Alleles of the Human β_2 Adrenergic Receptor Gene and Possible Effects on the Phenotype

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There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory which states
that this has already happened.

Chapter 1

Introduction

The regulation of blood pressure is maintained by various complex feedback circuits. These control systems include the heart and vascular system, baro- and chemoreceptors, the peripheral vegetative nervous system and vegetative nuclei in the central nervous system, the kidney, and several hormone systems, including both endocrine and paracrine pathways. The vegetative nervous system controls short-term adjustments ("short feedback-loop"), which enable physical activity as the reflex action, the renin-angiotensine system is the prominent medium-term control system. Long-term blood pressure adjustment is mediated by the kidneys by controlling the circulating volume; various hormone systems, including atrial natriuretic hormone, aldosterone, antidiuretic hormone, and medullolipids are involved in this pathways. There is general consent that essential hypertension is a multifactorial disease caused by both genetic and environmental factors. The hereditary nature of essential hypertension in humans is well established, both from epidemiological surveys [1–3], and from family studies, including the analyses of twin models [4–6]. The influence of genetic variance on blood pressure has been repeatedly demonstrated also in animal models of hypertension [7–9]. Due to the complexity of the regulatory system involved in blood pressure control, several candidate genes have been postulated. There exists good evidence from linkage studies and association studies, that polymorphisms of the angiotensine converting enzyme gene and the angiotensinogen gene are related to essential hypertension [10–12].

The adrenergic system appears to be a second control system which might be linked to the etiology of essential hypertension. Recent studies of our group favoured the hypothesis that vascular adrenoceptors play an important role in the etiology of hypertension. In a precursor state of essential hypertension, namely salt sensitivity (see section 1.2 on page 8), a reduced amount of vasodilatatory β_2-adrenergic receptors both on circulating lymphocytes and on cultured skin fibroblasts was demonstrated [13–18]. In addition, preliminary experiments revealed a reduced β_2-adrenoceptor density on cultured skin fibroblasts of hypertensive subjects as compared to matched normotensive controls. The present master's thesis was undertaken in order (a) to search for mutations of the β_2-adrenoceptor gene,

and (b) characterize alleles of the human β_2-adrenergic receptor through sequencing of the structure gene cloned into pUC-18 vectors. This study represents a necessary first step to perform association studies and linkage studies in subjects with and without hypertension.

1.1 Adrenergic System, Adrenoceptors – Classes, Functionality, Physiology

1.1.1 The Adrenergic System

The catecholamines dopamine, L(–)-epinephrine and L(–)-norepinephrine (Figure 1.1-1.3) act in the body as neurotransmitters as well as as hormones. Norepinephrine is the predominant neurotransmitter, whereas epinephrine the major hormone. Catecholamines are released from the nervous system following neuronal depolarization. The ratio of L(–)-epinephrine and L(–)-norepinephrine is about 80:20 in humans. Both transmitters put the body into a condition coined as "fight and flight reaction":

- there is an increased heart frequency and contractility
- bronchial dilatation
- decreased blood supply to the splanchnic bed, the skin and the kidneys
- important metabolic changes include increased hepatic glycogenolysis
- and increased lipolysis in lipocytes.

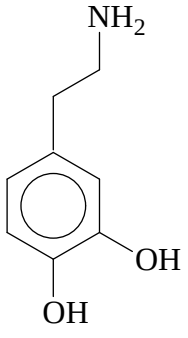

Figure 1.1: Dopamine

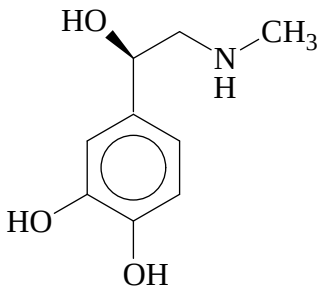

Figure 1.2: L(–)-Epinephrine

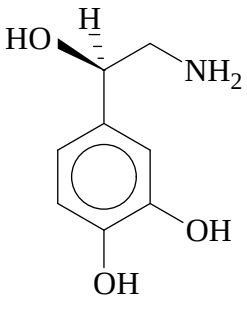

Figure 1.3: L(–)-Norepinephrine

Catecholamines exert their cellular action via binding to membrane proteins, the so-called adrenergic receptors or adrenoceptors. The term 'adrenoceptor' was first introduced by AHLQUIST to explain the different physiological effects of catecholamines [17]. In 1948 Ahlquist [19] further differentiated α- and β-receptors due to their response to L(–)-epinephrine. In vascular smooth muscles the degree of contraction induced by these substances follows the order *L(–)-epinephrine* $\geqslant$ *L(–)-norepinephrine* $\gg$ *isoprenaline* (α-receptors). The vaso-dilatatory effect and the positive chronotropic effects (i.e., rise of heart rate) follow the order *isoprenaline* $>$ *L(–)-epinephrine* $\geqslant$ *L(–)-norepinephrine* (β-receptors).

These types of receptors were further splitted into several subgroups. Table 1.2 on the next page summarizes their pharmacological differentiation and physiological effects.

The existence of these receptor subtypes has been proven by radioligand assays. It has been shown, that a number of tissues may contain both β-receptors. This distribution is often unbalanced, as table 1.1 shows.

Tissue:	β_1-*subtype*	β_2-*subtype*
heart	80%	20%
kidney	70%	30%
placenta	50%	50%
lungs	30%	70%
liver		$\leqslant$100%
leukocytes	0%	100%

Table 1.1: Distribution of β-receptors

1.1.2 The Adrenergic Receptor

A number of receptors transmit signals from the extracellular space to the intracellular compartment by means of a guanine nucleotide binding protein (G-Protein) [20], e.g. adrenoceptors, muscarinic cholinergic receptors, rhodopsin and the receptors for the antidiuretic hormone. These receptors show certain similarities concerning their molecular structure [21,22] (see section 1.3 on page 9). They all possess seven membrane spanning domains, hence they are referred to as "seven membrane spanning domain receptors". These hydrophobic domains have a length of 20 to 24 amino acids. The N-terminus of the receptor and three loops are located extracellularly (EI – EIII), the C-terminus and three loops extend intracellularly (CI – CIII). The β_1 and β_2 adrenoceptors share 54% of their sequence. The extracellular N-terminus contains several N-glycosylation sites, the inner loop CIII and the C-terminus carry phosphorylation sites (see figure 1.7 on page 15). The membrane crossing sections are ligand binding domains, the intracellular parts are bound to the G-protein. The high variability of the C-III loop and the C-terminus seems to be the main cause of the receptor specific aspects of binding the G-protein.

α_1-adrenoceptors
Selective agonists: phenylephrine, methoxamine, amidephrine, cirazoline *Selective antagonists:* prazosin, terazosin, BE 2254 *Occurrence and effects:* salivary glands (stimulation of K^+ and H_2O secretion), smooth muscles of arterioles (constriction) and bronchi (contraction), uterus, sphincters of the digestive system and the bladder, musculus dilatator pupillae (contraction).
α_2-adrenoceptors
Selective agonists: α-methyl-arterenone , B-HT 920, B-HT 933 *Selective antagonists:* yohimbine, rauwolscine, RX 781094 *Occurrence and effects:* presynaptical membranes (decreases via a feedback the release of norepinephrine), parotis, pancreas (decreases insulin secretion), uterus, kidney (decreases renin secretion), central nervous system, fatty cells (decreases lipolysis), platelets (aggregation).
β_1-adrenoceptors
Selective agonists: norepinephrine *Selective antagonists:* practolol, metoprolol, atenolol, bisoprolol, betaxolol, CGP 20712A *Occurrence and effects:* heart muscle (positive chronotrope, dromotrope and inotrope), kidney (increased renin secretion), salivary glands (increased amylase secretion).
β_2-adrenoceptors
Selective agonists: salbutamol, fenoterol, terbutaline, procaterol *Selective antagonists:* ICI 118, ICI 551 *Occurrence and effects:* vascular system and bronchi (dilatation), digestive system (relaxation of intestine mobility), fatty cells (lipolysis), liver (increase of glycolysis and gluconeogenesis), muscles (increase of glycolysis), pancreas (increase of insulin and glucagon secretion)

Table 1.2: Pharmacological and physiological properties of adrenergic receptors

Molecular biological studies revealed further subtypes of adrenergic receptors [22], such as the α_{1A}, α_{1B} [23, 24] types of α_1-receptors or the α_{2A}, α_{2B}, α_{2C2}, α_{2C4}, α_{2C10} [25–32] and α_{2D} types of the α_2-receptor. Recently a β-receptor of the fatty tissue (termed β_3 adrenoceptor) lacking phosphorylation sites was discovered [33] . All these subtypes can readily be divided into three groups by their molecular biological characteristics.

α_1-receptors: binding G_h protein, activating inositole triphosphate and diacylglycerole as second messengers,

α_2-receptors: binding the inhibitory G_i protein, restraining the adenylcylase system, cAMP as second messenger,

β-receptors: binding the adenylate cyclase stimulating G_s protein, cAMP as second messenger.

The human adrenoceptor subtypes are located on distinct genloci, which happen to be rather close for α_{1B} and β_2 as well as α_{2A} and β_1 (see table 1.3).

Subtype	*Genlocus*	*Incidence*
α_{1A}	unassigned	brains
α_{1B}	chromosome 5q23–q32	
α_{1C}	chromosome 8p21–p11.2	
α_{1D}	unassigned	
α_{2A}	chromosome 10q24–q26	platelets
α_{2B}	chromosome 2p13–q13	kidney
β_1	chromosome 10q24–q26	
β_2	chromosome 5q31–q32	
β_3	chromosome 8p12–p11.1	adipocytes

Table 1.3: Genloci of various adrenergic receptor subtypes

1.1.3 Functionality

As mentioned above, the β_2-adrenoceptor is linked to a guanine nucleotide regulatory protein. Several types of these proteins have been described [21] (see table 1.4 on the next page).

