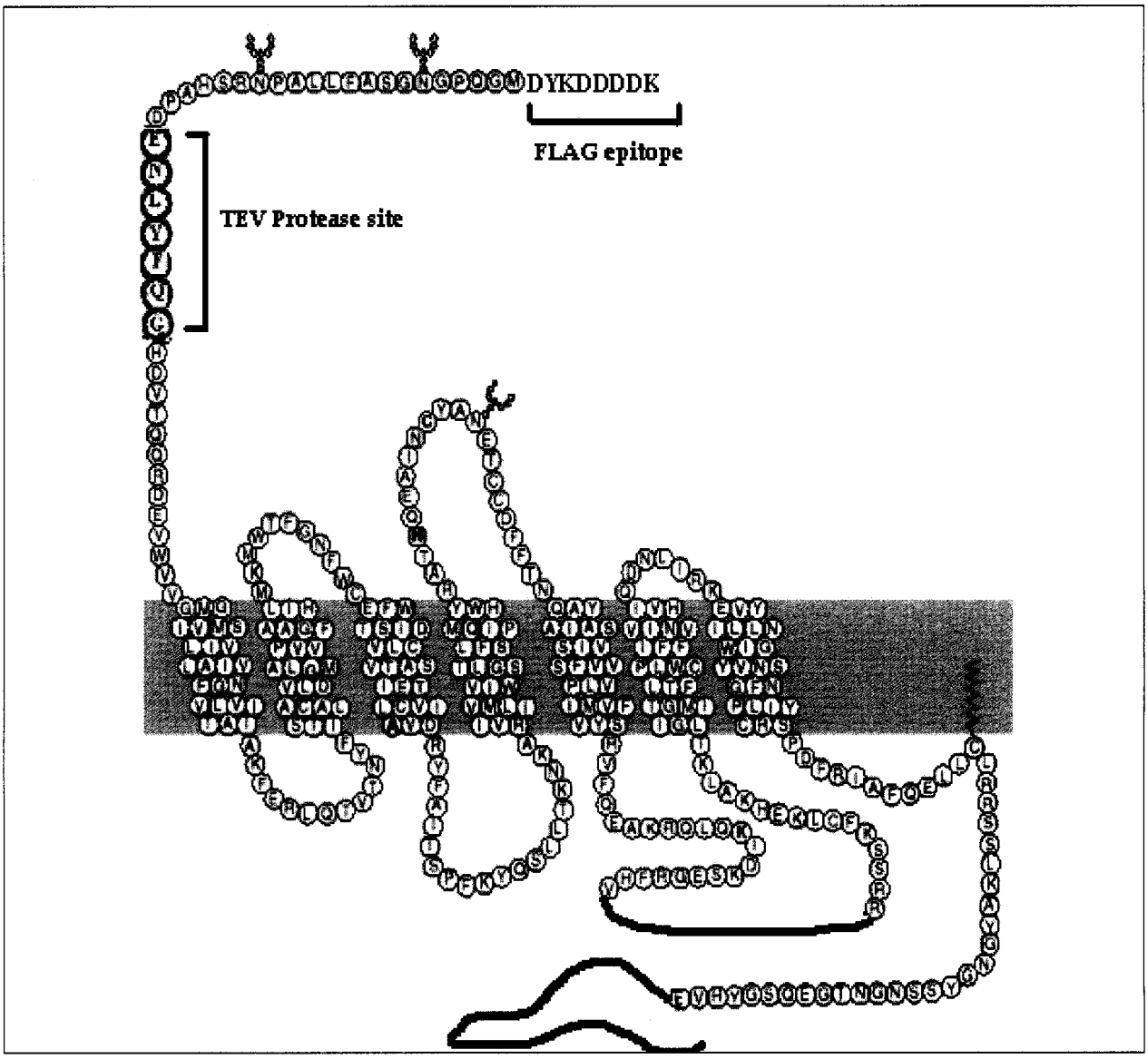


Figure 9. Two-dimensional structure of construct TR1, showing the deletion in the V loop and the truncation in the C-terminus. The deleted amino acids are shown in black line. TR1, like the parental construct has the N-terminal FLAG epitope shown in red color and TEV protease site shown in red color within circles.

Truncation-2-Beta 2-Adrenergic Receptor (TR-2-β2AR)

The second β2-AR construct (referred to as truncation-2- β2-AR, or TR2) was created by removing 25 aminoacids within the third intracellular loop, and truncating the C-terminus. TR1 and TR2 are nearly identical constructs; the only difference is that the V loop in TR2 is 7 aminoacids shorter than that in TR1. The V-loop was further shortened in TR2 to more closely mimic the structure of bovine rhodopsin, the only GPCR that has been crystallized thus far. The two-dimensional structure of TR-2 is shown in Figure 10.

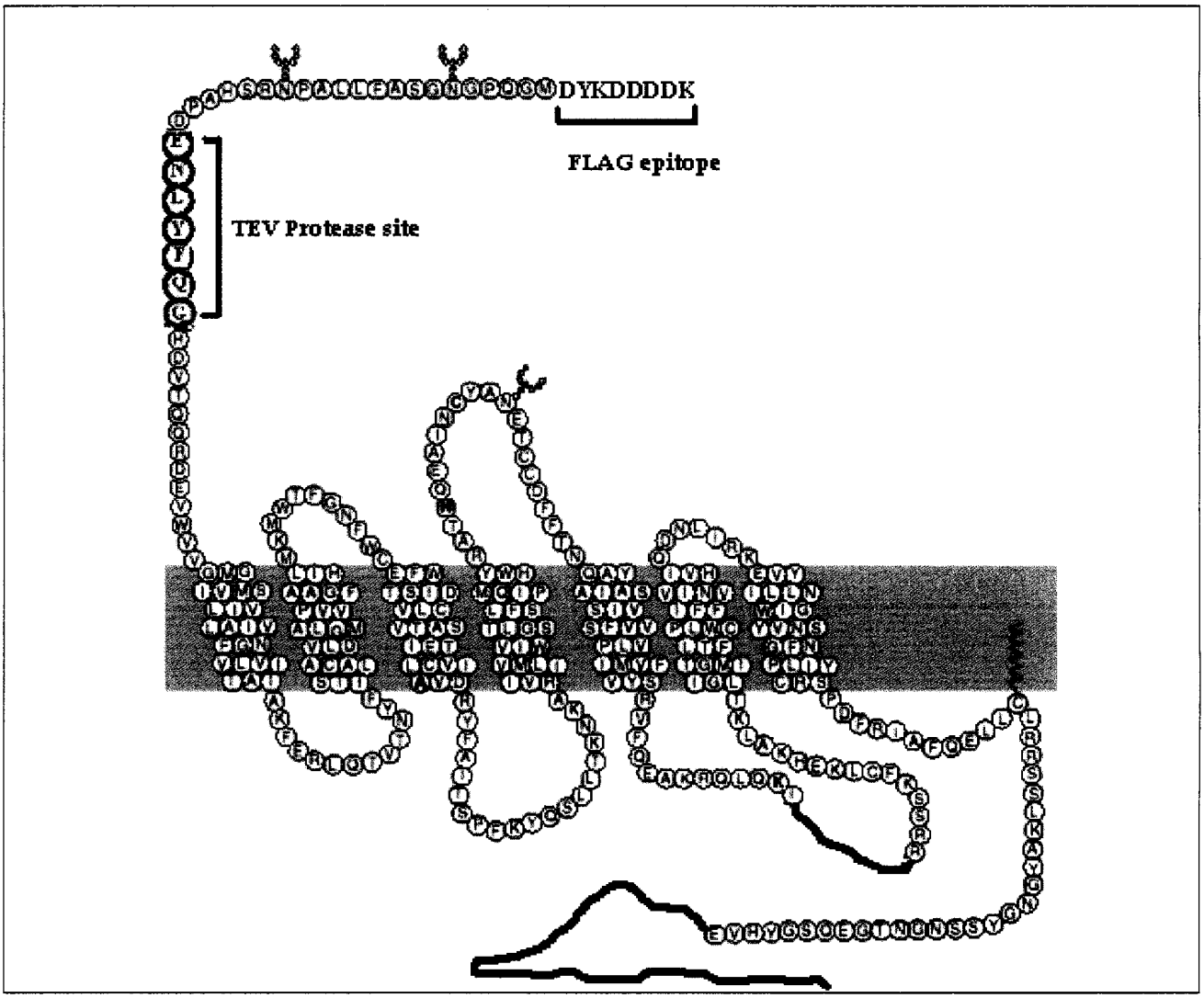


Figure 10. Two-dimensional structure of construct TR-2 showing the deletion in the V loop and the truncation of the C-terminus. The deleted amino acids are represented by black line. Like TR1, TR2 has the N-terminal FLAG epitope and TEV protease site.

Multiple sequence alignments of TR-1, TR-2, and the parental β2-AR are shown in appendix B.

PCR Amplification of Wild Type Beta-2 AR

The parental β2-AR in Pvl vector was amplified using two sets of primer. The forward primer was available in the lab and contained the PstI restriction site. It was a 24mer oligo whose sequence is shown below-

GTCATCACAGCCATTGCCAAGTTC.

The reverse primer was designed using the software program Primer-3. A portion of this primer was designed such that it showed some mismatch to the parental beta-2 AR sequence (shown in red), which was introduced to create a BglII restriction site. This primer was a 30mer whose sequence is shown below. The underlined portion of the oligo is the BglII restriction site. The red colored sequence in the oligo shows the mismatched nucleotide.

AGCTAGATCTAATCTTCTGGAGCTGCCTTT

The template DNA that was used for amplification was the TR-1 truncation construct in PGEM vector at a concentration of 100ng. The forward and reverse primers were the PstI restriction site containing primer and BglII restriction site containing primer at the concentration of 10pmoles. Pfu polymerase from stratagene was used for extension at a concentration of 1000units/ul reaction mixture. PCR reaction was conducted by denaturing the template at 95 degrees for a minute, followed by additional denaturation for 35 seconds. The primer was annealed at 58 degrees for 35 seconds followed by

extension at 68 degrees for two minutes. The cycles were repeated 28 times followed by a final extension for 12 minutes. The size of the PCR fragment was confirmed by running the PCR product on a 1% agarose gel. The gel scan is shown in the results section. The PCR products were excised from the gel and purified using qiagen`s QIA gel purification kit.

Cloning Amplified PCR Product into PGEM Vector

The amplified and purified PCR product that had the PstI and BglII site was transferred into PGEM vector. A schematic diagram of the entire cloning process is shown in Figure 10. The PGEM vector containing the parental β2-AR (TEV- β2AR) and the amplified PCR product was digested with KpnI and BglII at 37 degrees for two hours. To avoid recyclization the vector arms were treated with shrimp alkaline phosphatase at 37 degrees for one hour. The shrimp alkaline phosphatase was deactivated by heat treatment at 65 degrees for 15 minutes. The vector arms were run on an agarose gel, excised, extracted, and purified from the gel using qiagen`s gel purification kit.

The vector arms and the PCR products with sticky ends were ligated together using T4 DNA ligase in presence of ligation buffer (660 mM Tris-HCl (Ph7.6), 66mM $MgCI_2$, 100mM DTT, 1 mM ATP) at 14 degrees for an hour. The ligated mix was then transformed into DH10B cells by incubating the competent cells and the ligation mix on ice for two minutes followed by a heat shock treatment at 42 degrees for 45 seconds. The transformed competent cells were plated on LB agar plates containing ampicillin and allowed to grow overnight at 37 degrees. The colonies were hand picked and grown in luria broth media containing ampicillin at 37 degrees overnight. Miniprep was done

using the quiagen miniprep kit to extract the plasmid DNA. The concentration of DNA was determined by measuring absorbance at 260nm in a UV spectrophotometer and the purity of DNA was determined by the ratio of OD260/OD280. A restriction mapping was done to determine the positive clone.

C-Terminus Truncation

The positive clone that was obtained as described earlier had a short third intracellular loop and had the long C-terminus. To shorten the C-terminus, this construct was treated with EcoRV and BamH1. This portion of C-Terminus got chopped away by the restriction enzymes and a linker was ligated with the construct. The sequence of the linker is shown.

EcoRV-BamH1-5`

ATCACCATCATCACCATCACTAGG

EcoRV-BamH1-3`

GATCCCTAGTGATGGTGATGATGGTGAT

Ligation was done using T4 DNA ligase at 14 degrees for an hour in the presence of the ligation buffer that contained Mg^{2+} and ATP.

Subcloning into PVL Vector

The Pvl vector and the TR-2 PGEM were digested with NcoI and BamH1 at 37 degrees for two hours. The vector arms were dephosphorylated using shrimp alkaline phosphatase at 37 degrees for an hour. The phosphatase enzyme was deactivated by heat treatment at 65 degrees for 15 minutes. The PvL vector arms and the TR-2 that had sticky ends were ligated using T4 DNA ligase at 14 degrees for an hour in the presence of

cofactors Mg^{2+} and ATP. The ligated mixture was transformed into DH10B cells by mixing the ligation mix and the competent bacterial cells. This mix was incubated on ice for two minutes followed by a heat shock treatment at 42 degrees for 45 minutes. They were plated on LB- agarose plates containing ampicillin, overnight at 37 degrees. The colonies were hand picked and grown in luria broth media containing ampicillin at 37 degrees overnight. A miniprep was done using the Quiagen miniprep kit to extract the plasmid DNA. The concentration of DNA was determined by measuring absorbance at 260nm in a UV spectrophotometer and the purity of DNA was determined by the ratio of OD260/OD280. A restriction mapping was done to determine the positive clone. The purified DNA was sent out for sequencing using the anti HpaI and the PstI sequencing primers.