The G-proteins are located at the cytoplasmic side of the cell membrane and consist of three subunits: α(39–52kDa), β(35–36kDa) and γ(8–10kDa). The sequence of events leading to signal transduction by the β_2-adrenergic receptor can be described as follows [20, 21, 34]:

The binding of an agonist to the binding domain of the receptor is followed by binding of the receptor to the α-subunit of the G protein. As a consequence the G protein is activated (substitution of GTP for GDP) and the receptor is phosphorylated by a specific kinase. This phosphorylated receptor dissociates from the

G_s	(stimulating) signal transfer of β-receptors
G_i	(inhibitory) α_2 and muscarinic cholinergic receptors (subtype M2)
G_h	(contributes to activation of phospholipase C) α_1-receptors and muscarinic cholinergic receptors (subtype M1, H1)
G_t	(transducine 1 and transducine 2) rhodopsin
G_0	very common type in the brain, function yet unknown

Table 1.4: Guanine Nucleotide Binding Protein Classes

cell membrane and translocates into the cell. The G protein dissociates to its subunits. The α subunit, now bound to GTP, stimulates the adenylate cyclase which is located at the cytoplasmic side of the cell membrane, resulting in a plasmatical cAMP concentration rise. The G protein can also interfere in ion channel regulation. The bound GTP is converted to GDP and inactivates the G protein. The activity of the adenylate cyclase decays. The receptor is dephosphorylated and re-enters the cell membrane, eventually completing the cycle. In stage this the receptor can again react with a ligand.

The cAMP acts as second messenger: by activating protein kinases which in turn phosphorylate various proteins. The specific cell response is determined by these phosphorylated proteins and the protein kinases being present.

1.2 Salt Sensitivity and Adrenergic Receptors

It appears from epidemiological surveys that hypertension is rare in populations with a traditionally low salt intake [35]. However, if a high salt intake plays a role in human hypertension, great inter-individual variability must exist in susceptibility to salt. The term *salt sensitivity* was introduced for the phenomenon of substantial blood pressure rise ($\geqslant$ 3 mmHg) under a high salt diet. In a previous study, the daily salt intake in normotensive medical students [36–42] was altered from 200 mmol Na^+/day to 50 mmol/day and back to 200 mmol/day. A decrease in blood pressure following dietary salt reduction predominantly in individuals with a positive family history of hypertension was observed. Figure 1.5 on page 11 shows the distribution of the change in blood pressure of those with positive and negative family histories respectively. These findings suggest that those individuals with a positive history of hypertension are more likely to be salt sensitive, a conclusion which is also consistent with the observation that hypertensive patients, as a group, are more likely to be salt sensitive than normotensive persons [43]. A high salt diet over two weeks was accompanied by an up-regulation of α_2 and a down-regulation of β_2 adrenergic receptors, resulting in a rise of the α_2/β_2 adrenoceptor ratio. Such a response would favour enhanced α_2 mediated vasoconstriction [44], and reduced β_2 mediated vasodilation during a high salt intake [45] (see figure 1.6 on page 12). Neither changes in α_2 nor in β_2 are alone predicted blood pressure responses to a

	Hypertensive	Normotensive
	subjects	
Number	9	9
β_2 Bmax(fmol/mg)	52.8±14.3	114.4±19.8
α_2/β_2	74.4±43.66	7.68±2.75

Table 1.5: β_2 adrenoceptor downregulation in hypertensive subjects

high salt diet. Since α_2 and β_2 adrenoceptors mediate not only opposing effects on resistance vessels, but also may mediate opposing effects on renal sodium handling [46–48], the concept of the α_2 and β_2 receptors (operative adrenoceptor ratio) was put forward.

The increase in α_2/β_2 adrenergic receptor ratio by high salt intake may trigger a cascade of intracellular events such as a decrease of intracellular cyclic AMP and an increase in cytosolic calcium [49]. Both events increase potassium conductance of cell membranes [50], thereby activating the sodium pump [51]. Evidence for these events has been obtained in hypertensive subjects [7, 38, 52, 53].

In order to avoid possible influences of the milieu interieur on adrenoceptor regulation in vivo, studies on cultured fibroblasts were performed.

Furthermore, in a subsequent study [18], β_2 receptors were measured in cultured fibroblasts obtained from hypertensive subjects and matched[1] controls. The age of the subjects (42 – 53 years) was chosen to reduce the chance for controls to develop hypertension and to make it unlikely that the hypertensive patients have hypertension related to atherosclerosis. As shown in figure 1.4 on the following page, hypertensive individuals show a distinct reduction of β-receptors. These findings favour the hypothesis that salt sensitivity is a precursor of hypertension, in a significant proportion of hypertensives. As displayed in table 1.5, hypertensive subjects express a lower number of β_2 receptors as compared to normotensives. This evidence suggests that a possible genetic factor of essential hypertension is a disturbed regulation of the β_2-adrenoceptor.

1.3 Genetics of the β_2 Receptor: Receptor Codon, Codon Mutations

The importance of the genetic background for blood pressure has been studied extensively. FEINLEIB [54] and ZIMMER [55] suppose that about 60% of blood pressure variance of adults is genetically determined. This big influence of genetics can easily be seen by comparing blood pressure of relatives. The correlation is highest for monozygotic twins and falls with decreasing genetic similarity.

Adrenergic receptors are members of the G protein family (see section 1.1.3 on page 7). They possess seven regions of 20 to 24 hydrophobic amino acids cross-

[1] The controls were matched for sex, age and BMI.

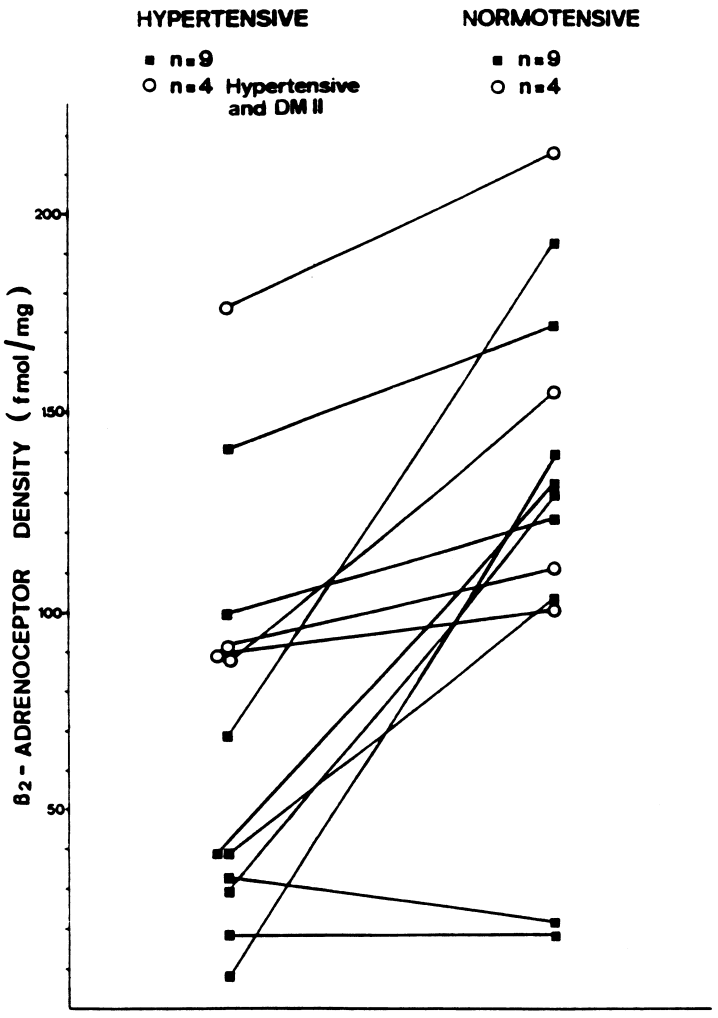

Figure 1.4: β_2 adrenoceptor densities of matched individuals

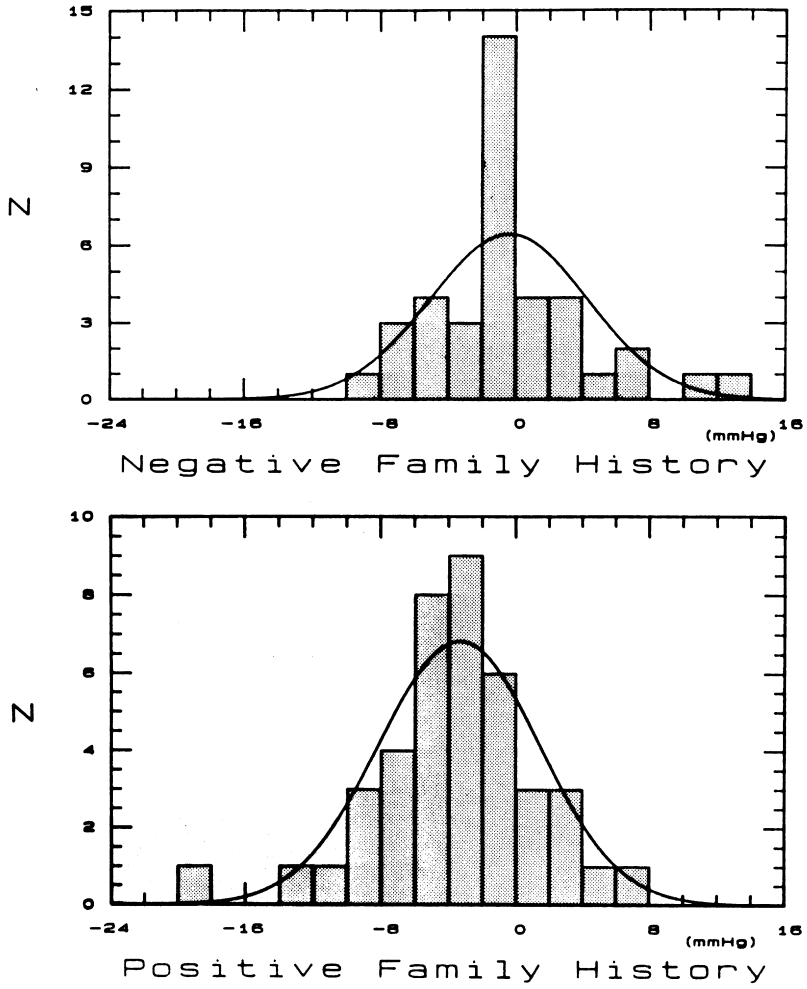

Figure 1.5: Histograms of blood pressure changes in 79 normotensive subjects after two weeks of salt restriction from 200 to 50 mmol per day. Subjects were classified according to family history of hypertension (positive, if either parents or grandparents had established hypertension prior to age of 65 years). Distribution of blood pressure changes was significantly shifted to the left in subjects with a positive family history ($p < 0.001$).

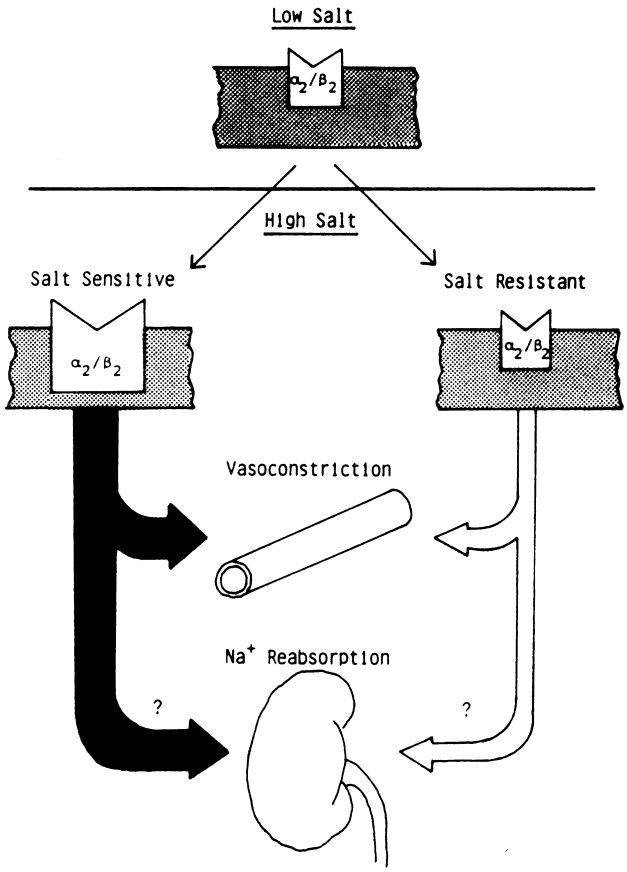

Figure 1.6: In genetically predisposed persons, a high salt intake may lead to enhanced up-regulation of the 'operative α_2/β_2 adrenoceptor ratio', thereby promoting vasoconstriction, reduced vasodilation, and enhanced renal Na^+ reabsorption. At low salt intake, subjects with or without genetic predisposition may be indistinguishable from one another.

ing the membrane in α-helixes [56]. Their amino acid homologies are strongest for these membrane spanning domains. The aminoterminus contains two sites for asparagine-linked glycosylation. With the help of deleted mutants of the β_2-adrenoceptor [57, 58] it has been shown that glycosylation deficient mutants do *not* change their agonist binding characteristics [59, 60] nor in the signal transduction, as binding to the G protein and stimulating the adenylate cyclase (see page 7), whereas the density of receptors is significantly reduced. The glycosylation therefore plays a major role in the translocation of the receptor to the plasma membrane. The binding site for agonists and antagonists results from interaction of the catecholamine group with the carboxylic group of Asp-113 in the 3rd hydrophobic domain of the β_2 receptor [61–65]. Agonistic effects are induced by hydrogen bonds of the hydroxy groups of Ser-204 and Ser-207 and the *meta*- or *para*-hydroxy groups of the ligand.

For stabilizing the binding box, cysteines in extracellular loops are essential. When Cys-106 and Cys-184 are substituted for valine, the ligand binding strength is dramatically reduced [26, 33]. Amino acid substitutions of the membrane domains MI, MII, MIII and MVII also decrease the ligand binding affinity. After glycosylation, the β_2-adrenoceptor is also modified by palmitoylisation at Cys-341 (N-terminal domain). This posttranslational modification is, as shown by deleted mutants [66], crucial for the interaction with the G protein.

The gene of the human β_2-adrenoceptor has been localized to chromosome 5q31–q32 (see table 1.3 on page 7) [67]. The gene is intronless throughout [68]. Its promoter region contains a variety of binding sites for regulatory elements, including a steroid receptor binding hexamer (TGTTCT) [68, 69], cyclic AMP responsive elements [70], a consensus TATA box and a consensus CAAT box [68]. Interestingly, a non-overlapping cistron in the β_2 receptor mRNA 5' leader region is translated and the resulting peptide inhibits receptor translation [71]. Despite the vast amount of literature on the molecular biology, structure and function of the human β_2 adrenergic receptor, little is known about the existence of alleles.

There was shown by LIGGETT [72], that substitution of an extracellular cysteine enhances receptor phosphorylation and desensitization. He [73] and others [74, 75] have shown, using site-directed mutagenesis, that mutations involving small regions of the β_2-adrenoceptor, including changes of a single amino acid, can markedly alter the functional properties of the receptor.

β_2-adrenoceptor cDNA was sequenced by several groups [76–78]. The derived sequence consists of 413 amino acids[2].

1.4 Objective

As outlined above, genetic variances of the human β_2 codon might be responsible for the expression of salt sensitivity and the development of hypertension.

[2]Throughout this thesis the CDNA sequence numbering of Strosberg [77] was used for the localization of nucleic acid substitutions and deletions.

This master's thesis was undertaken to search for mutations of the human β_2-adrenergic receptor codon and to characterize possible alleles through sequencing of the cloned structure gene (Chapter 3). The aim of this work was to develop a technique for fast detection of the mutations in order to gain information on the allelic frequencies and to be in the position to perform association and linkage studies (Chapter 4).

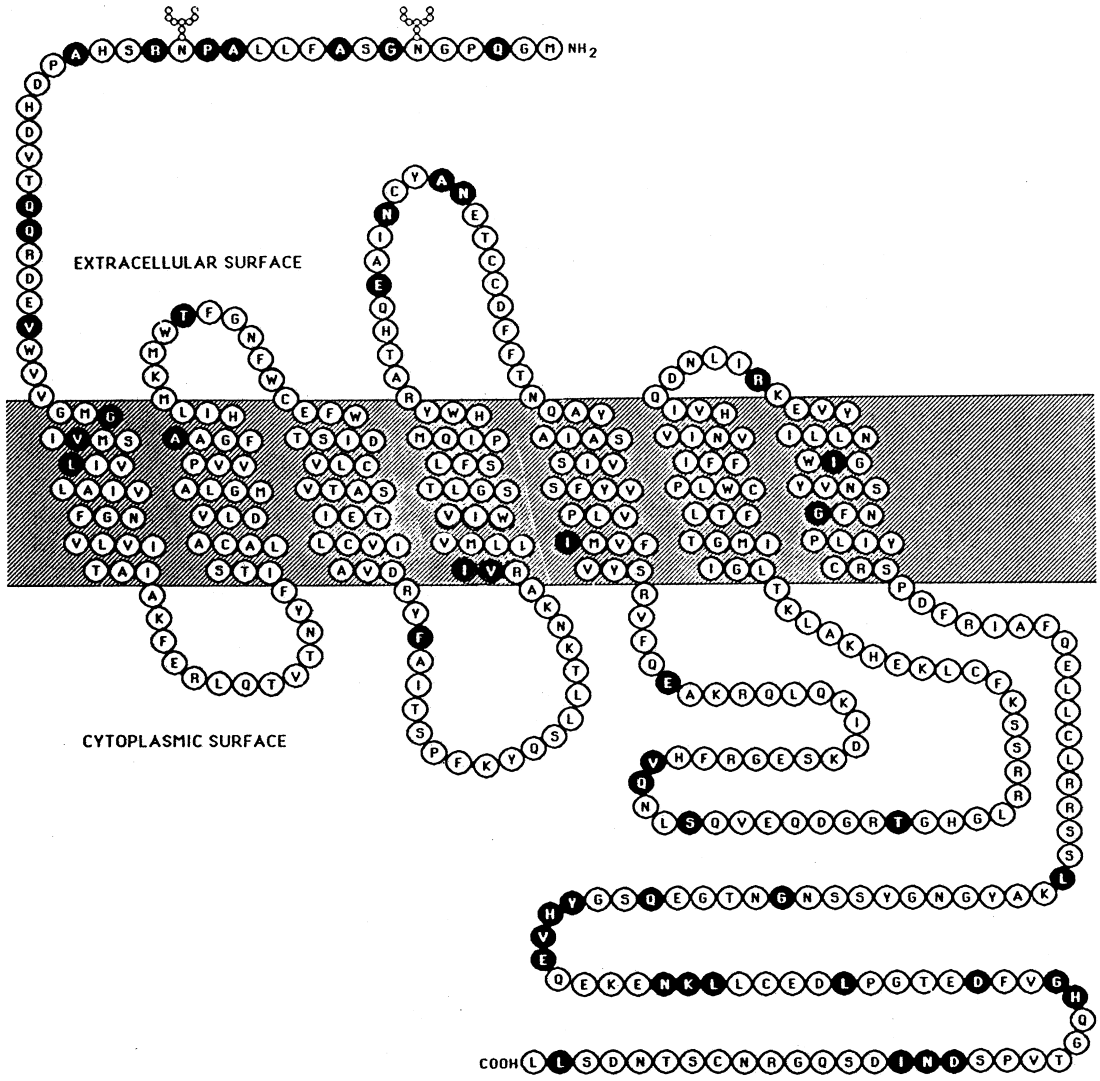

Figure 1.7: Proposed model for insertion of the β_2 adrenergic receptor in the cell membrane. The model is based on hydropathicity analysis of the human β_2 receptor according to the methods of Kyte and Doolittle [79]. The standard one-letter code for amino acid residues is used. Black circles indicate residues in the human sequence that differ from those in hamster. Also noted are the potential sites of N-linked glycosylation.

Chapter 2

Materials and Methods

2.1 Materials and Laboratory Apparatus

Plasticware. All experiments were performed in lockable sterilized polypropylene test tubes (capacity of 0.2 to 50 ml). Liquids were transferred by automatic pipettors (Gilson) using sterilized pipette-tips. For volumes larger than 1 ml, sterilized graduated plastic pipettes were used. All tubes were sterilized by steam autoclaving (45min, 121°C).

Centrifuges. Centrifugations were run in cooled or unrefrigerated microcentrifuges (Eppendorf tubes) and refrigerated tabletop centrifuges.

Refrigeration. A refrigerator, a –20°C freezer and a –70°C freezer were used. A refrigerated cabinet (4°C) was used for microcentrifugation and ligation. Liquid nitrogen and crashed ice was also necessary for this study.

Light measurement. A UV/Vis spectrophotometer was used for DNA quantification. An ultraviolet (UV) transilluminating light source (302 nm) was necessary to visualize reaction products. A dark room equipped with a Polaroid camera and a developing unit was used for documentation and for sequencing gel procedures.