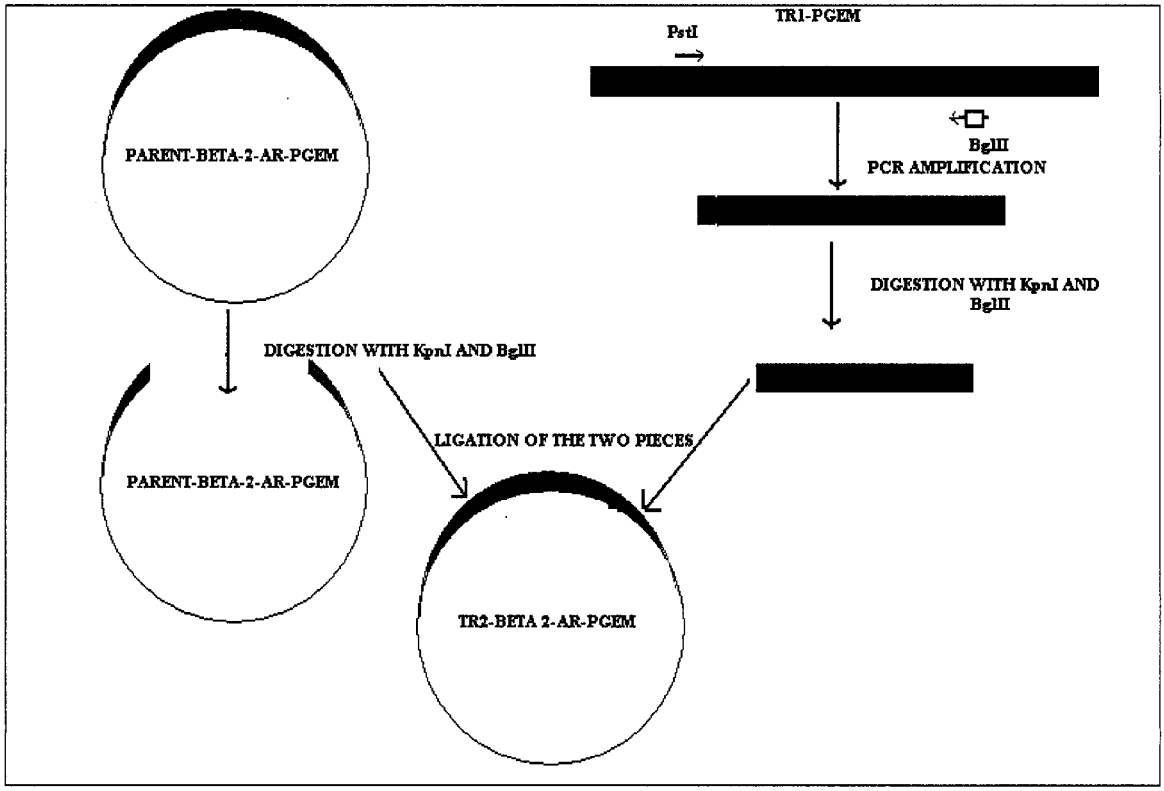


Figure 11. Schematic diagram of the steps involved in the process of cloning construct TR2-β2-AR.

Expressing the Truncated Receptors in Insect Cells

The insect cell line Sf9, which was originally derived from the ovarian tissues of *Spodoptera frugiperda* larva, was used to express the receptors. Healthy Sf9 cells when grown in TNM FH media attach to the bottom of the flask and grow as monolayer. They can also grow as suspension cells in spinner cultures. They double every 18-24 hours. Sf9`s were grown in 27 degrees in suspension culture in TNM FH media supplemented with antibiotic 50ug/ml gentamicin sulfate (Invitrogen) and 2.5ug/ml of Amphotericin B (fungizone) (Invitrogen).

Fifty to eighty percent confluent Sf9 cells ($5X10^6$ cells) were co-transfected with 5 ug of β2-AR-TR2-Pvl DNA and Baculo-gold DNA (PharMingen) using the Insectin Plus transfection kit (Invitrogen). The β2-AR-TR2 in Pvl vector (5ug) was mixed with 0.5ug of baculo gold DNA in a microfuge tube for 5 minutes. The insectin transfection reagent was added to the mix and incubated for fifteen minutes. This mixture was added drop wise to the flask containing insect cells and incubated at 27 degrees for four hours. After four hours fresh media was added to the flask and the cells were incubated in a humidified environment at 27 degrees. After two days 2ml of fresh media was added. After 5-7 days the media containing the virus was pipeted and stored at 4 degrees usually for less than two weeks in the dark. This viral supernatant was called P1.

The expression of the receptor was visually confirmed under a fluorescent microscope. To the cells attached to the bottom of the flask was added 1ml of PBS-Ca^{2+} and 2.5ul of Alexa tagged M1 antibody. When incubated for 20 minutes at room temperature, the M1 antibody binds to the FLAG epitope and was seen as bright membranes under the fluorescent microscope.

Amplification of the Virus

The viral stock from P1(500ul) was used to infect 50ml of Sf9 cells at $5X10^6$ cells/ml in the shaker at 28 degrees for 4 days. The receptor expression was checked using the Alexa tagged M1 antibody. The viral supernatant was collected after centrifuging the cells at 4000 rpm for 10 minutes in a SW30 rotor using a Beckmann centrifuge (radius $_{min}$ 75.3mm; radius $_{max}$ 123mm; k-factor 7768.7). The supernatant was stored at 4 degrees in the dark. This was the viral stock P2 that was used to infect the Sf9

cells to make membranes. The insect cells were infected for 48 hours and the infected cells were harvested to make membranes.

Preparation of Membranes From the Cells

Membrane preparation was done for all the three constructs-parental type TEV, TR-1 and TR-2. The membranes that were prepared was a mixture of Sf9 plasma membranes expressing the β2-AR, mitochondrial membranes, nuclear membranes, golgi membranes, and the endoplasmic reticulum membranes.

Fifty mililiters of cells at $5X10^6$ cells/ml were pelleted down after infection by spinning them at 500g for 5 minutes at 4 degrees. The pellet was washed twice with phosphate buffered saline (PBS). The pellet was lysed using 10mM Tris/HCl-pH 7.4; 1mM EDTA; 0.2M PMSF; in the presence of 10ug/ml Leupeptin and 10ug/ml Benzamidine as protease inhibitors. The lysed contents were homogenized using a glass 25ml dounce homogenizer fitted in with a type "A" pestle for 25 strokes. The dounced contents were then spun again at 500g for 10 minutes at 4 degrees to remove nuclear debris and unbroken cells. The supernatant was collected in Sorvall centrifuge tubes. The tubes were spun in a sorvall centrifuge at 11,500g for 30 minutes. The resulting pellet was resuspended in 20 ml of 10mM Tris-HCl, pH 7.5 in the presence of protease inhibitors and rescentrifuged. The pellet was finally resuspended in 10ml of binding buffer (75mM Tris-HCl, pH 7.6). Protein estimation was done as described below and the aliquots of membranes were stored at –80 degrees.

Protein Estimation using Bio-Rad`s DC Protein Assay Kit

The Bio-Rad *DC* (detergent compatible) protein assay is a colorimetric assay for determining protein concentration following detergent solubilization. The assay here is designed for use in microtiter plates. From the stock concentration of Bovine serum albumin (BSA) (2 mg/ml), a series of dilutions of BSA (0.4mg/ml, 0.8mg/ml; 1.2mg/ml; 1.6mg/ml; 2mg/ml) was made using the binding buffer (75mM Tris-HCl, pH 7.5). The receptor membranes whose protein concentration was to be determined was diluted 1:10 using binding buffer. Reagent S was mixed with reagent A (20ul of S to 940ul of B respectively). The standards and samples were transferred into a clean 96 well microtiter plate. The S:A mixture (20ul) was added into each well followed by 200ul of reagent B was added into each well. The plate was incubated for at least 15 minutes at room temperature and the absorbance was recorded at 595 nm using a microplate spectrophotometer. A standard curve was plotted using the absorbance values of BSA standards and the concentration of the receptor membranes was determined from the graph by extrapolation.

Determination of Specific Activity of the Membranes

Specific activity of receptor is defined by the ability of the receptor to bind to its ligand at saturable concentrations of substrate/ligand concentration per mg of protein. The specific activity of adrenergic receptor was determined by subtracting the total binding represented by cpm of 10nM DHA bound from non-specific binding represented by cpm of 10nM DHA bound in the presence of 10uM alprenolol per mg of total protein. The parental beta-2 adrenergic receptor and the truncated receptors were incubated with

10nM antagonist [^{3}H] dihydroalprenolol (DHA) to determine total binding (set in triplicates). The non-specific binding was determined by incubating the protein with 10nM [^{3}H] DHA supplemented with 10uM cold alprenolol. The specific binding was calculated by subtracting the total binding from the non-specific binding. The specific activity values are shown in the results section.

Western Blotting

Immunoblot analysis of the parental type β2-AR and the truncated β2-AR mutants was done using the membranes that were prepared from the transfected Sf9 insect cells as described previously. Seventy femetograms of protein was loaded into each well. The protein samples were mixed with 4X sample loading buffer containing SDS. The samples were not heated prior to loading into the polyacrylamide gel as this was a seven transmembrane protein. Samples were run on a 10% SDS-polyacrylamide discontinuous electrophoresis gel. Electrophoresed proteins were transferred to nitrocellulose membrane and blotted overnight with 5% dry milk, 2% horse serum, 20mM Tris (pH7.6), 137mM NaCl, and 0.05% Tween-20. The β2 receptors were labeled with M1 antibody that recognizes the FLAG epitope at the N-terminus (IBI, New Haven, CT) at 1:500 in blocking solution for one hour at room temperature. The nitrocellulose membrane was rinsed three times with PBS containing 0.05%Tween and labeled with goat anti-mouse secondary antibody conjugated with horseradish peroxidase at 1:1000 (Amersham Pharmacia Biotech) in blocking buffer. Following five rinses of the nitrocellulose membrane the membrane was treated with ECL detection kit for protein (Amersham Pharmacia Biotech).

Membrane Binding Assays

a) Saturation Binding Experiments

Antagonist binding assays were done with the parental β2-AR, TR1- β2-AR, and TR2- β2-AR expressed on the Sf9 membranes. Thirty-five milligrams of membrane protein was resuspended in 500ul of binding buffer (75mM Tris-HCl, pH7.5) supplemented with various concentrations of [^{3}H] dihydroalprenolol, ranging from 100pM to 20nM. This assay defined the amount of total membrane binding as a function of [^{3}H] dihydroalprenolol concentration. Non-specific binding was determined in the presence of 10uM unlabelled alprenolol. The assay reaction was performed at room temperature for an hour of incubation in a shaker at 250 rpm. The time for equilibrium was determined to be one hour from previous experiments conducted in the lab. The unbound ligand was removed by filtration using a filter harvester. The membranes were trapped into glass fiber filters by washing with binding buffer. Four washes were performed and complete removal of unbound ligand was presumed. The amount of radioactivity attached to the membranes on the filters was used as a measure of number of receptors present. The filter discs were transferred into vials to which was added the scintillation cocktail. The tubes were counted in a scintillation counter. Specific binding was determined by subtracting out non-specific binding from the total binding. Data was analyzed using the nonlinear regression analysis with Prism program (GraphPad Software, SanDiego, CA).

b) Competition Binding Experiments

Agonist binding assays were done with the parental β2-AR, TR1-β2-AR, and TR2-β2-AR receptor expressed on the Sf9 membranes. Competition binding assays were done on these membranes using 1nM [^{3}H] dihydroalprenolol in the presence of increasing concentrations of isoproterenol, an agonist. The concentrations of isoproterenol employed ranged from 0.01uM to 200uM. Thirty five milligrams of insect membrane protein was used for each of the three constructs. At each concentration of isoproterenol, binding was measured in a set of triplicates. In triplicate tubes total binding was measured in the absence of isoproterenol and alprenelol. In triplicates tubes non-specific binding was measured in presence of 10uM alprenolol. The assay reaction was performed at room temperature for an hour of incubation in a shaker at 250 rpm. The unbound radioligand was removed by filtration using a filter harvester and the membranes were trapped into glass fiber filters by washing with binding buffer. The amount of radioactivity attached to the membranes on the filters was used as a measure of number of receptors present. The filter discs were transferred into vials to which was added the scintillation cocktail. The filters were read in a scintillation counter. The specific binding was calculated by subtracting the nonspecific binding from total binding and this was used to normalize the competition data in terms of percent binding at a particular isoproterenol concentration. The data was analyzed using the nonlinear regression analysis using the prism program to fit for one site competition.

Studying the expression pattern of the constructs using immunoflorescence

Sf9 cells were infected using the viral supernatant P2 that was collected earlier for the

parental type and the truncated β2-AR receptors. The flasks were incubated overnight at 37 degrees in an incubator. Under the microscope the transfected cells looked sick with scalloped edges compared to the control cells. Five hundred microliters of infected cells was transferred into a chamber slide that was coated with poly-D-lysine to which 250ul of fresh media was added to the chambers and incubated overnight at 37 degrees.

Following the incubation the old media was removed and the infected cells on the slide were washed very gently with PBS supplemented with 1mg/ml Calcium (Ca^{2+}) and 1mg/ml Magnesium (Mg^{2+}). The cells were fixed with 4% paraformaldehyde (made in PBS containing calcium) at room temperature for 5 minutes. Following fixation cells were rinsed 4 times with PBS.

The paraformaldehyde fixed cells on one half of the chamber slide were treated with blocking buffer (5% dry milk, 50mM HEPES, pH 7.4 in PBS) to reduce nonspecific antibody binding. The cells on the other half of the chamber slide were treated with 0.1% Saponin to permeabilize cells. Saponin acts by solubilizing the cholesterol layer on the membranes and thereby making the cells permeable. The slides were incubated for 30 minutes at room temperature.

The primary antibody used for labeling the β2-adrenergic receptors was the M1 antibody that was conjugated with the flurophore alexa. This antibody recognizes the FLAG epitope at the N-Terminus. DAPI (Molecular Probes) was used at 1:500 as a control stain to visualize the nuclear compartment for all the three constructs. All antibody applications of the fixed cells were done in the presence of blocking buffer for 1 hour at room temperature. For the permeabilized samples the primary antibody was

made in blocking agent that contained Saponin. The antibodies were used at 1:500 for M1 and DAPI respectively. Following the incubation the slides were rinsed with PBS four times. Saponin was used in PBS to rinse the permebealized samples. Pictures were taken using the Olympus confocal fluorescent microscope under the guidance of Dr Carl Hurt. Adobe photoshop was used to edit and format images.

RESULTS

Cloning of Truncation 2-β2 Adrenergic Receptor

The PCR amplification of the β2-AR using site directed mutagenesis primer containing BglII restriction site gave a product of 539 base pairs. The PCR products were run on a 1% agarose gel. The gel picture is shown in Figure 12.

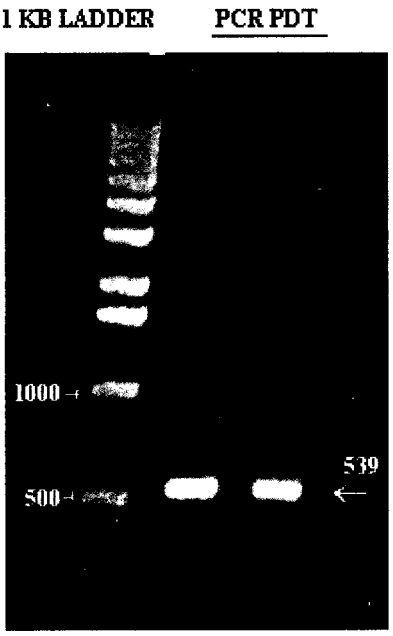


Figure 12. Ethidium bromide stained 1% agarose gel showing the PCR product. The PCR product shows a band around 539 bases which is the expected size range.

The PCR products were purified using Qiagen`s QIA quick gel purification kit. The purified PCR products and the parental β2-AR in pGEM vector were digested using KpnI and BglII. After ligation and transformation of this plasmid into DH10B cells the colonies were selected for miniprep of DNA. A restriction digest was set up using NcoI and BglII to identify positive clone. The samples were run on a 1% agarose gel that is shown in Figure 13.

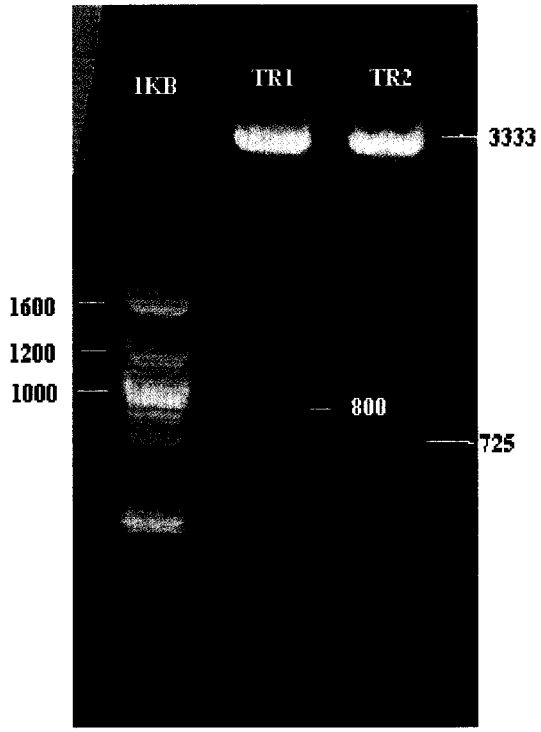


Figure 13. An ethidium bromide stained 1% agarose gel showing restriction digest products with NcoI and BglII. The size fragments expected are 3333bp fragment and 800bp for TR2 and 3333bp fragment and 725bp fragment for TR2.

The positive clone was sent for sequencing at the Stanford PAN facility. No mutations were identified in the positive clone. The beta-2 adrenergic receptor gene was completely sequenced using sequencing primers available in the lab.

Protein Expression

Protein estimation for the three constructs suggests that the expression of the receptors decreases with the degree of truncation. The total protein was determined using the DC protein assay kit (Bio-Rad, Hercules, CA).

Table 3. Total protein determination done for the three constructs using the DC protein assay kit

Constructs	Total protein in ug/ul
Parental-β2-AR	5.5
TR1-β2-AR	4.0
TR2-β2-AR	3.8

Somewhat different results were observed from the western blot. The blot shows a very low level of truncated receptor-2 compared to truncated receptor1, a difference far greater than that seen in the Bradford assay. In contrast the relative differences between the wild type and TR1 seem to correspond closely with the Bradford data.

The results from the immunofluorescence experiments confirm that the expression of TR2 was lower than TR1 and the expression of TR1 was lower when compared to the parental β2-AR. Also the immunofluorescence studies suggest that some of the TR2 receptors and TR1 receptors were confined intracellularly as a pool.

Although most of the receptors were seen on the surface of cell some of these mutant receptors where not transported to the plasma membranes. The immunofluorescence images are shown in Appendix D.

Determination of the specific activity of protein suggested that it decreases with the degree of truncation. Specific activity of receptor is defined as number of pmoles of DHA bound per mg of total protein. TR-2 had a lower specific activity in comparison to TR-1 and TR-1 had a lower specific activity when compared to the parental type. The specific activity of all the three constructs is shown in the table.

Table 4. The specific activity of the three constructs in pmol/mg of protein determined by binding assay.

Constructs	Specific activity in pmol/mg of protein
Parental-β2-AR	3.2
TR1-β2-AR	1.6
TR2-β2-AR	0.5

Binding Experiments

Saturation binding experiments showed that the affinity of the β2-AR increased nearly 3.5 times with the first truncation. Also these experiments showed that further truncating the loop lead to a decrease in affinity. A comparison of KD value for the three constructs is shown below in the table.

Table 5. Summary table showing the affinity of the three constructs and the Bmax in pmol/mg of protein determined by saturation binding experiments

Constructs	KD in nM	Bmax in pmol/mg
Parental-β2-AR	1.763±0.17	5
TR1-β2-AR	0.5154±0.059	0.62
TR2-β2-AR	5.163±1.817	0.22

The loop truncation in TR1 causes a decreased expression and/or maturation and/or translocation to the plasma membrane (giving rise to an 8-fold decrease in total number of receptors expressed/cell), while simultaneously resulting in a nearly 3.5 fold increase in ligand affinity.

The saturation binding data was analyzed using the prism from Graph pad software. Scatchard plots and semi log plots for the three constructs are shown in the Appendix G. Scatchard plots linearize the data by plotting bound versus bound/free. Since most data points in the graph below are crunched at lower concentrations, a graph plotted with log concentration and specific binding spreads the data evenly.

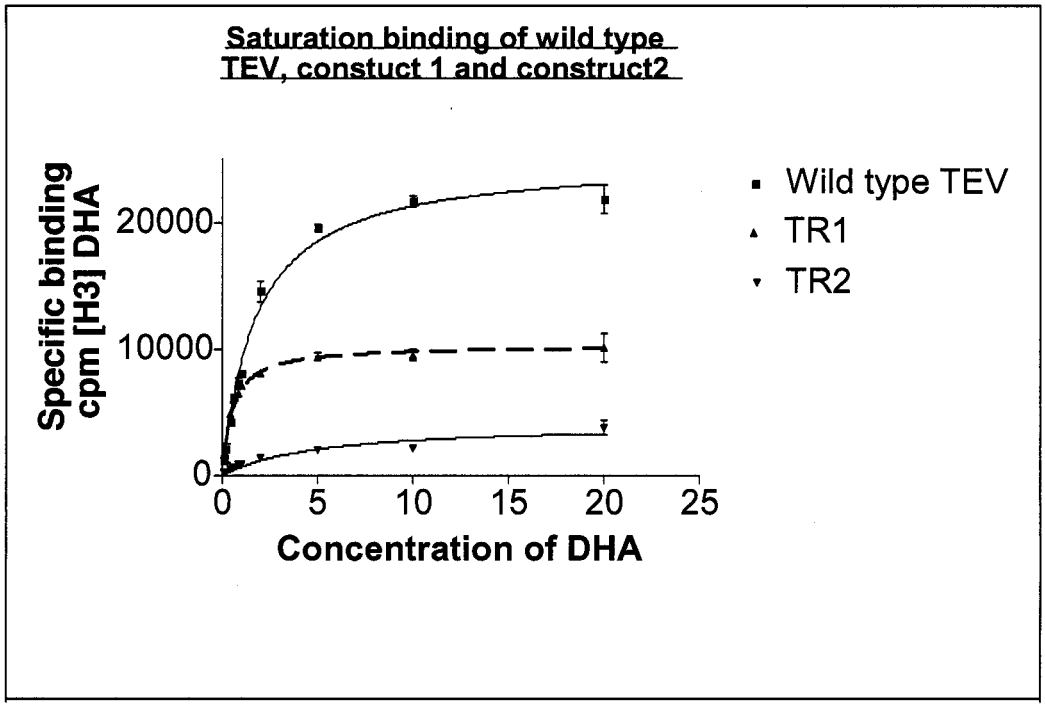


Figure 14. Graph depicting the saturation binding curves for three constructs-Parental β2-AR, TR1, and TR2. The Bmax value decreases as the extent of truncation increases. Each curve was constructed from three data points.

Competitive binding experiments measure the binding of a single concentration of labeled ligand, DHA in the presence of various concentrations of unlabeled ligand, here isoproterenol. The top of the curve is a plateau at a value equal to radioligand binding in the absence of the competing unlabeled drug. The bottom of the curve is a plateau equal

to nonspecific binding. The concentration of unlabeled drug that produces radioligand binding half way between the upper and lower plateaus is called the EC50 (effective concentration 50%). Competition binding experiments suggests that the EC50 value for the three constructs do not change much. A comparison of Ki and EC50 for the three constructs is shown below in the Table 6. The Ki is the concentration of the competing ligand, here isoproterenol that will bind to half the binding sites at equilibrium, in the absence of radioligand or other competitors. If the Ki is low, the affinity of the receptor for the inhibitor is high.

Table 6. Summary table showing the pharmacological properties of the three constructs determined by competition binding assays

Constructs	$EC_{50(nM)}$	KI(nM)	Hill Slope
Parental-β2-AR	170	5900	-0.84±0.21
TR1-β2-AR	270	9438	-1.2±0.2
TR2-β2-AR	174	6889	-0.7±0.2

A hill coefficient of 1 indicates completely independent binding of ligand to its receptor, regardless of how many additional ligands are already bound. Numbers greater than one indicate positive cooperativity, while numbers less than one indicate negative cooperativity. From this data it looks like ALP binds to beta-2 receptor following the priniciples of negaive cooperativity.

The competition binding data was analyzed using Prism from the Graph pad software.

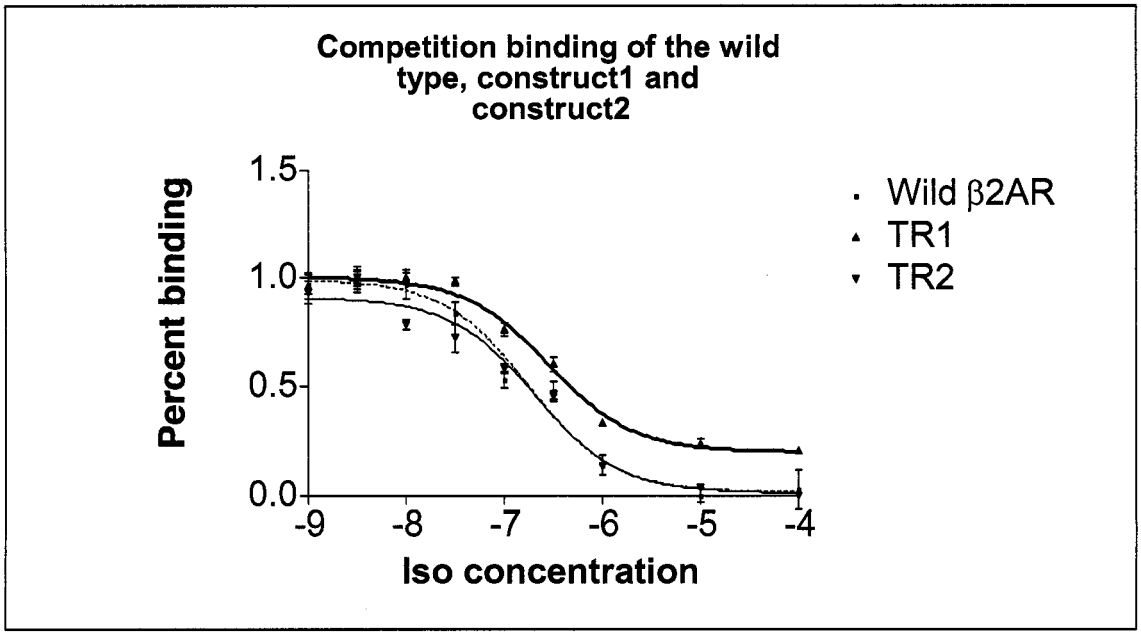


Figure 15. Graph depicting the competition binding curves for three constructs-Parental β2-AR, TR1, and TR2. The IC50 value of the curve is very similar to the parental type. The pharmacological properties are unaffected.

DISCUSSION

This section summarizes the technical problems that were encountered during the course of this project; interprets the data obtained from the binding and immunofluorescence experiments and discusses the questions that remain to be answered in the future.

Problems Encountered

a) Cloning of TR2

Cloning of the truncated receptor was the longest part of the project. This construct was very difficult to clone. Initially it was decided that the cloning of the second construct, TR-2 would be done by using the first truncation, TR-1 as the starting point. This turned out to be a wrong choice as the first and the second truncation constructs only differed by 18 bases. The positive clones were not visualized by agarose gel electrophoresis and sequencing each of the clones was not feasible. So the wild type construct was used as the starting point. This was relatively easy as the positive clones could be identified by restriction mapping and by running the fragments in an agarose gel. The positive clones were sent out for sequencing. All except one clone had a single base pair mutation that led to a stop codon right in the middle of the sequence.

b) Expression of TR-2 in Sf9 Cells

It was difficult to visualize the size difference between the two truncated constructs by western blotting as the difference between the two constructs was only six amino acids. A band that was shorter than the wild type was detected but the exact size

was not resolvable through the polyacrylamide gel electrophoresis. There were multiple minor bands that were seen in the western blot. These bands may be seen due to receptor aggregation and or differential glycosylation. One could try a higher strength detergent during SDS-PAGE and deglycosylate to reduce glycosylation. This was beyond the scope of this project.

What does the result mean?

a) Saturation binding experiments

The K_D value of the ligand/antagonist binding to the receptor in case of parental type was 1.763± 0.17nM. This agrees with the results shown in the literature, 2±1.3nM (Seifert et al,1998). The first truncation TR1 leads to an increase in the affinity. The K_D value was found to be 0.5154±0.05nM. As discussed earlier the affinity of the receptor is reciprocal of K_D value. The affinity for the ligand to the receptor increased about 3.5 fold in TR-1 when compared to the wild type beta-2 adrenergic receptor.

The second truncation TR-2 had a KD value of 5.163±1.8nM. This means that the affinity for the ligand/antagonist for the receptor decreased about three fold compared to the wild type receptor.

This shift in binding affinity could be explained by proposing that in the first truncation the third intracellular loop (V interloop) was shortened such that it brought the two portions on either side of the truncation, domains (I-V) and (VI-VII) just close enough such that the receptor was better able to interact and bind with the ligand. In the second truncation the affinity of the ligand to bind to its receptor decreased suggesting that the segment in the third intracellular loop was shortened so much that the two

portions (I-V) and (VI-VII) had come very close and were unable to bind and interact with the ligand as effectively as the parental type or the TR1. This could be due to steric hinderance between these two portions; domains I-V and VI-VII.

The affinity for the antagonist binding increased almost 10 fold for TR-1 when compared to the truncation, TR-2 (Table.5). This would suggest we could have gone far enough in shortening the third intracellular loop (V loop) and that any further truncation of the loop would lead to very low binding efficiency. The two portions on either side of the truncation in TR-2 may have changed in conformation due to steric interference such that they are unable to interact with the ligand as effectively as the TR-1. A schematic diagram depicting the different degrees of truncations is shown in Figure 16. This gives an idea of the structural constraints introduced with the truncations.