Electrophoresis systems. Two power supplies and three different gel-systems (vertical sequencing unit, submerged minigels and horizontal polyacrylamide cleangels) were used together with the necessary auxiliary equipment.

Common laboratory equipment. A pH meter, hotplate-stirrer, vortex mixer, balances for weighting reagents, laboratory timers and desiccators were required. All PCR steps were performed in a laminar flow.

2.2 Chemicals

All used chemicals were ACS grade or better (biochemical grade). Chemicals were obtained from Sigma, Merck and Gibco. Biochemicals, enzymes and markers were obtained from Sigma, Boehringer Mannheim, New England Biolabs, Pharmacia,

Promega and AGS (Angewandte Gentechnologie Systeme). Radiochemicals were obtained from NEN (DuPont, New England Nuclear).

2.3 Buffers

All solutions and buffer stocks were prepared using chemicals of analytical grade or better. Aqueous solutions were prepared using bidistilled water only. All solutions were sterilized by autoclavation (121°C, 30min) or filtration (pore size 0.22 μm).

2.3.1 Stock Solutions

$CaCl_2$, 1M: 147 g $CaCl_2 \cdot H_2O$ dissolved in 1 liter H_2O. Autoclaved, stored at room temperature.

EDTA, 0.5M: 186 g/l ethylenediamine tetraacetic acid, disodium salt and 10 N NaOH to pH 8.0, autoclaved.

Glucose, 20%: 20 g glucose dissolved in 100ml water, sterile filtered.

3M KAc/5M HAc: 29.4 g potassium acetate and 11.5 ml acetic acid in 100 ml of water. Autoclaved.

NaAc, 3M: 408 g $CH_3COONa \cdot 3H_2O$ in 1 liter H_2O. Acetic acid to pH 5.2, autoclaved.

PEG, 20%: 20 g Polyethylenglycol 6000 dissolved in 100ml water, sterile filtered.

SDS 20%: 200 g SDS (Sodium dodecyl sulfate) dissolved in 1 liter water at 60°C, not autoclaved!

TAE buffer, 40×: 193.6 g Tris-base, 15.2 g EDTA-$Na_2 \cdot 2H_2O$, 108.9 g Na acetate $\cdot 3H_2O$. Acetic acid to pH 7.2, H_2O to make 1 liter. Stored at room temperature.

TBE buffer, 10×: 60.5 g Tris-base, 30.8 g boric acid and 3.72 g EDTA-$Na_2 \cdot 2H_2O$, H_2O to make 1 liter. Stored at room temperature. Remade if major residues appear in solution.

Gel loading buffer, 5×: 50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in 5× TAE.

2.3.2 Other Solutions

Xgal, 2%: 2 mg Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, for formula see figure 3.1 on page 38) in 1 ml of dimethylformamide (DMF). Stored dark at –20°C.

IPTG, 100mM: 23.8 mg isopropyl-β-D-thiogalactopyranoside (for formula, see figure 3.2 on page 38) in 1 ml H_2O, stored at –20°C.

Ethidium bromide solution: 10 mg/ml. Used to visualize DNA: emits fluorescence when excited at 250–320nm (for formula, see figure 2.4 on page 33). Stored at 4°C in dark. Handle with gloves and avoid inhalation.

Proteinase K: 30 mg Proteinase K are dissolved in 1ml H_2O and stored at –20°C.

Lysis buffer I: 50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA. pH adjusted to 8.0, stored at 4°C.

Lysis buffer II: 0.2 M NaOH and 1% SDS in water, always prepared freshly.

TE buffer: 10 mM Tris (pH 7.4) and 0.1 mM EDTA–Na_2 (pH 8.0), stored at room temperature.

Sevac: 24 parts chloroform mixed with 1 part isoamyl alcohol.

Phenol: Phenol was redistilled at 160°C and 8-hydroxyquinoline added to a final concentration of 0.1%. It was then extracted several times with an equal volume of buffer (1M Tris [pH 8.0], followed by 0.1M Tris [pH 8.0] and 0.2% β-mercaptoethanol). Stored at 4°C in dark under equilibration buffer.

2.4 Culture Media

All culture media were sterilized by autoclavation (121°C, 40 min) before usage.

2.4.1 Liquid Media

LB medium (Luria-Bertani)**:** 10 g/l bacto-tryptone
5 g/l bacto-yeast extract
10 g/l NaCl
pH adjusted to 7.4 with NaOH

2.4.2 Solid Media

The following method was used to make hard agar-based culture plates for growth of bacterial colonies. To obtain solid media, 15 g agar were added to the liquid medium (LB) before autoclavation. After cooling to 50°C, the agarose-medium was poured directly into petri dishes, allowing about 30–35 ml per 85mm dish. To avoid bubbles, the surface of the medium was flamed with a bunsen burner before the gel hardened.

2.4.3 Antibiotics

To obtain selective media, ampicillin (50 mg/l), was added as antibiotic to the liquid media after autoclavation and cooling to 50°C. Ampicillin (6-[D-(–)-α-amino-α-phenylacetamido]-penicillanic acid) is a derivative of penicillin that kills growing cells by interfering with a terminal reaction in bacterial cellular synthesis.

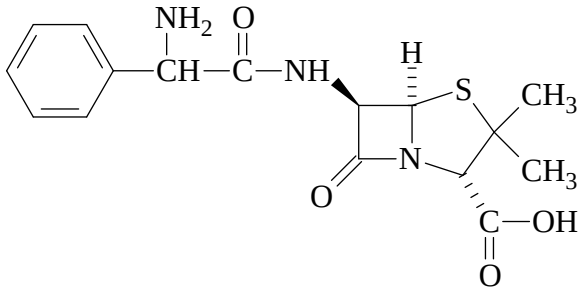

Figure 2.1: Ampicillin

2.4.4 Top-Agar

8 g agar were added to the liquid LB medium before autoclavation. After cooling to 50°C, the medium was poured into plates.

2.5 General Methods

2.5.1 DNA Preparation from Whole Blood

This method provides a high-quality preparation of high molecular weight genomic DNA from whole blood. Proteinase K is used to liberate nucleic acids from cells.

10 ml collected EDTA blood is mixed with 30 ml DNA lysis buffer and left in a 50 ml polypropylene screw-cap tube (Falcon) on crashed ice for one hour. After centrifuging for 15 minutes at 1500 rpm (4°C), the supernatant is decanted, the pellet (white blood cells) resuspended in 10 ml DNA lysis buffer and centrifuged again for 10 minutes at 1500 rpm (4°C). The supernatant is decanted again and the pellet resuspended in 5 ml 1× SE buffer. 0.25 mg Proteinase K and 250 μl 20% SDS are added and incubated overnight at 37°C.

1.5ml saturated NaCl (6M) are added, shaken and centrifuged for 20 minutes at 17000 rpm. The supernatant is decanted into a fresh tube, centrifuged once again and transferred to a fresh tube again. Two volumes absolute alcohol are added to precipitate DNA[1]. Large pieces of DNA can be removed by collecting fibres with a glass rod and transferring them to a new tube. Again, 1 ml absolute ethanol is added, centrifuged and the supernatant decanted. The pellet is washed with 200μl 70% ethanol, centrifuged shortly, decanted and desiccated in a vacuum evaporator (Speed Vac).

2.5.2 Nucleic Acid Quantification

Nucleic acids absorb UV light of 250 to 270 nm wavelength, with a maximum at 260 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 μg/ml for double-stranded DNA and 40 μg/ml for single-stranded DNA and RNA. The ratio between the readings at 260 nm and 280 nm ($A_{260/280}$ ratio) provides an estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 to 1.95, respectively. DNA producing a ratio below 1.6 should be re-purified[2].

2.5.3 Restriction Endonucleases and Their Use

Restriction endonucleases (REs) are bacterial enzymes that cleave double-stranded DNA. Type I REs are important in bacterial function but do not cleave DNA at specific sequences. Type II restriction endonucleases require highly specific sites for DNA cleavage. These enzymes allow cloning and purification (see section 3.3 on page 36) as well as sequence determination (RFLP, see section 4.2 on page 57). The 400 or so known REs are typically isolated from a variety of bacterial strains and available commercially [80].

[1] DNA should be a fibrous white material.

[2] ratio $\leqslant$ 1.6 indicates presence of protein and/or phenolic impurities.

Restriction endonucleases are present in bacteria presumably to destroy DNA from foreign sources (e.g., infecting bacteriophage) by cleaving the foreign DNA at specific restriction sites. The host bacteria DNA is protected from cleavage because specific recognition sites are modified, usually by methylation at one of the bases in the site, making the site no longer a substrate for RE cleavage. Host bacteria used to propagate cloned DNA in the laboratory are usually mutant in the host restriction genes; thus their intracellular enzyme activities will not destroy the foreign recombinant sequences.

The cleavage site specifities for many REs have been defined. They cut DNA within or near to their particular recognition sequences, which typically are four to six nucleotides in length with a twofold axis of symmetry. For example, the RE *Eco*RI, used for cloning of the β_2-adrenoceptor gene (see section 3.3 on page 36), requires that six base pairs occur in the following specific order:

5'...GAATTC...3'
3'...CTTAAG...5'

*Eco*RI recognizes this sequence and cleaves it in a unique fashion, resulting in two termini with protruding 5' ends:

...G and AATTC...
...CTTAA G...

These ends are complementary ("sticky") and can be enzymatically reattached to any other *Eco*RI generated termini using T4 DNA ligase (see 3.3.4 on page 40). Many restriction enzymes, like *Eco*RI, generate fragments with protruding 5' tails; others (e.g., *Pst*I) generate fragments with 3' protruding, cohesive termini, whereas still others (e.g., *Bal*I) cleave at the axis of symmetry to produce blunt-ended fragments. Each restriction endonuclease has a specific sequence and number of nucleotides required to create the recognition site. Some REs do not require a specific nucleotide in every position of the recognition site.

REs are useful because their specifity and the resulting ligatable termini allow dissection, analysis, and restructuring of DNA in a controlled, predictable, site-specific manner. The amount of RE activity is not usually defined using classical enzyme kinetics. Rather, RE activity is defined in practical terms for use in the laboratory. One unit (U) of activity for an restriction endonuclease is defined as the amount of enzyme that will cut 1 μg at all specific sites in a DNA sample (usually bacteriophage λ) in 1 hour at 37°C (or the appropriate temperature maximizing enzyme activity). The actual activity achieved may be different if additional RE digestion sites are present.