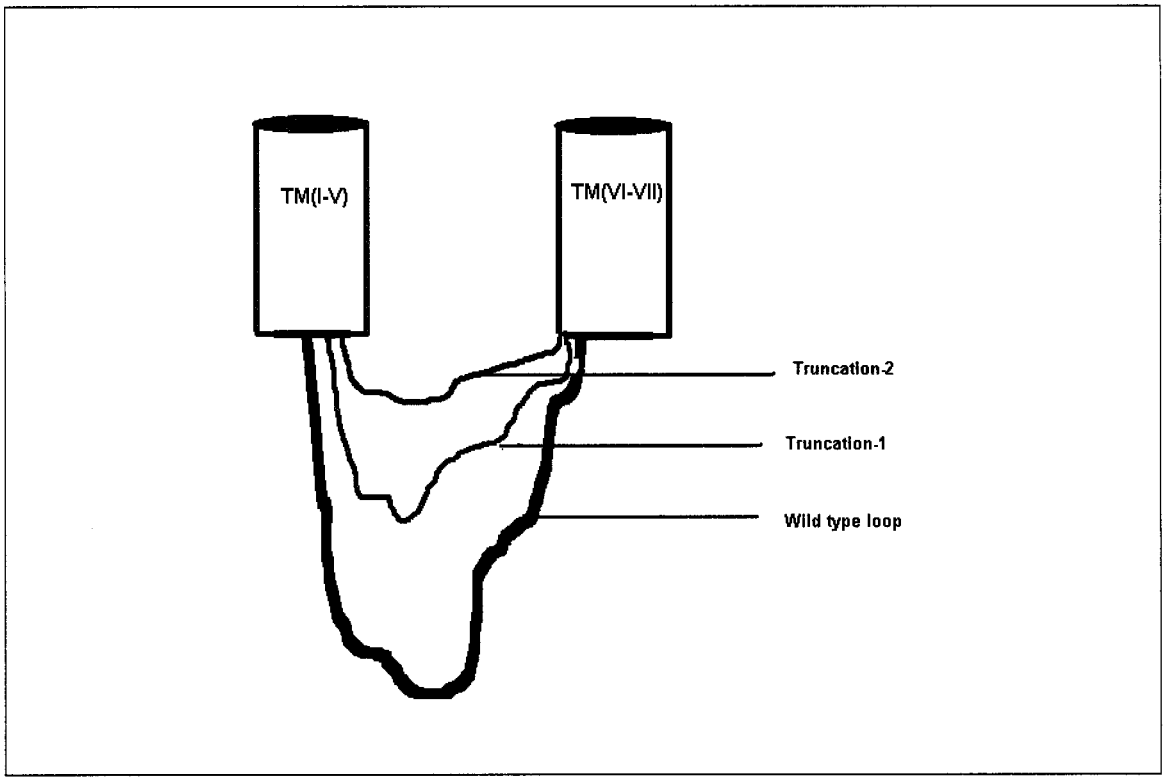


Figure 16. A schematic diagram showing different lengths of III interloop (V loop) that could affect free movement of the domains (I-V) and domains (VI-VII).

The B_{max} of the parental β2-AR was determined to be 5pmol/mg of protein. The B_{max} for TR1- β2-AR was found to be 0.62pmol/mg of protein. The B_{max} for truncation TR2- β2-AR further decreased to 0.22pmol/mg of protein. The B_{max} represents the total receptors present and the observation that the number of receptors decreases with truncation is in agreement with the results showing specific activity and the results from the western blot. We found a lower degree of expression of TR2- β2-AR when compared to TR1- β2-AR. TR1- β2-AR had a lower expression profile when compared to the

parental type receptor. It is possible that most of the mutant, truncated versions of receptors are non functional, sequestered in the ER or golgi, or misdirected to the endosomal compartment and thus rendered unavailable to extracellular ligand.

The affinity of the receptor for its ligand also reflects on the structural stability of the receptor. The two portions of the parental β2-AR, domains I-V and domains VI to VII, that are connected by a long third intracellular loop can move and rotate freely. As we shorten this third intracellular loop (as seen in TR1- β2-AR), domains I-V and domains VI to VII, on either side of the loop would come closer enough and are less likely to move randomly. This brings in some sort of structural and conformational orderliness that leads to increased stability of the receptor. The stability therefore increases approximately 3.5 fold with the first truncation. In the case of second truncation the third intracellular loop has been shortened to the extent that there is a structural restraint introduced between the two portions, domains I-V and domains VI to VII, that probably lead to the loss of any free movement thereby decreasing the stability of the receptor (Figure 15).

Competition Binding Experiments

The EC50 value of the parental β2-AR is around 170nM. This agrees with the EC50 values published in the literature, 150+40nM (Ghanouni et al). The Ki value for the first truncation TR1-β2-AR is around 9.4uM. The Ki value for the third truncation TR2- β2-AR is around 7uM. There was no major alteration in the Ki value for the three constructs. A slight increase in Ki observed in case of first truncation, TR1- β2-AR (5.9 to 9.4uM) can be explained by the fact that the affinity of the anatagonist for the receptor

increases four fold which means that the concentration of the unlabelled ligand, isoproterenol that needs to displace the radioligand from its binding site also increases. This increase is nevertheless not significant. The almost unaltered Ki values points out that the pharmacological property (the physiological and biological) of the parental receptor and the mutants remain the same. This means that the truncation probably has not destroyed the mutant receptor`s biological/functional properties.

Immunofluorescence Data

The results from the immunofluorescence data confirm the fact that the expression of truncated receptors on the plasma membranes remains lower than the parental type. The truncated receptors do not get completely cycled to the plasma membrane but instead some of the mutant receptors remain confined intracellularly as aggregated pools of receptor. This explains the lower expression of the receptors in the western blot but does not answer the possibility of the truncated receptors not being cycled to the plasmamembrane. These pools of intracellular receptors might be different from the ones that are seen on the surface of the membranes. These questions remain to be answered. The intracellular pool of truncated receptors may arise due to incorrectly folded truncated proteins. These misfolded proteins are generally non-functional and tend to aggregate in endoplasmic reticulum or in the golgi regions of the cell. It is possible that there are some signaling signature sequences in the wild type beta-2 adrenergic receptor that helps the receptor to efficiently target to the surface of plasma membrane and it is possible that somewhere along the truncation, either in the loop or in

the C-terminus, this signaling signature was removed and therefore these truncated versions do not find their way on to the plasma membrane as effectively as the wild type.

Future Experiments and Projects

The aim of this project was to make stable constructs that may be used to crystallize the protein. The binding data from the two constructs led to suggest that TR-1 is stable and has a higher affinity for the ligand. This construct may be used for stability studies such as testing the affinity of ligand to TR1 at different temperatures and also testing the binding affinities in the presence of different detergents. Once the stability data is obtained crystallization experiments could be conducted.

As the truncated receptor failed to completely cycle to the plasma membrane it would be interesting to put the C-terminus back to see if this particular region is responsible for targeting the receptors to plasma membrane and then conduct binding assays on these constructs to see if their pharmacological properties or affinities varies. To study the nature of the intracellular pool of receptors one could attempt to isolate the plasma membrane fractions from the endoplasmic reticulum fractions and study functional expression of these two fractions by radioligand binding.

It has been shown that misfolded proteins tend to be retained in the endoplasmic reticulum by binding protein (BiP) (Hammond et al., 1994). BiP is an ER chaperone protein and colocalization studies with BiP and M1 antibody would suggest if these proteins are indeed nonfunctional.

Conclusions

Affinity of the antagonist increased around four times for the first truncation, TR1- β2-AR when compared to the parental- β2-AR. The affinity for the antagonist decreased about three fold in TR2- β2-AR when compared to the parental type. Between the two truncations the affinity for the antagonist decreased ten fold in TR-2 when compared to TR-1.

The Ki value of the agonist isoproterenol remained the same. The expression level of the truncated receptors was low when compared to the wild type. The expression patterns of the truncated receptor also indicate that some of the membranes are retained internally.

The results from my experiments prove that truncating the receptor (TR1) leads to increase in stability and that any further truncation may not be tolerated well. The TR2 does not have a good affinity for the antagonist.

LITERATURE CITED

1. Abe, K., Kusakabe, Y., Tanemura, K., Emori, Y., and Arai, S. (1993). Multiple genes for G protein-coupled receptors and their expression in lingual epithelia. *FEBS Lett* 316: 253-256.

2. Bargmann, C.I. (1997). Olfactory receptors, vomeronosal receptors and the organization of olfactory information. *Cell* 90: 585-587.

3. Ando, H., Seidou, M., and Kito, Y. (1991). Light induced, GTP binding protein mediated membrane currents of *Xenopus* oocytes injected with rhodopsin of cephalopods. *Vision res* 31: 1087-1091.

4. Kobilka, B.K. (1995). Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. *Anal Biochem* 231: 269-271.

5. Kobilka, B.K., Dixon, R. A., Frielle, T.,Dohlman,H.G., Bolanowski, M.A., sigal, I.S., Yang-Feng, T.L., Francke,U., Caron, M.G., Lefkowitz, R.J. (1987). CDNA for the human beta2-adrenergic receptor: a protein with multiple membrane spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet derived growth factor. *Proc Natl Acad Sci U S A* 84: 46-50.

6. Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., and Diehl, R.E. (1986). Cloning of the gene and the cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* 321: 75-79.

7. Okada, T., Le Trong, I., Fox, B.A., Behnke, A., Stenkamp, R.E., and Palczewski, K. (2000). X-ray diffraction analysis of three dimensional crystals of bovine rhodopsin obtained from mixed micelles. *J Struct Biol* 130: 73-80.

8. Hammond, C., and Helenius, A. (1994). Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment and Golgi apparatus. J Cell Biol 126: 41-52.

9. Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E., and Downing, K.H. (1990). Model for the structure of bacteriorhodospin based on high resolution electron cryo microscopy. *J Mol Biol* 213: 899-929.

10. Mizobe, T., Maze, M., Lam, V., Suryanarayana, S., and Kobilka, B.K. (1996). Arrangement of transmembrane domains in adrenergic receptors, similarity to bacteriorhodopsin. *J Biol Chem* 271: 2387-2389.

11. Emorine, L. J., Marullo, S., Briend-Sutren, M. M., Patey, G., Tate, K., Delavier-Klutchko, C., and strosberg, A. D. (1998). Molecular characterization of the human beta3-adrenergic receptor. *Science* 245: 1118-1121

12. Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995). The family of G-protein- coupled receptors. *FASEB J* 9: 745-754.

13. Wu, D., Katz, A., Lee, C.H., and Simon, M.I. (1992). Activation of phospholipase C by alpha 1- adrenergic receptors is mediated by the alpha subunits of G_q family. *J Biol Chem* 36: 25798-25802.

14. Kurose, H., Regan, J.W., Caron, M.G., and Lefkowitz, R.J. (1991). Functional interactions of recombinant alpha 2 adrenergic receptor subtypes and G proteins in reconstituted phospholipid vesicles. *Biochemistry* 13: 3335-3341.

15. Frielle, T., Collins, S., Daniel, K.W., Caron, M.G., and Lefkowitz, R.J. (1987) Cloning of the c-DNA for the human beta 1-adrenergic receptor. *Proct Natl Acad Sci USA* 22: 7920-7924.

16. Strosberg, A.D. (1997). Structure and function of the beta 3-adrenergic receptor. *Annu Rev Pharmacol Toxicol* 37: 421-450.

17. Cerione, R. A., Staniszewski, C., Benovic, J. L., Lefkowitz, R. J., Caron, M. G., Gierschik, P., Somers, R., Spiegel, A. M., Codina, J., and Birnbaumer, L. (1985). Specificity of the functional interactions of the beta adrenergic receptor and rhodopsin with guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J Biol Chem* 260: 1493-1500.

18. Starder,C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994) Structure and function of G protein coupled receptors. *Annu Rev Biochem* 63: 101-132.

19. Bourne, H. (1997). How receptors talk to trimeric G proteins. *Curr Opin Cell Biol* 9:134-142

20. Wall, M. A. (1995). The structure of G protein heterotrimer $Gi\alpha_1\beta_1\gamma_1$. *Cell* 83: 1047-1058.

21. Coleman, D. E. (1994). Structure of active conformations of Giα1 and the mechanism of GTP hydrolysis. *Science* 265: 1405-1412.

22. Hamm, H. E. (1998). The many faces of G protein signaling. *J Biol Chem* 273: 669-672.

23. Kolakowski Jr, L. F. (1994). GPCRDb: a G-protein coupled receptor database. Receptors Channels 2: 1-7.

24. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992). Sequence alignment of the G-protein coupled superfamily. *DNA Cell Biol* 11: 1-20.

25. Kobilka, B. K. (1992). Adrenergic receptors as models for G-protein-coupled receptors. *Annu Rev Neurosci* 15: 87-114.

26. Motulsky, H. The GraphPad guide to analyzing radioligand binding data.

27. Motulsky, H. J., Insel, A. P. the study of cell surface receptors with radioligand binding: methodology, data analysis and experimental problems.

28. Okada, T., Ernst, O. P., Palczewski, K., and Hoffmann, K. P. (2001). Activation of rhodopsin: New insights from structural and biochemical studies. *Trends Biochem Sci* 26: 318-324.

29. Tota, R. T., and Strader, C. D. (1990). Charaterization of the binding domain of the beta-adrenergic receptor with the fluorescent antagonist carazolol. *J Biol Chem* 265: 16891-16897.

30. Samama, P., Cotecchia, S., Costa, T., Lefkowitz, R. J. (1993). A mutation induced activated state of the β2-adrenergic receptor. *J Biol Chem* 268: 4625-2636.

31. Gether, U., Ballesteros, J. A., Seifart, R., Sanders-Bush, E., Weinstein, H., and Kobilka, B. K. (1997). Structural instability of a constitutively active G-protein coupled receptor. Agonist independent activation due to conformational flexibility. *J Biol Chem* 272: 2587-2590.

32. Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999). Mutation of a highly conserved aspartic acid in the β2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment. Mol Pharmacol 56: 175-184.

33. Gether, U., Lin, S., and Kobilka, B. K. (1995). Fluorescent labeling of purified beta 2-adrenergic receptor: evidence for ligand specific conformational changes. *J Biol Chem* 270: 28268-28275.

34. Gether, U., Lin, S., Ghanouni, P., Ballesteros, J. A., Weinstein, H., and Kobilka, B. K. (1997). Agonists induce conformational changes in transmembrane domains III and VI of the β2 adrenergic receptor. EMBO J 16: 6737-6747.

35. Hebert, T. E., Moffett, S., Morello, J. P., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996). A peptide derived from a β2 adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* 271: 16384-16392.

36. Gether, U., Kobilka, B. K. (1998). G Protein coupled receptors: Mechanism of agonist binding. J Biol Chem 273: 17979-17982.

APPENDIX A

Sequence of beta-2 adrenergic receptor

MGQPGNGSAF LLAPNRSHAP DHDVTQQRDE VWVVGMGIVM SLIVLAIVFG NVLVITAIAK
FERLQTVTNY FITSLACADL VMGLAVVPFG AAHILMKMWT FGNFWCEFWT SIDVLCVTAS
IETLCVIAVD RYFAITSPFK YQSLLTKNKA RVIILMVWIV SGLTSFLPIQ MHWYRATHQE
AINCYANETC CDFFTNQAYA IASSIVSFYV PLVIMVFVYS RVFQEAKRQL QKIDKSEGRF
HVQNLSQVEQ DGRTGHGLRR SSKFCLKEHK ALKTLGIIMG TFTLCWLPFF IVNIVHVIQD
NLIRKEVYIL LNWIGYVNSG FNPLIYCRSP DFRIAFQELL CLRRSSLKAY GNGYSSNGNT
GEQSGYHVEQ EKENKLLCED LPGTEDFVGH QGTVPSDNID SQGRNCSTND SLL

LEGEND:

- Membrane spanning domain-I
- Membrane spanning domain-II
- Membrane spanning domain-III
- Membrane spanning domain-IV
- Membrane spanning domain-V
- Membrane spanning domain-VI
- Membrane spanning domain-VII
- The sequences underlined represents N-glycosylation sites predicted using the web service http://www.cbs.dtu.dk/services/NetNGlyc/ that allows to identify 86% of the glycosylated and 61% of the non-glycosylated □equins, with an overall accuracy of 76%.

APPENDIX B

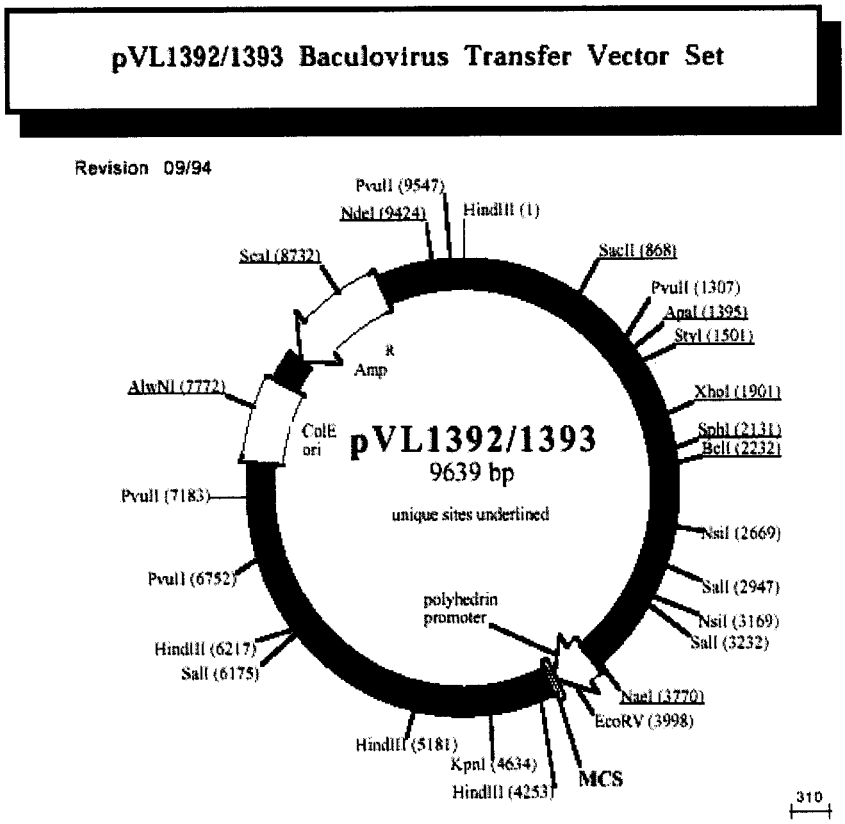
Multiple sequence alignment of wild type beta2-adrenergic receptor, TR1, TR2, and bovine rhodopsin

```
Multalin version 5.4.1
Copyright I.N.R.A. France 1989, 1991, 1994, 1996
Published research using this software should cite
Multiple sequence alignment with hierarchical clustering
F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890
Symbol comparison table: blosum62
Gap weight: 12
Gap length weight: 2
Consensus levels: high=90% low=50%
Consensus symbols:
 ! is anyone of IV
 $ is anyone of LM
 % is anyone of FY
 # is anyone of NDQEBZ

 MSF:    420    Check:   0          ..
 Name: Wild           Len:   420  Check: 9180  Weight:  0.79
 Name: TR1-beta2-adre Len:   420  Check: 7710  Weight:  0.79
 Name: TR2-beta2-adre Len:   420  Check: 3662  Weight:  0.84
 Name: bovine         Len:   420  Check: 2894  Weight:  1.57
 Name: Consensus      Len:   420  Check: 1806  Weight:  0.00

//

            1                                                  50
      Wild     MGQPGNG SAFLLAPNRS HAPDHDVTQQ RDEVWVVGMG IVMSLIVLAI
TR1-beta2-     MGQPGNG SAFLLAPNRS HAPDHDVTQQ RDEVWVVGMG IVMSLIVLAI
TR2-beta2-     MGQPGNG SAFLLAPNRS HAPDHDVTQQ RDEVWVVGMG IVMSLIVLAI
    bovine  MNGTEGPNFY VPFSNKTGVV RSPFEAPQYY LAEPWQFSML AAYMFLLIML
 Consensus  ...mgqPgng saFllapnrs haPdhdvtqq rdEvWvvgMg ivmslivlai

            51                                                 100
      Wild  VFG-NVLVIT AIAKFERLQT VTNYFITSLA CADLVMGLAV VPFGAAHILM
TR1-beta2-  VFG-NVLVIT AIAKFERLQT VTNYFITSLA CADLVMGLAV VPFGAAHILM
TR2-beta2-  VFG-NVLVIT AIAKFERLQT VTNYFITSLA CADLVMGLAV VPFGAAHILM
    bovine  GFPINFLTLY VTVQHKKLRT PLNYILLNLA VADLFMVFGG FTTTLYTSLH
 Consensus  vFg.NvLvit aiakferLqT vtNYfitsLA cADLvMglav vpfgaahiLm
```

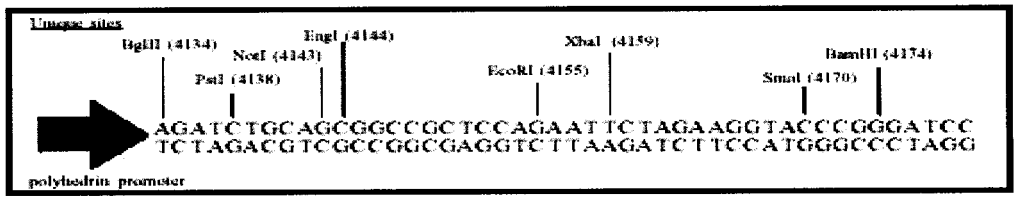
```
           101                                                   150
      Wild KMWTFGNFWC EFWTSIDVLC VTASIETLCV IAVDRYFAIT SPFKYQSLLT
TR1-beta2- KMWTFGNFWC EFWTSIDVLC VTASIETLCV IAVDRYFAIT SPFKYQSLLT
TR2-beta2- KMWTFGNFWC EFWTSIDVLC VTASIETLCV IAVDRYFAIT SPFKYQSLLT
    bovine GYFVFGPTGC NLEGFFATLG GEIALWSLVV LAIERYVVVC KPMS-NFRFG
 Consensus kmwtFGnfwC #fwtsidvLc vtasietLcV iA!#RYfa!t sPfky#sllt

           151                                                   200
      Wild KNKARVIILM VWIVSGLTSF LPIQMHWYRA THQEAINCYA NE-TCCDFFT
TR1-beta2- KNKARVIILM VWIVSGLTSF LPIQMHWYRA THQEAINCYA NE-TCCDFFT
TR2-beta2- KNKARVIILM VWIVSGLTSF LPIQMHWYRA THQEAINCYA NE-TCCDFFT
    bovine ENHAIMGVAF TWVMALACAA PPLVGWSRYI PEGMQCSCGI DYYTPHEETN
 Consensus kNkArvi!lm vW!vsgltsf lPiqmhwyra thqeainCya #e.Tcc#fft

           201                                                   250
      Wild NQAYAIASSI VSFYVPLVIM VFVYSRVFQE AKRQLQKIDK SEGRFHVQNL
TR1-beta2- NQAYAIASSI VSFYVPLVIM VFVYSRVFQE AKRQLQKIDK SEGRF-----
TR2-beta2- NQAYAIASSI VSFYVPLVIM VFVYSRVFQE AKRQLQKI-- ----------
    bovine NESFVIYMFV VHFIIPLIVI FFCYGQLVFT VKEAAAQQ-- ----------
 Consensus N#a%aIass! VsFy!PL!!m vFvYsrvfqe aKrqlqki.. ..........

           251                                                   300
      Wild SQVEQDGRTG HGLRRSSKFC LKEHKALKTL GIIMGTFTLC WLPFFIVNIV
TR1-beta2- ---------- ---RRSSKFC LKEHKALKTL GIIMGTFTLC WLPFFIVNIV
TR2-beta2- ---------- ----RSSKFC LKEHKALKTL GIIMGTFTLC WLPFFIVNIV
    bovine ---------- ---QESATTQ KAEKEVTRMV IIMVIAFLIC WLPYAGVAFY
 Consensus .......... ....rSskfc lkEhkalktl gIimgtFtlC WLP%fiVniv

           301                                                   350
      Wild HVIQDNLIRK EVYILLN-WI GYVNSGFNPL IYCR-SPDFR IAFQELLCLR
TR1-beta2- HVIQDNLIRK EVYILLN-WI GYVNSGFNPL IYCR-SPDFR IAFQELLCLR
TR2-beta2- HVIQDNLIRK EVYILLN-WI GYVNSGFNPL IYCR-SPDFR IAFQELLCLR
    bovine IFTHQGSDFG PIFMTIPAFF AKTSAVYNPV IYIMMNKQFR NCMVTTLCCG
 Consensus hviq#nlirk e!%illn.wi gyvnsg%NPl IYcr.sp#FR iafqelLClr

           351                                                   400
      Wild RSSLKAYGNG YSSNGNTGEQ SGYHVEQEKE NKLLCEDLPG TEDFVGHQGT
TR1-beta2- RSSLKAYGNG YSSNGNTGEQ SGYHVE
TR2-beta2- RSSLKAYGNG YSSNGNTGEQ SGYHVE
    bovine KNPL---GDD EASTTVSKTE TSQVAPA
 Consensus rssLkayG#g ysSngntge# sgyhve.... .......... ..........

           401               420
      Wild VPSDNIDSQG RNCSTNDSLL
TR1-beta2-
TR2-beta2-
    bovine
 Consensus .......... ..........
```