Analytical digests were performed in a volume of 20 μl (approximately 0.2 to 1 μg DNA). For preparative digests, the amount of DNA and the used digestion volume was increased appropriately. The amount of added enzyme and the incubation length was adjusted to the amount of digestible DNA. Manufacturers recommendations for incubation temperature were followed strictly. Only the shipped tenfold

buffers were used in digestion assays. For the specific restriction sites of the used enzymes, see section 4.2.3 on page 58.

2.5.4 Phenol Extraction

Perhaps the most basic of all procedures in molecular cloning is the purification of nucleic acid. The key step, the removal of proteins, can often be carried out simply by extracting aqueous solutions of nucleic acids with phenol. Such extractions are used whenever it is necessary to inactivate or remove enzymes (e.g., restriction endonucleases) that are used in one step of an operation before proceeding to the next.

The standard way to remove proteins from nucleic acid solution is to extract once with phenol and once with chloroform (Sevac). This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Furthermore, although phenol denatures proteins efficiently, it does not completely inhibit RNase activity [81]. The final extraction with chloroform also removes any lingering traces of phenol from the nucleic acid preparation.

The sample is diluted to a volume of about 400 to 500 μl. One volume TE-saturated phenol is added, mixed by vortexing[3]. A centrifugation step is performed to separate layers (room temperature, 2min, 13000 rpm). The upper aqueous layer is removed and transferred to a new tube. The lower organic layer and interface[4] is discarded. The aqueous layer is mixed with 0.5 volume Sevac (chloroform:isoamyl alcohol 24:1) and centrifuged for 2 minutes at 13000 rpm. The aqueous layer is removed and collected in a new tube [82, 83].

2.5.5 Ethanol Precipitation

The most widely used method for concentrating DNA is precipitation with ethanol. The precipitate of DNA, which is allowed to form at low temperature (–20°C or less) in the presence of moderate concentrations of monovalent cations, is recovered by centrifugation and redissolved in an appropriate buffer. The technique is rapid and is quantitative even with nanogram amounts of DNA.

0.1 volume of 3M sodium acetate (pH 5.2) are added to one volume of the aqueous solution. This gives a final concentration of 0.3 M sodium acetate[5,6]. 2 to 2.5 volumes of cold (–20°C) absolute ethanol are added. The tube is left at –70°C for

[3] Mix gently for large fragments of DNA to avoid shearing. These samples are mixed by repeated gentle inversions. Do not vortex.

[4] A small amount of aqueous layer may be lost with each extraction. Volumes are adjusted accordingly.

[5] Instead of sodium acetate, 5 M NaCl can be used. Salt is necessary to form a nucleic acid precipitate at low nucleic concentrations. Sodium acetate may be preferred over NaCl for its buffering capacity.

[6] If the DNA concentration is less than 1 μg/ml, addition of 10 μg glycogen increases recovery by facilitating precipitation.

one hour or at –20°C for several hours (overnight). DNA is precipitated by centrifugation for 20 minutes at 4°C (13000 rpm), the supernatant is discarded carefully, the remaining pellet washed with 300 μl of 80% ethanol to remove residual salt, centrifuged again for 5 minutes at 4°C, the supernatant discarded again and the pellet dried in a vacuum evaporator (Speed Vac, Savant). The pellet is resuspended appropriately[7]. It can also be stored as a pellet stably at –20°C [82, 83].

2.5.6 Oligonucleotide Purification

Oligonucleotide synthesis was carried out both, by a PCR-MATE EP391 DNA synthesizer, and a commercial manufacturer (MWG–Biolabs). The commercial product was delivered ready to use, whereas the non-commercial ones were delivered as crude elution products in concentrated ammonia. The protecting groups were cleaved off by overnight incubation at 55°C. A vacuum evaporation of ammonia and ethanol precipitation (see section 2.5.5 on the preceding page) was performed to purify short primers, whereas longer oligonucleotides were purified by Oligonucleotide Purification Cartridges (Applied Biosystems) using the following protocol:

The cartridge was flushed with 5 ml acetonitrile, followed by 5 ml 2.0M triethyl amine acetate. About 50 to 100 OD units of the crude, deprotected oligonucleotide still in concentrated ammonia were diluted with one third volume of bidistilled water to give a final volume of 4 ml. This solution was slowly pushed through the cartridge. The eluted fraction was saved and pushed through the cartridge once again. The loaded cartridge was slowly washed with 5 ml 1.5M ammonia hydroxide three times, and flushed with 5 ml water two times. The oligonucleotide was now detritylated with 5 ml of 2% trifluoroacetic acid, flushed with 2$\times$ 5 ml water again and the purified, detritylated oligonucleotide eluted by slowly washing the cartridge with 1 ml 20% acetonitrile.

2.5.7 Heat Denaturation of DNA

To produce single-stranded DNA for some reactions (see section 3.5 on page 43), the double-stranded nucleic acids had to be separated by heating to 95°C for 3 to 5 minutes. By rapid cooling to 0°C (crashed ice), a renaturation is inhibited. Heat denaturation also removes secondary structures, disturbing a sequencing electrophoresis.

[7] Suspension of DNA is often in TE buffer for its buffering capacity, for restriction digest usually in water.

2.6 PCR Techniques

The PCR[8] (Polymerase Chain Reaction) is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 20 cycles of PCR yields about a million-fold (2^{20}) amplification. This method, which was invented by Kary Mullis [84, 85] was originally applied by a group in the Human Genetics Department at Cetus to the amplification of human β-globin DNA and to the prenatal diagnosis of sickle-cell anemia [86–88].

Initially, the PCR used the Klenow fragment of *E. coli* DNA polymerase I [84, 86] to extend the annealed primers. This enzyme was inactivated by the high temperature required to separate the two DNA strands at the outset of each PCR cycle. The introduction of the thermostable DNA polymerase (*Taq* polymerase [89]) isolated from *Thermus aquaticus*[9] transformed the PCR into a simple and robust reaction which could now be automated by a thermal cycling device. The effect of varying the reaction parameters (e.g., enzyme, primer and Mg^{2+} concentration as well as the temperature cycling protocol) is discussed below. Although, for any given pair of oligonucleotide primers, an optimal set of conditions can be established, there is no single set of conditions that will be optimal for all possible reactions.

The initial PCR method based on DNA synthesis by the Klenow enzyme at 37°C was not highly specific. The use of the *Taq* polymerase not only simplified the PCR procedure but significantly increased the specifity and the overall yield of the reaction. The higher temperature optimum for the *Taq* polymerase ($\sim$ 75°C) allowed the use of higher temperatures for primer annealing and extension, thereby increasing the overall stringency of the reaction and minimizing the extension of primers that were mismatched with the template.

Although the PCR is considered primarily a method for producing copies of a specific sequence, it is also a very powerful and precise way of altering a particular template sequence. Since the oligonucleotide primers become physically incorporated into the amplified product and mismatches between the 5' end of the primer and *initial* template[10] are tolerated, it is possible to introduce new sequence infor-

[8] The PCR process is covered by US patents 4,683,195 and 4,683,202 and foreign equivalents owned by Hoffman-La Roche.

[9] *T. aquaticus* YT1, a thermophilic, eubacterial microorganism capable of growth at 70°- 75°C, was isolated from a hot spring in Yellowstone National Park and first described in [90] 25 years ago.

[10] After the first few cycles, virtually all of the templates have been synthesized in previous cycles and, therefore, contain the primer sequences.

mation adjacent to the target sequence via the primers. Thus, for cloning a given sequence, one is no longer constrained by the naturally occurring restriction sites and one may add any restriction enzyme recognition sequence to the 5' ends of the primer [91] creating a new restriction site in the double-stranded amplification product. Furthermore, specific nucleotide substitutions, insertions, and deletions can also be introduced into the amplified product with the appropriate primers.

2.6.1 Standard PCR Conditions

In the few years since its introduction [84–86], the polymerase chain reaction has become a widespread research technique. This popularity of the PCR is primarily due to its apparent simplicity and high probability of success. In fact, the PCR is a relatively complicated and, as yet, incompletely understood biochemical brew; where constantly changing kinetic interactions among the several components determine the quality of the products obtained. Although good results will be obtained in most cases, there are a number of parameters that can be explored if better results are required or if the reaction fails altogether.

Because of the great variety of applications in which PCR is used, it is probably impossible to describe a single set of conditions that will guarantee success in all situations. Nevertheless, the reaction outlined below proved to be adequate for most amplifications and in those cases where problems are encountered, it provides at least a starting point from which modifications can be attempted (e.g., the Mg^{2+} concentration should be checked in every new PCR setup).

The standard PCR is typically done in a 50 μl volume and, in addition to the sample DNA, contains 50mM KCl, 10mM Tris $\cdot$ HCl (pH 8.4), 1.5mM $MgCl_2$, 100 μg/ml gelatin, 0.25 μM of each primer, 200 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 2.5 units of *Taq* polymerase. The type of the DNA sample will be variable, but it will usually have between 10^2 and 10^5 copies of template (e.g., 0.1 μg human genomic DNA).

2.6.2 Primer Selection

Unfortunately, the approach to the selection of efficient and specific primers remains somewhat empirical. There is no set of rules that will ensure the synthesis of an effective pair of primers. Yet it is the primers that more than anything else determine the success or failure of an amplification reaction. The following guidelines aid primer design:

1. Whenever possible, select primers with a random base distribution and a GC content similar to that of the amplified fragment.

2. Avoid sequences with a significant secondary structure (e.g., hairpin loops), particularly at the 3' end of the primer.

3. Check the pair of primers for mutual complementary. In particular, avoiding primers with 3' overlaps will reduce incidence of "primer dimer".

Most primers will be between 20 and 30 bases in length and the optimal amount to use in an amplification will vary. Sequences not complementary to the template can be added to the 5' end of the primers. These exogenous sequences become incorporated into the double-stranded PCR product and provide a means of introducing restriction sites [91] or regulatory elements at the ends of the amplified target sequence [92].

"Primer dimer" is an amplification artifact often observed in the PCR product, especially when many cycles of amplification are performed on a sample containing very few initial copies of template. It is a double-stranded fragment whose length is very close to the sum of the two primers and appears to occur when one primer is extended by the polymerase over the other primer. The resulting concatenation is an extremely efficient PCR template that can, if it occurs at an early cycle, easily overwhelm a reaction and become the predominant product.