APPENDIX C

pVL1392 vector map



MCS
pVL1392

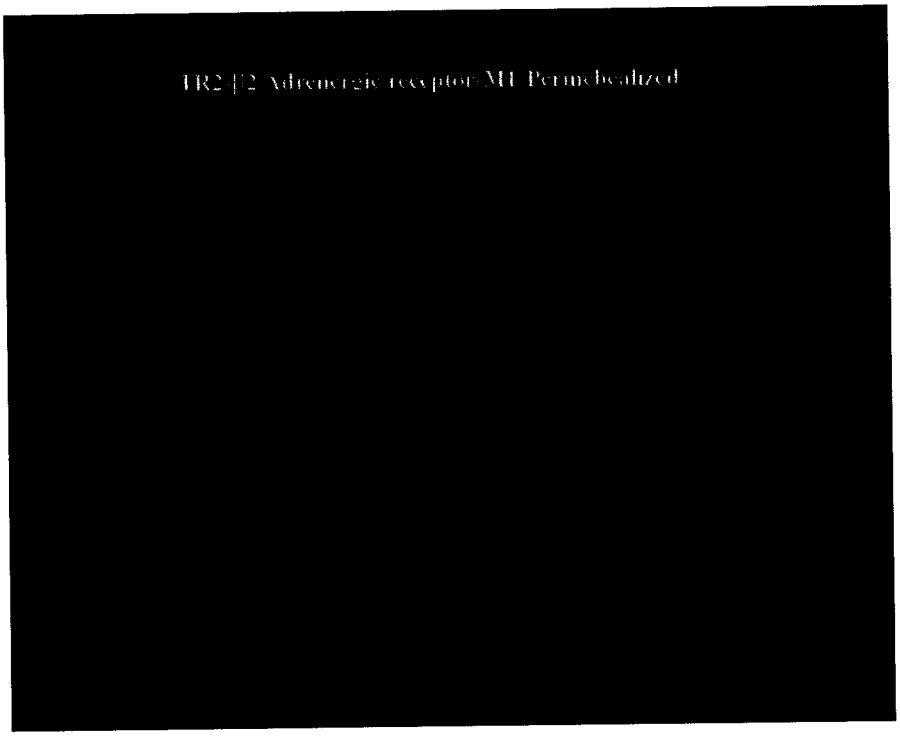


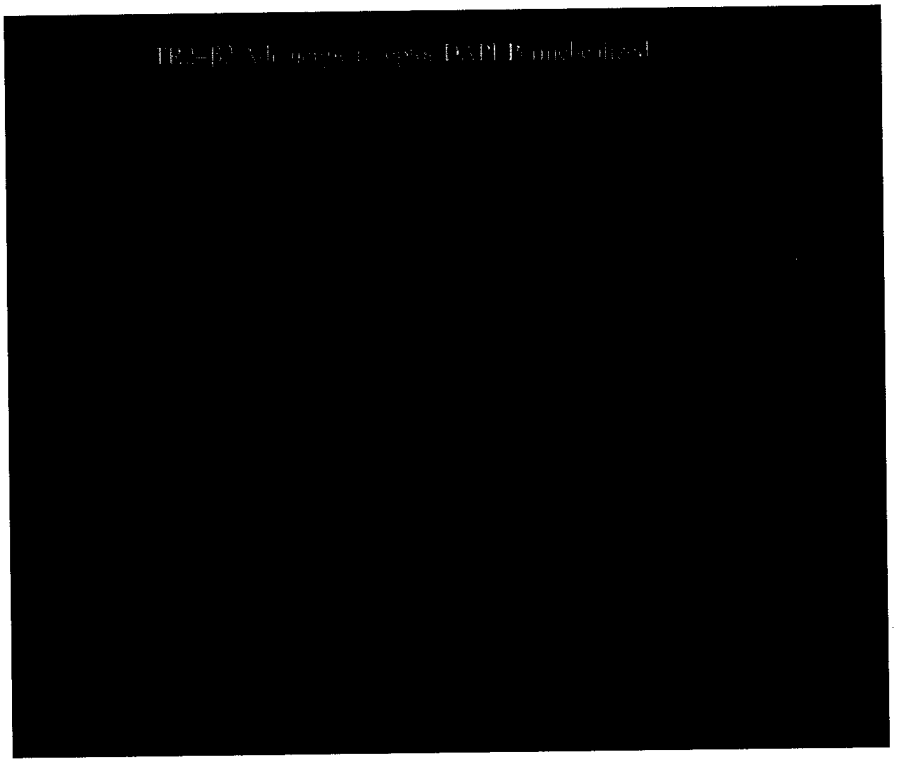
APPENDIX D

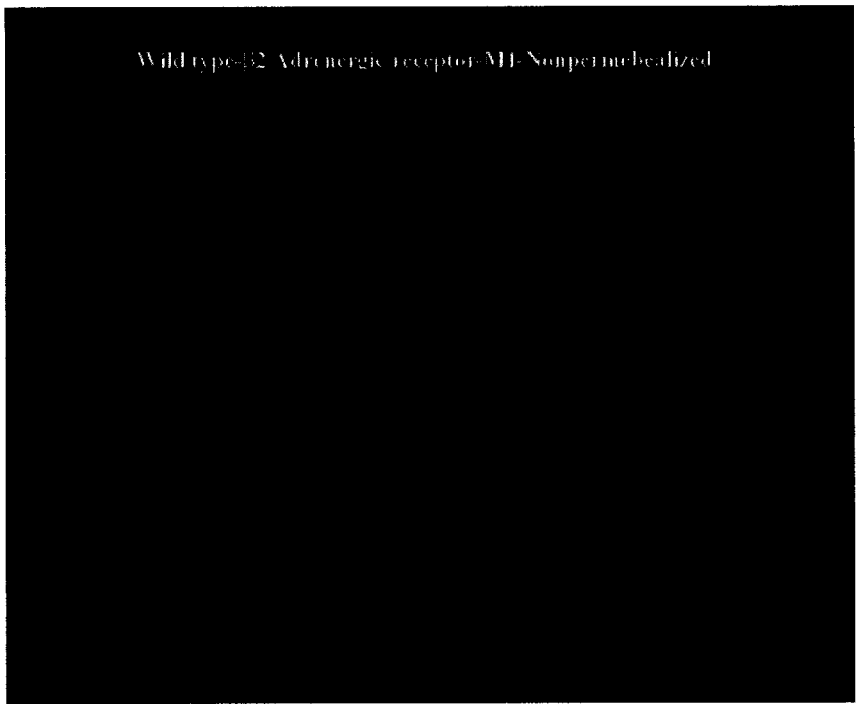
Immunofluorescence images

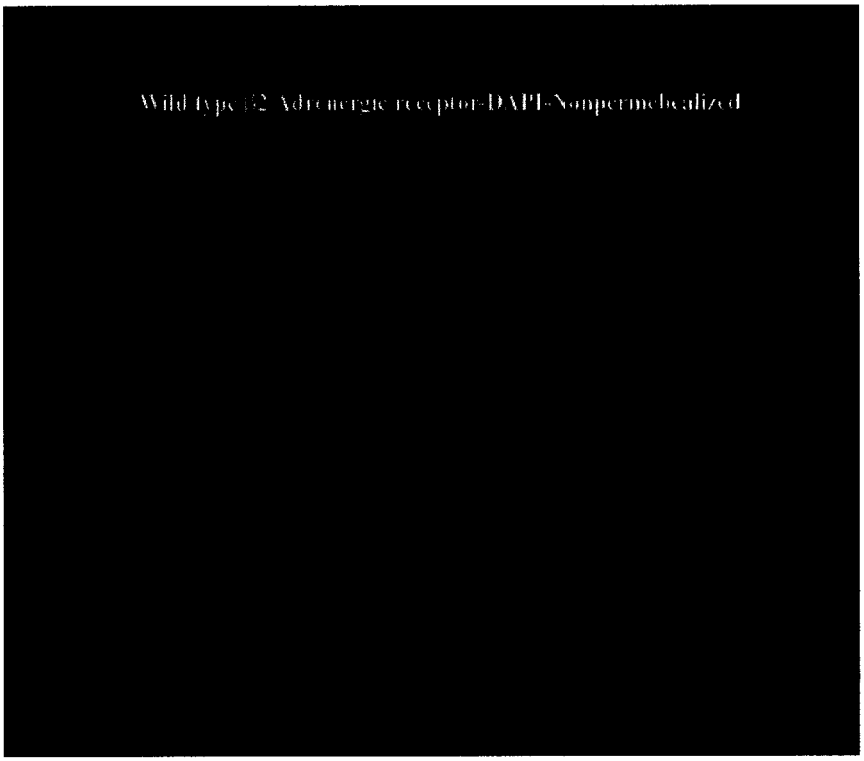

ERI β2 Adrenergic receptor-MH-Permeabealized

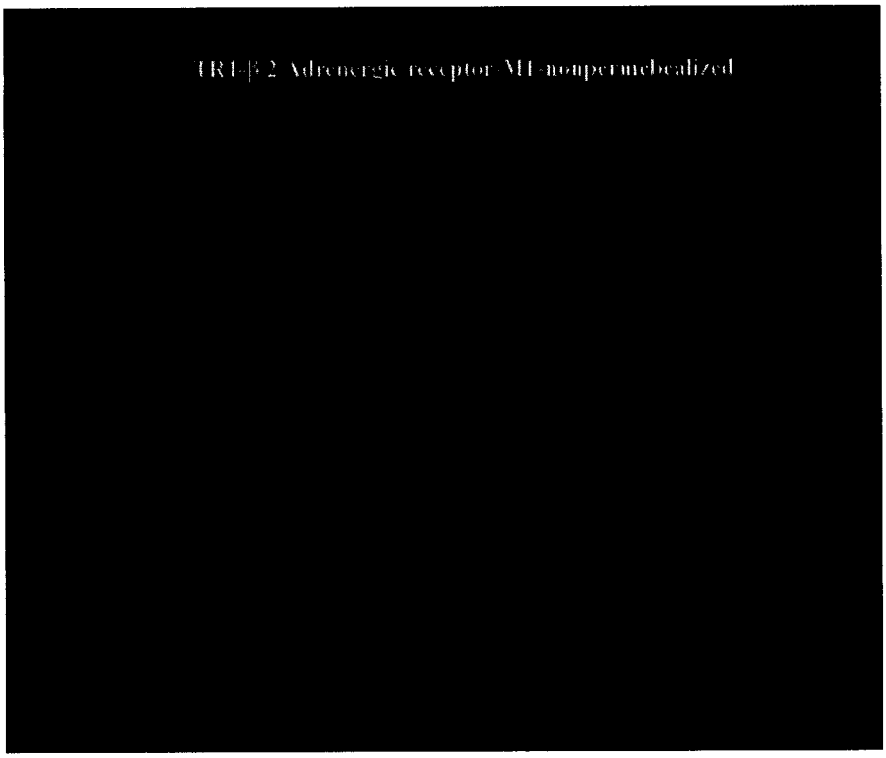

ERI β2 Adrenergic receptor-DAPI-Permeabealized

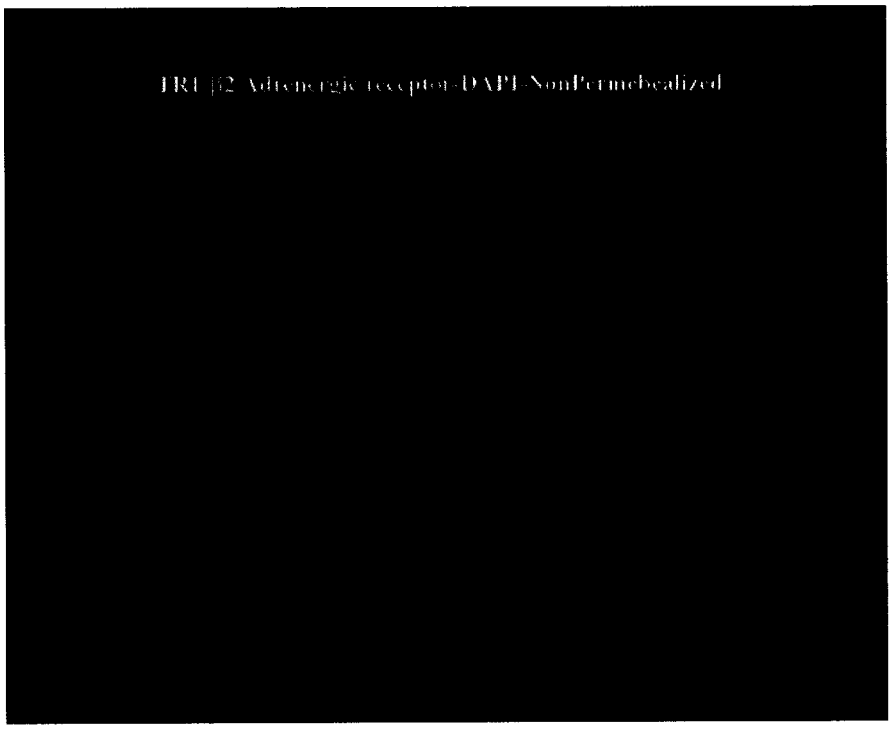

TR2 β2 Adrenergic receptor MI Permeabilized

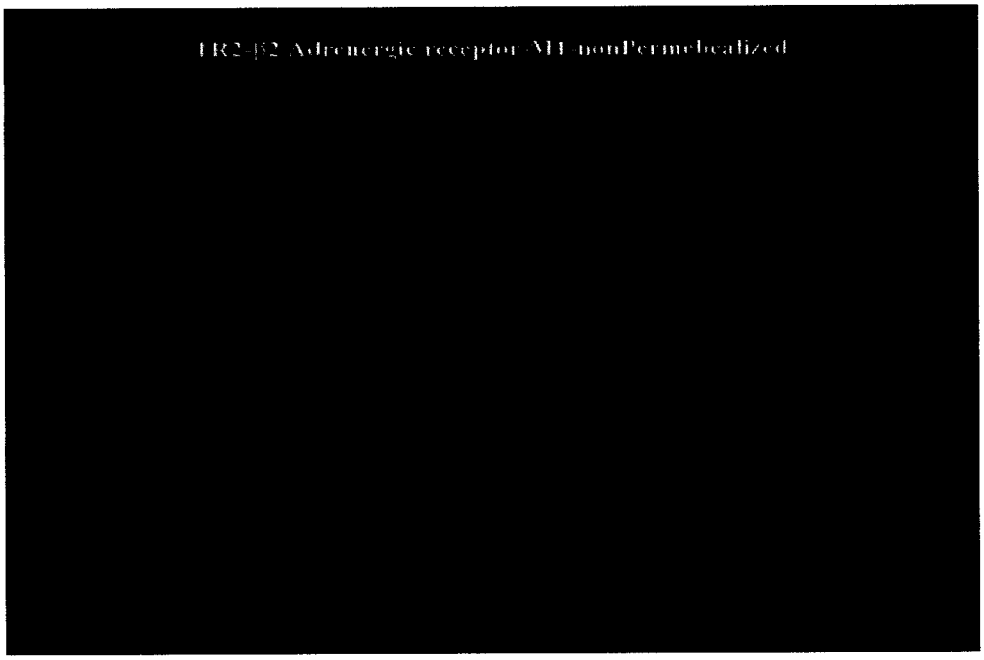

TR3 β3 Adrenergic receptor DAPI Permeabilized

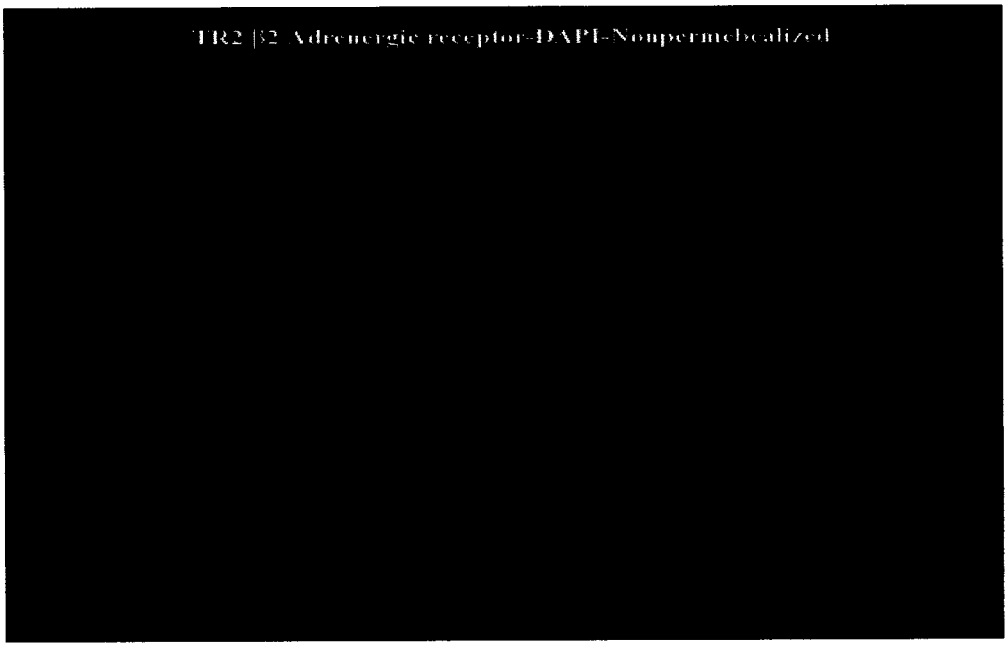

Wild type β2 Adrenergic receptor-MII-Nonpermeabalized


Wild type β2 Adrenergic receptor-DAPI-Nonpermeabalized


TRI-β 2 Adrenergic receptor-MH-nonpermeabealized

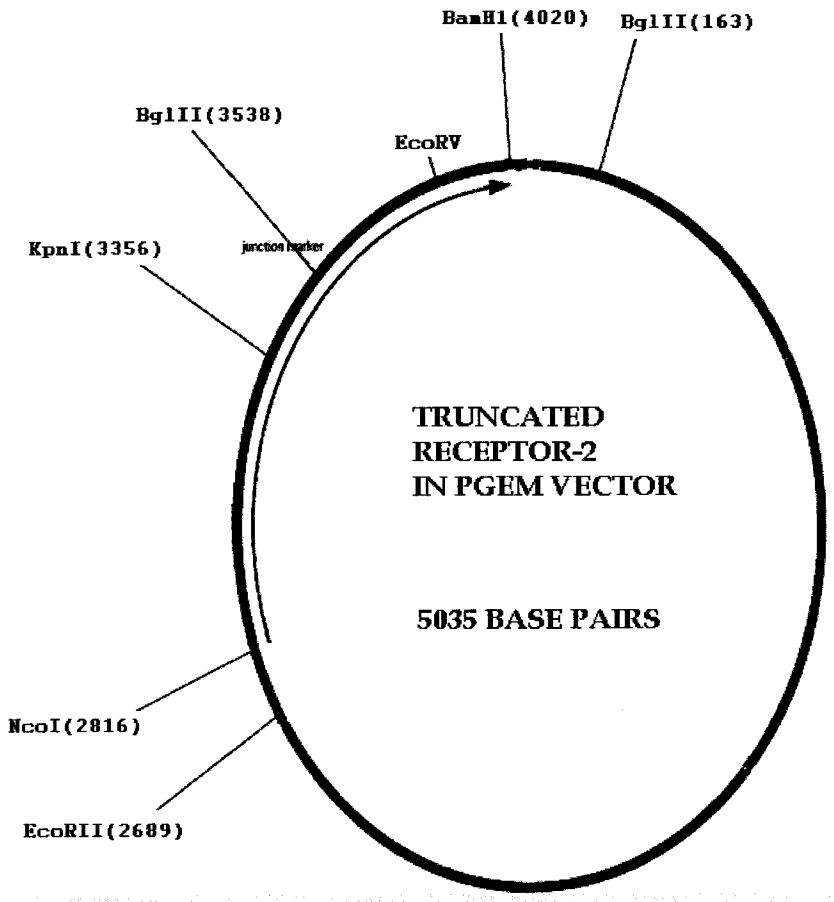

TRI-β2 Adrenergic receptor-DAPI-NonPermeabealized

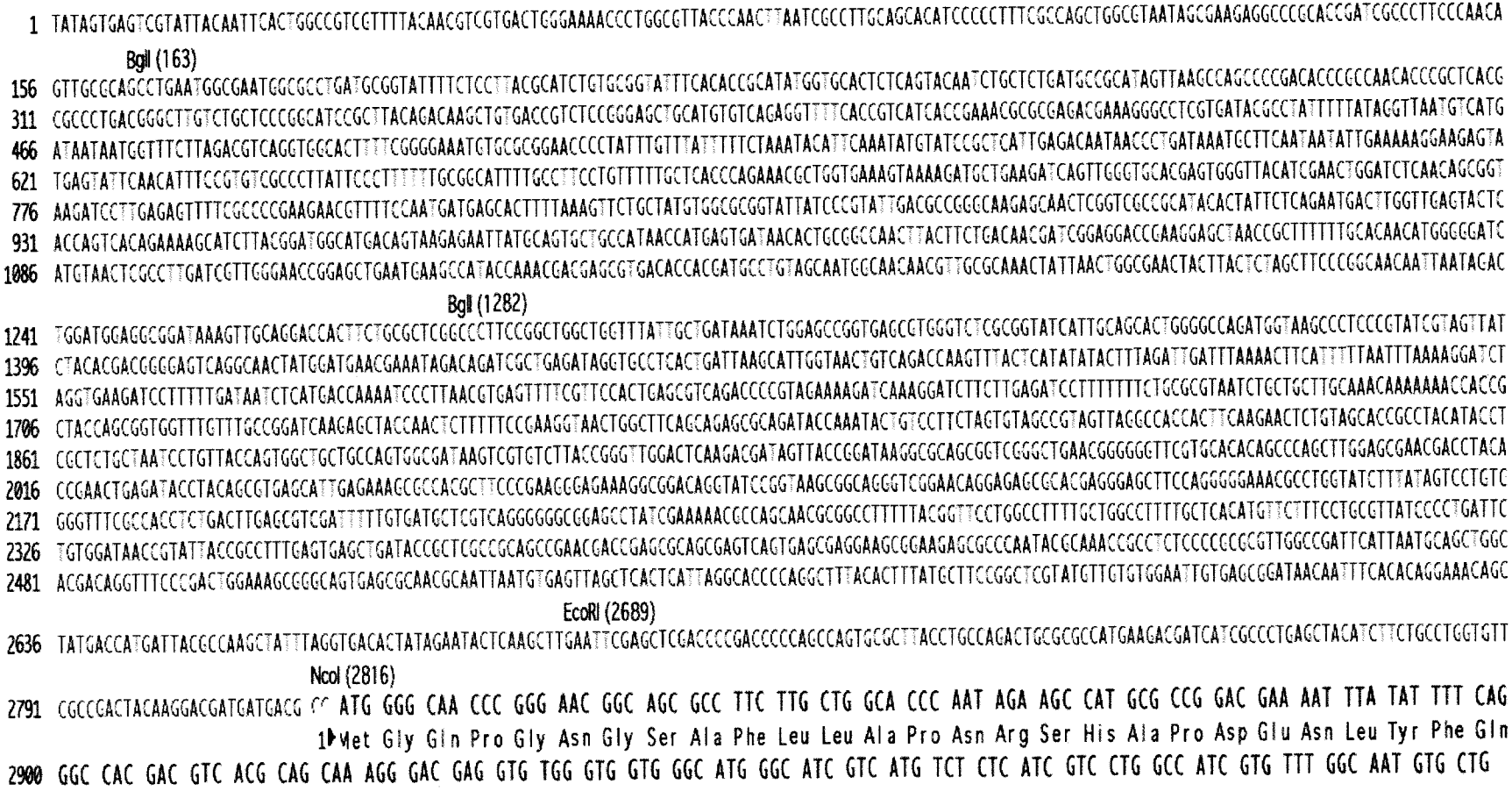

TR2-β2 Adrenergic receptor-NH-nonPermeabolized

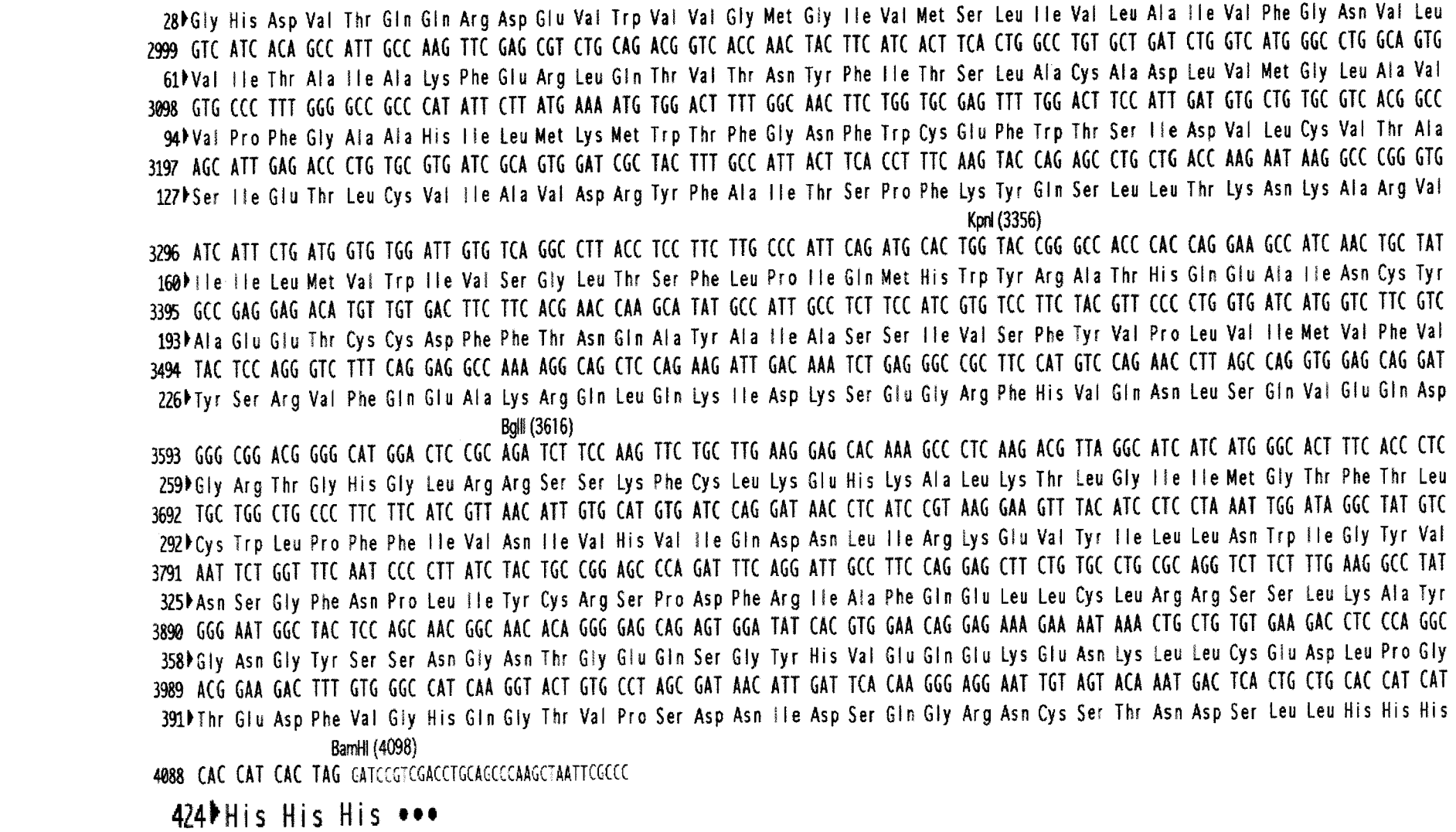

TR2 β2 Adrenergic receptor-DAPI-Nonpermeabolized

APPENDIX E

Vector sequence of clone TR2




BglI (163)
BglI (1282)
EcoRI (2689)
NcoI (2816)
1 TATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACA
156 GTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTCACG
311 CGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATG
466 ATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATTGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTA
621 TGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGT
776 AAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTC
931 ACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATC
1086 ATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGAC
1241 TGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTAT
1396 CTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCT
1551 AGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCG
1706 CTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCT
1861 CGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACA
2016 CCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTC
2171 GGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATCGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTC
2326 TGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCCCGCGTTGGCCGATTCATTAATGCAGCTGGC
2481 ACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC
2636 TATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTTGAATTCGAGCTCGACCCCGACCCCCAGCCAGTGCGCTTACCTGCCAGACTGCGCGCCATGAAGACGATCATCGCCCTGAGCTACATCTTCTGCCTGGTGTT
2791 CGCCGACTACAAGGACGATGATGACG CC ATG GGG CAA CCC GGG AAC GGC AGC GCC TTC TTG CTG GCA CCC AAT AGA AGC CAT GCG CCG GAC GAA AAT TTA TAT TTT CAG
1 Met Gly Gln Pro Gly Asn Gly Ser Ala Phe Leu Leu Ala Pro Asn Arg Ser His Ala Pro Asp Glu Asn Leu Tyr Phe Gln
2900 GGC CAC GAC GTC ACG CAG CAA AGG GAC GAG GTG TGG GTG GTG GGC ATG GGC ATC GTC ATG TCT CTC ATC GTC CTG GCC ATC GTG TTT GGC AAT GTG CTG


28 Gly His Asp Val Thr Gln Gln Arg Asp Glu Val Trp Val Val Gly Met Gly Ile Val Met Ser Leu Ile Val Leu Ala Ile Val Phe Gly Asn Val Leu
2999 GTC ATC ACA GCC ATT GCC AAG TTC GAG CGT CTG CAG ACG GTC ACC AAC TAC TTC ATC ACT TCA CTG GCC TGT GCT GAT CTG GTC ATG GGC CTG GCA GTG
61 Val Ile Thr Ala Ile Ala Lys Phe Glu Arg Leu Gln Thr Val Thr Asn Tyr Phe Ile Thr Ser Leu Ala Cys Ala Asp Leu Val Met Gly Leu Ala Val
3098 GTG CCC TTT GGG GCC GCC CAT ATT CTT ATG AAA ATG TGG ACT TTT GGC AAC TTC TGG TGC GAG TTT TGG ACT TCC ATT GAT GTG CTG TGC GTC ACG GCC
94 Val Pro Phe Gly Ala Ala His Ile Leu Met Lys Met Trp Thr Phe Gly Asn Phe Trp Cys Glu Phe Trp Thr Ser Ile Asp Val Leu Cys Val Thr Ala
3197 AGC ATT GAG ACC CTG TGC GTG ATC GCA GTG GAT CGC TAC TTT GCC ATT ACT TCA CCT TTC AAG TAC CAG AGC CTG CTG ACC AAG AAT AAG GCC CGG GTG
127 Ser Ile Glu Thr Leu Cys Val Ile Ala Val Asp Arg Tyr Phe Ala Ile Thr Ser Pro Phe Lys Tyr Gln Ser Leu Leu Thr Lys Asn Lys Ala Arg Val
KpnI (3356)
3296 ATC ATT CTG ATG GTG TGG ATT GTG TCA GGC CTT ACC TCC TTC TTG CCC ATT CAG ATG CAC TGG TAC CGG GCC ACC CAC CAG GAA GCC ATC AAC TGC TAT
160 Ile Ile Leu Met Val Trp Ile Val Ser Gly Leu Thr Ser Phe Leu Pro Ile Gln Met His Trp Tyr Arg Ala Thr His Gln Glu Ala Ile Asn Cys Tyr
3395 GCC GAG GAG ACA TGT TGT GAC TTC TTC ACG AAC CAA GCA TAT GCC ATT GCC TCT TCC ATC GTG TCC TTC TAC GTT CCC CTG GTG ATC ATG GTC TTC GTC
193 Ala Glu Glu Thr Cys Cys Asp Phe Phe Thr Asn Gln Ala Tyr Ala Ile Ala Ser Ser Ile Val Ser Phe Tyr Val Pro Leu Val Ile Met Val Phe Val
3494 TAC TCC AGG GTC TTT CAG GAG GCC AAA AGG CAG CTC CAG AAG ATT GAC AAA TCT GAG GGC CGC TTC CAT GTC CAG AAC CTT AGC CAG GTG GAG CAG GAT
226 Tyr Ser Arg Val Phe Gln Glu Ala Lys Arg Gln Leu Gln Lys Ile Asp Lys Ser Glu Gly Arg Phe His Val Gln Asn Leu Ser Gln Val Glu Gln Asp
BglII (3616)
3593 GGG CGG ACG GGG CAT GGA CTC CGC AGA TCT TCC AAG TTC TGC TTG AAG GAG CAC AAA GCC CTC AAG ACG TTA GGC ATC ATC ATG GGC ACT TTC ACC CTC
259 Gly Arg Thr Gly His Gly Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys Ala Leu Lys Thr Leu Gly Ile Ile Met Gly Thr Phe Thr Leu
3692 TGC TGG CTG CCC TTC TTC ATC GTT AAC ATT GTG CAT GTG ATC CAG GAT AAC CTC ATC CGT AAG GAA GTT TAC ATC CTC CTA AAT TGG ATA GGC TAT GTC
292 Cys Trp Leu Pro Phe Phe Ile Val Asn Ile Val His Val Ile Gln Asp Asn Leu Ile Arg Lys Glu Val Tyr Ile Leu Leu Asn Trp Ile Gly Tyr Val
3791 AAT TCT GGT TTC AAT CCC CTT ATC TAC TGC CGG AGC CCA GAT TTC AGG ATT GCC TTC CAG GAG CTT CTG TGC CTG CGC AGG TCT TCT TTG AAG GCC TAT
325 Asn Ser Gly Phe Asn Pro Leu Ile Tyr Cys Arg Ser Pro Asp Phe Arg Ile Ala Phe Gln Glu Leu Leu Cys Leu Arg Arg Ser Ser Leu Lys Ala Tyr
3890 GGG AAT GGC TAC TCC AGC AAC GGC AAC ACA GGG GAG CAG AGT GGA TAT CAC GTG GAA CAG GAG AAA GAA AAT AAA CTG CTG TGT GAA GAC CTC CCA GGC
358 Gly Asn Gly Tyr Ser Ser Asn Gly Asn Thr Gly Glu Gln Ser Gly Tyr His Val Glu Gln Glu Lys Glu Asn Lys Leu Leu Cys Glu Asp Leu Pro Gly
3989 ACG GAA GAC TTT GTG GGC CAT CAA GGT ACT GTG CCT AGC GAT AAC ATT GAT TCA CAA GGG AGG AAT TGT AGT ACA AAT GAC TCA CTG CTG CAC CAT CAT
391 Thr Glu Asp Phe Val Gly His Gln Gly Thr Val Pro Ser Asp Asn Ile Asp Ser Gln Gly Arg Asn Cys Ser Thr Asn Asp Ser Leu Leu His His His
BamHI (4098)
4088 CAC CAT CAC TAG GATCCGTCGACCTGCAGCCCAAGCTAATTCGCCC
424 His His His •••

APPENDIX F

Protocols and calculations

PCR reaction

Template DNA was the wild type, parental beta-2 adrenergic receptor construct in PGEM vector. Forward primer used was pre pstI primer and reverse primer was the BglII site containing primer as discussed in the methods section.