2.6.3 Determination of Melting Temperatures

The melting temperature of a specific oligonucleotide primer (T_m) can be calculated by several different ways. The used primer pairs should be constructed to have a similar T_m. The simplest equation, often used, is

$$T_m = 2(A+T) + 4(G+C)$$

where A, C, G, and T symbolize the count of the particular nucleotide in the primer. This formula was developed for hybridization assays with oligonucleotides at a salt concentration of 1 M [93]. The equation is inaccurate for calculation of T_m of primers longer than 20 nt. Usually, the chosen annealing temperature is about 5°C below the calculated T_m and is determined by trial and error.

Other equations used are

$$T_{m(gc)} = 81.5 + 16.6(\log[J^+]) + 0.41(\%G+C) - (600/l)$$

where J^+ is the concentration of monovalenced cations and l is the length of the oligonucleotide [94]. This formula is correct for oligonucleotides with a length between 14 and 70 nucleotides.

$$T_p = 22 + 1.46(l_n)$$

This formula calculates T_p, the optimal annealing temperature ± 2–5°C. l_n is the effective length of the primer: $2(G+C)+(A+T)$ [95]. This equation should result in correct values for oligonucleotides with 20 to 35 nucleotides.

2.6.4 The PCR Buffer

Changes to the PCR buffer will usually affect the outcome of the amplification. In particular, the concentration of $MgCl_2$ can have a profound effect on the specifity and yield of an amplification. Concentrations of about 1.5 mM are usually optimal

(with 200 μM each dNTP), but in some circumstances, different amounts of Mg^{2+} may prove to be necessary. For that reason, every established PCR in this thesis was preceded by an $MgCl_2$ optimization assay. Generally, excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield.

The deoxynucleotide triphosphates are usually present at 50 to 200 μM of each. Higher concentrations may tend to promote misincorporations by the polymerase and should be avoided [96]. At 50 and 200 μM, there is sufficient precursor to synthesize approximately 6.5 to 25 μg of DNA, respectively.

Taq polymerase is available from a number of vendors. For amplification reactions involving DNA samples with high sequence complexity, such as genomic DNA, there is an optimum concentration of *Taq* polymerase, usually 1 to 4 units per 100 μl. Increasing the amount of enzyme beyond this level can result in greater production of non-specific PCR products and reduce yield of the desired target fragment.

2.6.5 Factors Affecting Specifity

There are a number of factors that can affect the specifity of the amplification reaction. The stringency of the annealing step can be controlled to some extent by adjusting the annealing temperature. Minimizing the incubation time during the annealing and extension steps will limit the opportunities for mispriming and extension by molecules of otherwise idle DNA polymerase. Reducing primer and enzyme concentrations also serves to limit mispriming, particulary the type that leads to dimerization. Finally, changing $MgCl_2$ levels can further improve specificity, either by increasing the stringency of the reaction or by direct effects on the polymerase itself (activity, processitivity, etc.).

2.6.6 Fidelity of Amplification

The DNA polymerase makes errors at a low but finite rate that varies depending on the enzyme, condition of the reaction, and the sequence [97]. For example, the errors produced by *Taq* polymerase are primarily single-base substitutions. That rate can be higher than 10^{-3} per nucleotides at high Mg^{2+} and high nucleotide concentrations, and less than 10^{-6} per nucleotide under other conditions.

During the PCR, amplification products serve as templates for the subsequent cycles. Therefore, sequence changes caused by errors of the DNA polymerase are "inherited". Since there is no selection based on the functional significance of sequence information, these sequence changes can accumulate. Consequently, a significant percentage of the amplified fragments may carry "mutations". The percentage of fragments with a correct sequence can be mathematically estimated, assuming a random distribution of errors, and that both mutated and correct sequences are amplified with the same efficiency [98, 99]. If amplification proceeds with an efficiency of k, then the amount of old (O, present at the beginning of the

cycle) and new (N, synthesized during the cycle) strands at the end of each cycle have the following relationship:

$$O = \frac{1}{k+1}$$
$$N = \frac{k}{k+1}$$
$$0 < k < 1$$

The probability of producing fragments without error (p) in one cycle of amplification is given by the probability of no-hit in a Poisson distribution.

$$p = \exp(-mL)$$

where m is the error rate of the polymerase per nucleotide and L is the length of amplification unit in nucleotides.

Therefore, the fraction of strands with a correct sequence after n cycles, $F(n)$, can be estimated by

$$F(n) = f^n = \frac{[1 + k\exp(-mL)]^n}{(k+1)^n}$$

This estimate may not be appropriate if the reaction is initiated from a very small amount of DNA (e.g., less than 10^3 molecules), when the timing of occurrence of the first mutation can significantly alter the error rate.

When the errors during PCR are distributed evenly throughout the fragment, no particular mutated sequence constitutes a major subpopulation. In this case the predominant species at each nucleotide position in the product is that of the initial sequence, and direct sequencing of the product is an appropriate method. The situation is different, when PCR products are used as cloning substrates. Each clone originates from a single molecule in the PCR product mixture, and therefore may represent errors occurring during the amplification process.

2.6.7 Used Materials and Precautions

Because the polymerase chain reaction is capable of amplifying as little as a single molecule of DNA, precautions have to be taken to guard against contamination of the reaction mixture with trace amounts of DNAs that could serve as templates [100]. Contaminations can be controlled in several ways. DNA for PCR was isolated and purified in a different area to the PCR sample preparation. PCR sample preparation was performed in a laminar flow only, using a separate set of automatic pipettors. 0.45 ml eppendorf tubes (sterilized) were used for the PCR, run in a three block thermocycler (Biometra TRIO) with block control. A negative control was always run with the cycled samples. The water used in PCR was heat sterilized, distilled water subjected to an additional UV sterilization step. It has to be emphasized that all three major steps of PCR (DNA preparation, sample preparation and PCR) were performed in different rooms.

2.6.8 Oligonucleotide Quantification

The molar concentration of the used oligonucleotide primers was determined by reading the absorption of a 1:100 diluted sample at 260 nm. The molar extinction coefficient was calculated using the following per nucleotide coefficients:

N	ε_{260} [cm^2/mol]
T	8400
A	15200
G	12010
C	7000

Table 2.1: Molar extinction coefficients of nucleotides

2.7 Gel Electrophoresis

Molecules in a mixture can be separated according to size by electrophoresis, a technique dependent on the fact that dissolved molecules in an electric field move at a speed determined by their charge-mass ratio. Many successful variations of electrophoresis are in general use; the separation of small molecules, such as amino acids and nucleotides, is one example.

Nucleic acids in solution generally have a negative charge because their phosphate groups are ionized; thus they migrate toward a positive electrode. However, nucleic acid molecules consisting of long chains have almost identical charge-mass ratios, whatever their length, because each residue contributes about the same charge and mass. Therefore, if the electrophoresis of nucleic acids were simply carried out in solution, little or no separation of molecules of varying lengths would occur.

Molecules are now most commonly subjected to electrophoresis in a *gel*, rather than a liquid solution. The size of the pores in such gels limits the rate at which molecules can move through them. Nucleic acids with identical charge-mass ratios separate according to length, with the longer ones moving more slowly. Even very long nucleic acids (chains containing 10,000 to 20,000 residues) that differ in length by only a few percentage points can be separated. In mixtures containing chains of 500 nucleotides or less (and separated on polyacryl amide gels), each chain length can be resolved, which made DNA sequencing possible.

2.7.1 Agarose Gel Electrophoresis

The standard method used to separate, identify, and purify DNA fragments is electrophoresis through agarose gels. The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragments that cannot be separated adequately by other sizing procedures. Furthermore, the location of DNA within the

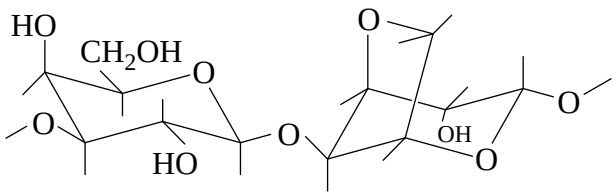

Figure 2.2: Agarose structure unit

gel can be determined directly: Bands of DNA in the gel are stained with the intercalating dye ethidium bromide; as little as 1 ng of DNA can be detected by direct examination of the gel in ultraviolet light [101].

Agarose, which is extracted from seaweed, is a linear polymer whose basic structure is shown in figure 2.2. Commercially available agarose is not completely pure, it is contaminated with other polysaccharides, salts, and proteins. These differences can affect both, the migration of the DNA and the ability of the DNA recovered from the gel to serve as a substrate to enzymatic reactions. Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The electrophoretic migration rate of DNA through agarose gels is dependent upon four main parameters, which will be discussed below.

The molecular size of the DNA. Molecules of linear, duplex DNA, which are believed to migrate in an end-on position [102, 103] travel through gel matrices at rates that are inversely proportional to the logarithm of their molecular weights [104].

The agarose concentration. A DNA fragment of given size migrates at different rates through gels containing different concentrations of agarose. There is a linear relationship between the logarithm of the electrophoretic mobility of DNA (μ) and gel concentration (τ), which is described by the equation:

$$\log \mu = \log \mu_0 - \mathrm{K}_r \tau$$

where μ_0 is the free electrophoretic mobility and K_r is the retardation coefficient, a constant that is related to the properties of the gel and the size of the migrating molecules. Thus, by using gels of different concentrations, it is possible to resolve a wide-range of DNA fragments.

The conformation of the DNA. Closed circular, nicked circular and linear DNA of the same molecular weight migrate through agarose gels at different rates. The relative mobilities of the three forms are dependent primarily on the agarose concentration in the gel but are also influenced by the strength of the applied current,

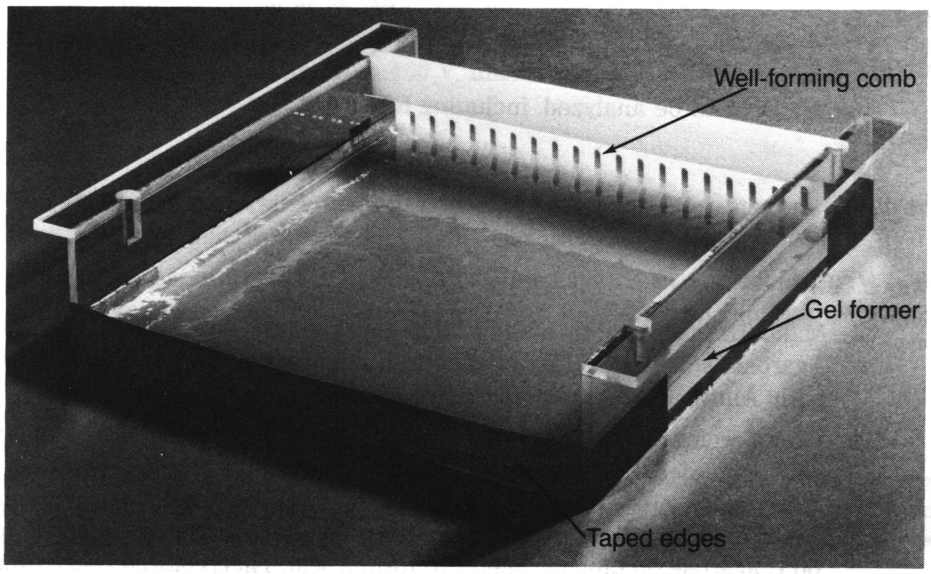

Figure 2.3: Photograph of agarose gel, showing taped edges and well-forming comb in position.

the ionic strength of the buffer, and the density of superhelical twists in the DNA.

The applied current. At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA is increased differentially. Thus, the effective range of separation of agarose gels decreases as the voltage is increased. Gels should be run at no more than 5 V/cm.

Base composition and temperature. The electrophoretic behavior of DNA in agarose gels (by contrast to polyacrylamide gels [105]) is not significantly affected either by the base composition of the DNA [106] or the temperature at which the gel is run. Thus, in agarose gels the relative electrophoretic mobilities of DNA fragments of different sizes do not change between 4°C and 30°C.

The agarose concentration is varied for different fragment ranges. For analyzing the complete β_2-adrenoceptor codon (1239 bp), a 1% agarose gel[11] is made by dissolving agarose in $1\times$ TAE buffer by heating in a microwave oven. After cooling to about 60°C, ethidium bromide[12] is added to a final concentration of 0.5 μg/ml. The agarose solution is poured into a taped gel former mold to make the gel. A well-forming comb (12 slots for minigels) is placed near one edge of the gel. The gel is cooled to harden until it becomes milky and opaque (approximately one

[11] 1% agarose gel is good for DNA pieces that range in size from 0.6 to 3 kb. For smaller pieces of DNA (150–700 bp), 2.5 to 3% agarose was used.

[12] Ethidium bromide is a cancerogen, handle with care!

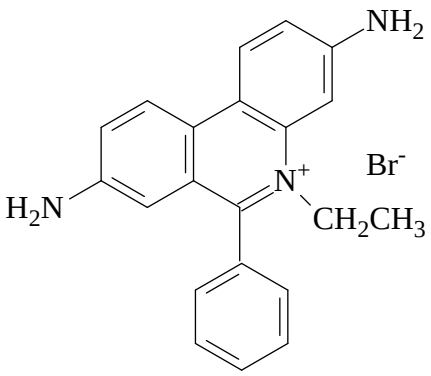

Figure 2.4: Ethidium bromide

hour). The gel mold is placed horizontally into the electrophoresis tank, which is filled with 1× TAE [13] (0.5 μg/ml ethidium bromide).

The gel loading buffer is applied to the samples and they are carefully added to individual wells[14]. The electrophoresis is run by 70–100 V/20–80 mA for about an hour or at 20 to 30 V overnight. The size of fragments can be determined by calibrating the gel, using known standards (e.g., λ DNA *Eco*RI / *Hind*III digest, Boehringer Mannheim, or 100bp ladder, BioVentures, Inc.), and comparing the distance the unknown fragment has migrated.

The most convenient method of visualizing DNA in agarose gels is by use of the fluorescent dye ethidium bromide [101] (2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide). This substance (see figure 2.4) contains a planar group that intercalates between stacked bases of DNA. The fixed position of this group and its close proximity to the bases causes dye bound to DNA to display an increased fluorescent yield compared to dye in free solution. UV-irradiation absorbed by the DNA at 260 nm and transmitted to the dye, or irradiation absorbed at 300 nm and 360 nm by the bound dye itself, is emitted at 590 nm in the red-orange region of the visible spectrum.

Ethidium bromide can be used to detect both single- and double-stranded nucleic acids. However, the affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is poor.

[13] For small DNA fragments, TBE buffer is used for the preparation of the gel and the buffer to sharpen nucleic acids bands.

[14] Approximately 10 to 15 μg of genomic DNA, or 20 ng per single band of cloned DNA, is readily detected with ethidium bromide staining. If too much DNA is loaded, the band will be distorted.

2.7.2 Freeze–Squeeze–Technique

This method was used to extract DNA bands from agarose gels. The bottom of an 1.5 ml microfuge tube is pierced and stuffed with glass wool. The small cut gel pieces are filled into the tube and the tube submerged in liquid nitrogen. This destroys the gel structure. After shock-freezing, the tube is inserted into another one and centrifuged for 15 minutes at room temperature at 13000 rpm to collect the aqueous layer holding the DNA in the lower tube. The DNA solution can now be subjected to a phenol extraction (see section 2.5.4 on page 23) and ethanol precipitation (see section 2.5.5).

2.7.3 Polyacrylamide Gels

Polyacrylamide gels are used to analyze and prepare fragments of DNA less than 1 kb in length [107]. They may be cast in a variety of polyacrylamide concentrations, ranging from 3.5% to 20%, depending on the sizes of the fragments of interest (see table 3.3 on page 48).

In the presence of free radicals, which are usually supplied by ammonium persulfate and stabilized by TEMED (N,N,N',N'-tetramethylenediamine), a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains. When the bifunctional agent N,N'-methylenebisacrylamide is included in the polymerization reaction, the chains become cross-linked to form a gel, whose porosity is determined by the length of the chains and the degree of cross-linking.

The length of the chains is determined by the concentration of acrylamide in the polymerization reaction (between 3.5% and 20%): one molecule of cross-linker is included for every 29 monomers of acrylamide. Polyacrylamide gels are most commonly poured between two glass plates that are held apart by spacers. In this arrangement, most of the acrylamide solution is shielded from the exposure to the air, so that inhibition of polymerization by oxygen is confined to a narrow layer at the top of the gel. Sequencing gels are invariably run in the vertical position, whereas cleangels are run horizontally.

Polyacrylamide gels can range in length from 10 cm to 100 cm, depending on the separation required. However, they have three major advantages over agarose gels: Their resolving power is so great that they can separate molecules of DNA whose lengths differ by as little as 0.2% (i.e., 1 bp in 500 bp). They can further accommodate much larger quantities of DNA than agarose gels, and DNA recovered from polyacrylamide gels is extremely pure.

Sequencing gels (see section 3.5.3 on page 47) are polymerized in the presence of an agent (urea) that suppresses base pairing of nucleic acids. Denatured DNA migrates through gels at a rate that is almost completely independent of its base composition and sequence.

Chapter 3

Sequencing of the Coding Region

In a first step it was necessary to characterize the sequence of the human β_2-adrenergic receptor. Firstly, sequencing the complete structure gene of the receptor from both, salt resistant and salt sensitive subjects was performed. Secondly, the revealed sequences were compared with published gene sequences [77,78]. Based on the results obtained and the mutations discovered screening methods for rapid screening of these mutations had to be developed. Furthermore, the sequencing results were used as references for the scanning methods described later on (see chapter 4).

3.1 Sequencing Strategy

The complete structure gene of the human β_2-adrenoceptor of subjects with known phenotype (β_2-adrenoceptor density) was amplified using the established PCR protocols (see section 3.2 on the next page), and cloned into a pUC18 (see section 3.3 on page 37 and figure 3.3 on page 39) vector. As the transferred insert had a length of 1397 base pairs, it was not practicable to sequence it in one run. Although it would have been possible to sequence the insert using commercially available Forward and Reverse primers only, a smarter solution to the problem was to subdivide the coding region in easily reproducible overlapping 200 to 400 bp long sequence sections. This made it possible to read found mutations several times. The primers used and the starting points for the sequence reaction are summarized in table 3.1.

Table 3.1: Sequencing primers for the β_2-adrenoceptor

Primer	Sequence (5'→ 3')	size	from–to
Forward Primer[a]	CGC CAG GGT TTT CCC AGT CAC GAC	24	–47
β1490	CTT CAC TGG CCT GTG CTG	18	1481–1498
β1740	TCA TTC TGA TGG TGT GGA TT	20	1721–1740
β1980	ACA AAT CTG AGG GCC GC	17	1964–1980
Reverse Primer[a]	TCA CAC AGG AAA CAG CTA TGA C	22	–34

[a] pUC/M13 sequencing primers: the primer location refers to the distance from the polylinker site

3.2 PCR of the Coding Fragment

PCR was performed in 50 μl volumes using 0.5 μg of genomic DNA extracted from whole blood (see section 2.5.1 on page 21). Added were 4 μl of a dNTP mix (200 μM each, Pharmacia), 5 μl tenfold *Taq*-polymerase buffer (Promega), 1.5mM $MgCl_2$ (3 μl 25mM $MgCl_2$) and 2.5 units *Taq*-DNA polymerase (Promega). The polymerase buffer consisted of 50mM KCl, 10mM Tris · HCl (pH 9.0 at room temperature) and 1% Triton X-100. Both primers (β25 and β231) were used at a final concentration of 0.5 μM each (25 pmol added).

The reaction volume was mixed, shortly centrifuged and overlayed with 30 μl mineral oil. After amplification, the mineral oil was removed by extraction with 100 μl chloroform and the amplified DNA precipitated with two volumes absolute ethanol (see section 2.5.5 on page 23). The isolated pellet was then resuspended in 15 μl water and a 4 μl sample was visualized on an agarose-gel to ensure positive reaction together with a negative control (PCR mixture without added template).

The primers had an integrated *Eco*RI linker for subcloning:

5' primer: β25 GGG AAT TCT TAC CTG CCA GAC TGC G
3' primer: β231 GGG AAT TCA GAA GGA TGC CCT TCC TTC TGC
*Eco*RI linker site

The following table summarizes the characteristics of the used primer pair β25/β231. The theoretical melting point T_m can be calculated using different methods, all three referenced in section 2.6.3 on page 27 are given.