Constituents	Volume
Template DNA(100ng)	1ul
Fwd Primer(10 pmol)	1ul
Rev Primer(10 pmol)	1ul
10X PCR (cfu)	5ul
dntp`s (0.2mM)	4ul
Pfu Polymerase(1000 units/ul)	1ul
Water	37ul
	50ul

Conditions of PCR	
Lid 105 degrees	
Temperature of denaturation	95 degrees for 1minute
Temperature of denaturation	30 seconds
Temperature of annealing	58 degrees for 30 seconds
Temperature of elongation	68 degrees for 2 minutes
Repeat 28 cycles	
Temperature of elongation	68 degrees for 12 minutes
Hold at 4 degrees	

Cloning of TR-2 construct in PGEM

The PCR product and the parental PGEM vector were treated with KpnI and BglII to create sticky ends for ligation.

Constituents	Volume
Parental PGEM vector DNA(1ug)	1.4ul
KpnI (500units/ul)	2.0ul
BglII(500units/ul)	2.0ul
Buffer-2	3.0ul
Water	21.6ul
	30ul

Constituents	Volume
Purified PCR product	5.0ul
KpnI (500units/ul)	2.0ul
BglII(500units/ul)	2.0ul
Buffer-2	3.0ul
Water	18.0ul
	30ul

Incubated both vector arms and PCR products with the restriction enzymes at 37 degrees for two hours.

Dephosphorylation of vector arms

To 30ul of digested vector arms, 1ul of shrimp alkaline phosphatase was added and incubated at 37 degrees for 1 hour. After 1hour the alkaline phosphatases were inactivated by heat reating at 65-70 degrees for 10 minutes.

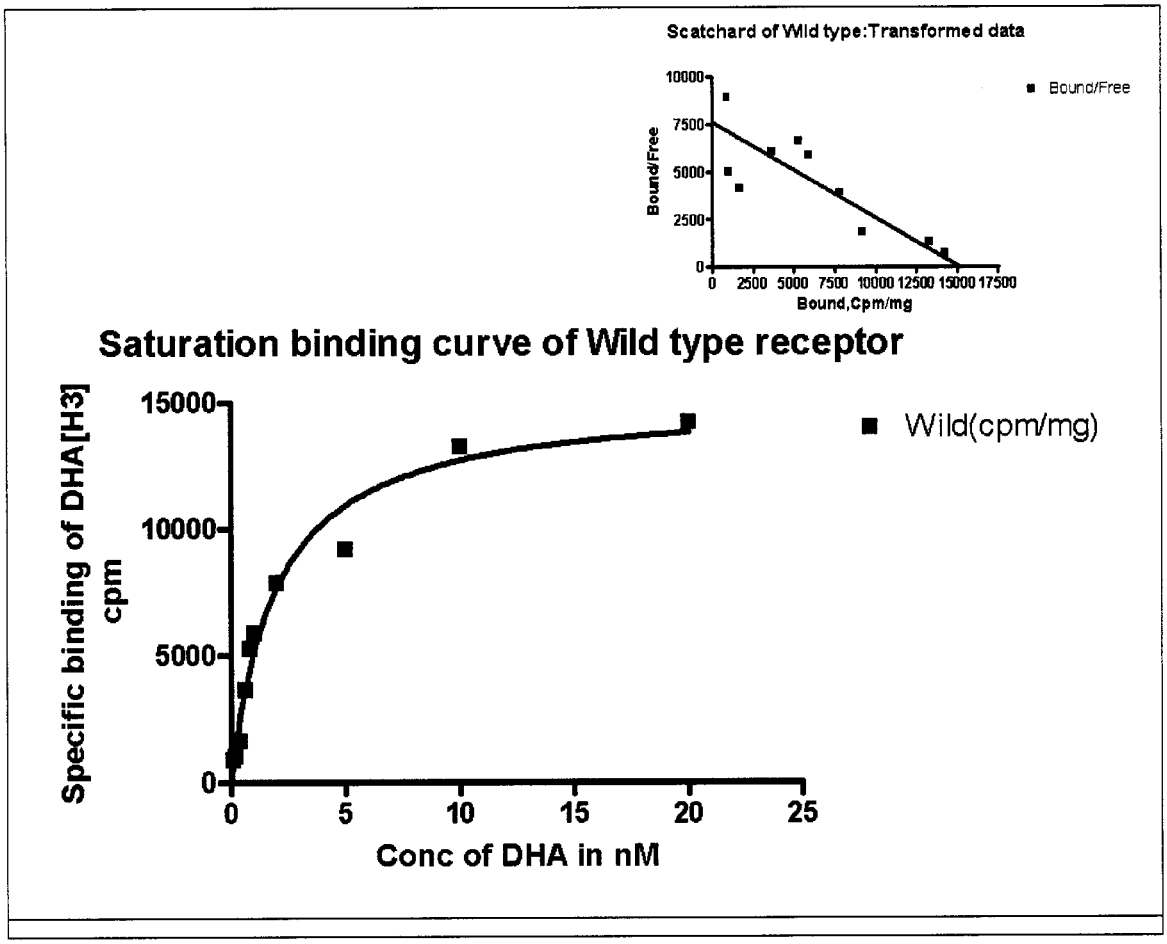
Ligation reaction

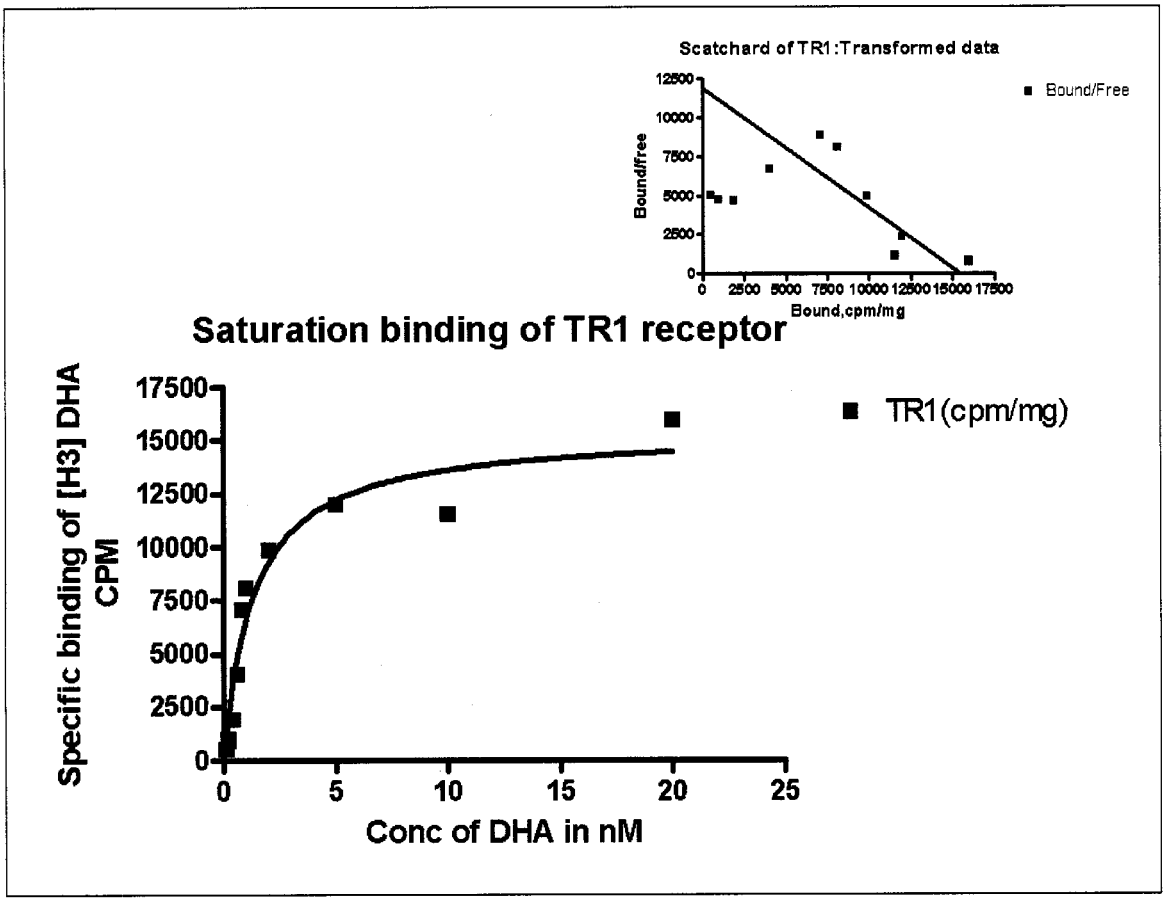
Constituents	Positive	Negative
Solution A	10ul	10ul
Solution B	2.5ul	2.5ul
Volume of 5X Tris Mg buffer	0.5ul	0.5ul
Volume of insert	1.5ul	
volume of vector	0.5ul	0.5ul
volume of water		1.5ul

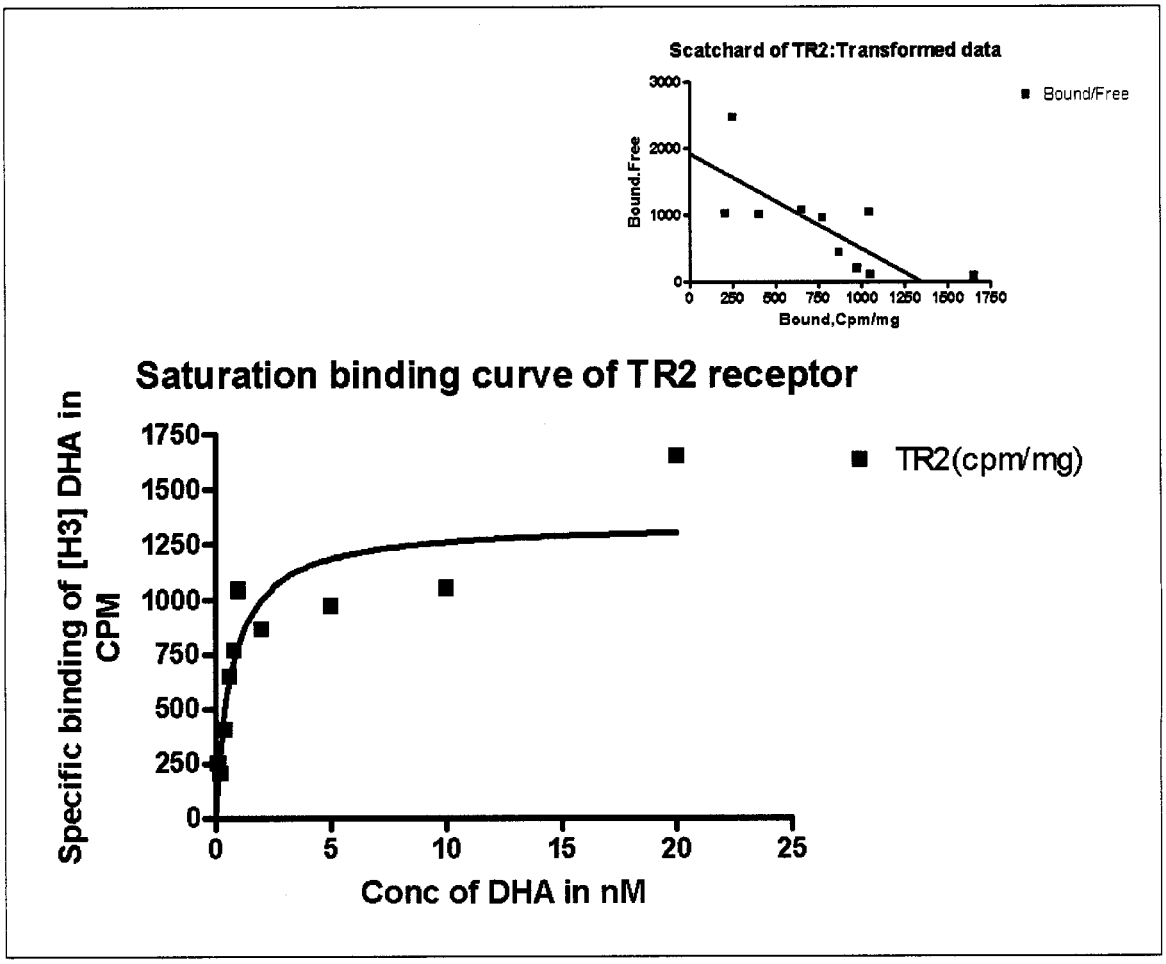
The tubes were incubated at 16 degrees for an hour.

APPENDIX G

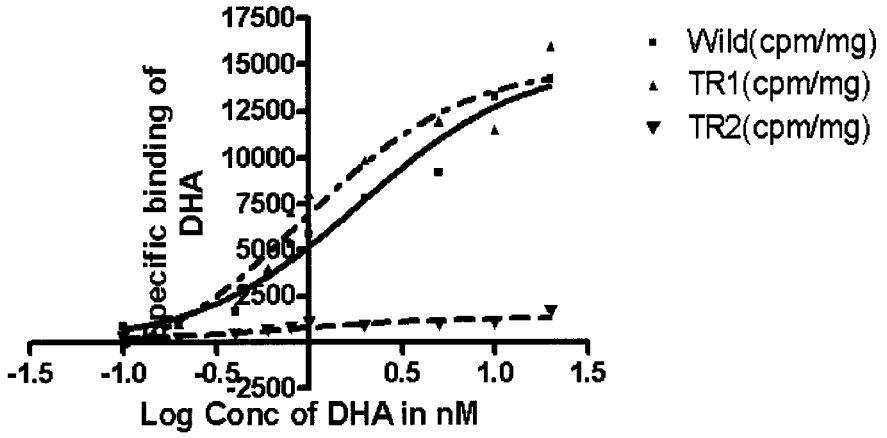
Scatchard plots and semi lograthmic plots of the three constructs




Scatchard of TR1:Transformed data
Bound/Free
Bound/free
Bound,cpm/mg
Saturation binding of TR1 receptor
Specific binding of [H3] DHA CPM
TR1(cpm/mg)
Conc of DHA in nM


Scatchard of TR2:Transformed data
Bound/Free
Bound.Free
3000
2000
1000
0
0 250 500 750 1000 1250 1500 1750
Bound,Cpm/mg
Saturation binding curve of TR2 receptor
Specific binding of [H3] DHA in CPM
1750
1500
1250
1000
750
500
250
0
0 5 10 15 20 25
Conc of DHA in nM
TR2(cpm/mg)

Semi log plot of Specific binding versus Log conc of DHA



	Wild(cpm/mg)	TR1(cpm/mg)	TR2(cpm/mg)
EC50	1.889	0.9135	0.7312