Primer	n	T_m [°C]	$T_{m(gc)}$ [°C]	T_p [°C]	GC Cont. [%]	Ann. T. [°C]	from–to	size
β25	18	58.0	57.89	64.34	61.0	53	1242–1259	1411
β231	23	72.0	63.29	74.56	56.5		2639–2617	

Table 3.2: Cloning primers characteristics

For cycle protocol, an initial denaturation step (95°C for 2 minutes) was followed by an annealing step (53°C), extension step (72°C) and denaturing step (94°C), each of one minute. After 35 cycles, a final extension step (72°C) of 5 minutes was added.

3.3 Subcloning the Insert in a pUC18 plasmid

Plasmids are extrachromosomal genetic elements found in a variety of bacterial species. They are double-stranded, closed circular DNA molecules that range in size from 1 kb to greater than 200 kb. Often, plasmids contain genes coding for enzymes that, under certain circumstances, are advantageous to the bacterial host. Among the phenotypes conferred by different plasmids are:

resistance to antibiotics
production of antibiotics
degradation of complex organic compounds
production of colicins
production of enterotoxins
production of restriction and modification enzymes

Under natural conditions, many plasmids are transmitted to new hosts by a process similar to bacterial conjugation. In the laboratory, however, plasmids can be transferred to bacteria by an artificial process, known as transformation, in which they are introduced into bacteria that have been treated in ways that make some of the cells temporarily permeable to small DNA molecules (see section 3.4 on page 41).

For the most part, replication of plasmid DNA is carried out by the same set of enzymes used to duplicate the bacterial chromosome. Some plasmids are under *stringent control*, which means that their replication is coupled to that of the host. Plasmids under *relaxed control*, on the other hand, have copy numbers of 10–200. More importantly, the copy number can be increased to several thousands per cell if host protein synthesis is stopped (e.g., by treatment with chloramphenicol) [108]. In the absence of protein synthesis, replication of relaxed plasmids continues, whereas replication of chromosomal DNA ceases.

A plasmid employed as a cloning vector should possess several properties. It should be relatively small and replicate in a relaxed fashion (like the used pUC vector). In addition, it should carry one or more selection markers to enable identification of transformants and to maintain the plasmid in the bacterial population. Finally, it should contain a single recognition site for one or more restriction enzymes in regions not essential for plasmid replication. Preferably, these restriction sites, into which foreign DNA can be inserted, should be located within the genes coding for selectable markers so that insertion of a foreign DNA fragment inactivates the gene.

The most commonly cited plasmid vector is pBR322. An alternative to pBR322 cloning of DNA is to use pUC plasmids. These are modified pBR322 vectors with the ampicillin resistance gene and an added polylinker site, similar to that in the M13mp vectors. Also included in pUC plasmids is the 5' end of the *lac Z* gene[1] (β-galactosidase), which is disrupted by insertion of cloned fragments into the polylinker site, allowing a colour selection of insert-bearing fragments. The amino-terminal portion of the β-galactosidase protein produced in the infected cells is able to complement ("α complementation") a defective β-galactosidase gene present on the F episome in the host cell. This complementation produces active β-galactosidase (*gal*$^+$), which gives rise to a blue colour when the phage and cells are grown in the presence of the inducer isopropyl-thiogalactoside (IPTG, see figure 3.2 on the following page) and the chromogenic substrate Xgal (see fig-

[1] it is a fragment of the *E. coli lac* operon, containing the regulatory region and the coding information of the first amino acids.

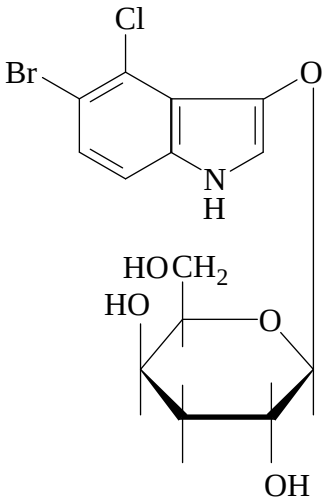

Figure 3.1: 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal)

ure 3.1). Insert-bearing plasmids will fail to complement the *gal*⁻ genotype of an α-complementing *E. coli* host. The plasmid transformant remains *gal*⁻, and cannot metabolize the chromogenic substrate Xgal to a blue pigment. Resulting colonies, grown on LB/ampicillin plates supplemented with IPTG and Xgal will remain white [109].

3.3.1 Preparation of the Vector

These small plasmids (2.7 kb, see figure 3.3 on the next page) have the pBR322 ampicillin resistance gene, the pBR322 origin of replication, and a portion of the *lac Z* gene of *E. coli*. Within the *lac* region is a polylinker sequence of unique RE

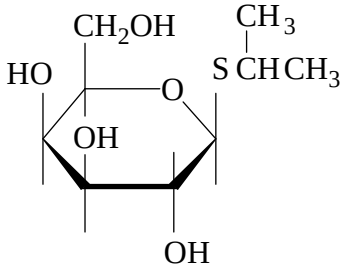

Figure 3.2: Isopropyl-β-D-thiogalactopyranoside (IPTG)

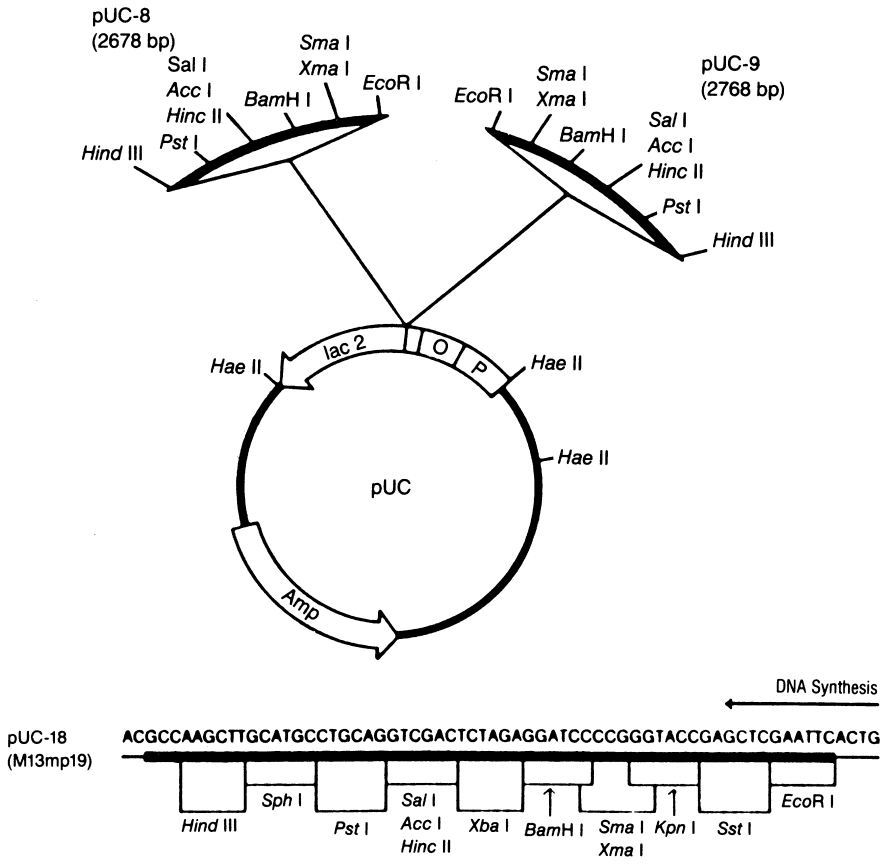

Figure 3.3: pUC plasmids, indicating restriction maps of polylinker regions of pUC8, 9 and 18

recognition sites identical to those in the multiple cloning sites of M13. They grow to a high copy number and can be amplified with chloramphenicol.

The pUC vector is linearized by *Eco*RI digest (see figure 3.3) to generate termini of the vector that are compatible with the cohesive termini found in the insert fragment to be subcloned.

6 μl pUC18 were digested[2] in a volume of 20 μl with 20 U (1 μl) *Eco*RI (New England Biolabs).

3.3.2 Dephosphorylation of the Linearized Vector

During ligation, DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group. Recircularization of plasmid DNA can there-

[2] see section 2.5.3 on page 21.

fore be minimized by removing the 5' phosphates from both ends of the linear DNA with calf intestinal phosphatase [110, 111]. As a result, neither strand of the duplex can form a phosphodiester bond. However, a foreign DNA segment with 5'-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open circular molecule containing two nicks. Because circular DNA (even nicked circular DNA) transforms much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids.

For dephosphorylization, the vector digest (see section 3.3.1), 5 μl CIP buffer (1mM $ZnCl_2$, 1mM $MgCl_2$, 10mM Tris, pH 8.3) and 1 μl calf intestinal phosphatase (Boehringer Mannheim) were added to an end volume of 50 μl and incubated at 37°C for 30 minutes. An additional batch of enzyme (1.5 μl) was added and incubated for further 30 minutes. The dephosphorylization was followed by a phenol extraction (see section 2.5.4 on page 23) and an ethanol precipitation (see section 2.5.5).

3.3.3 Preparation of the Insert

The PCR product has to be digested with the same restriction endonuclease as the vector in order to create cohesive ends, as the ligation of blunt-ended fragments yields poor results (K_m for the activity of T4 ligase on blunt-ended DNA is nearly 100 times higher than its K_m on DNA with cohesive ends). Furthermore, in contrast to blunt end cloning, it is not relevant in overhanging end cloning whether or not terminal dAs are added to the PCR product, since they are removed if present.

5 μl of the ethanol precipitated PCR product (see section 3.2 on page 36) are digested with 20 U (1 μl) *Eco*RI (New England Biolabs) in 20 μl volume for 90 minutes at 37°C. The digest is separated on agarose, and the insert band cut out. After eluating the DNA (see section 2.7.2 on page 34), a phenol extraction (see section 2.5.4 on page 23) and ethanol precipitation (see section 2.5.5) followed. The final DNA pellet was resuspended in 10 μl water and now ready for ligation.

3.3.4 Ligation of Insert into the Vector

After plasmid preparation, the fragment to be subcloned is ligated into the prepared vector. The ratio of prepared vector to prepared insert is estimated by agarose gel electrophoresis[3] of 2 μl of each. The ligation is performed using a T4 DNA ligase. The enzyme, a single polypeptide, catalyzes the formation of a phosphodiester bond between adjacent 3' hydroxyl and 5' phosphate termini in DNA [112].

The appropriate amounts of vector and insert were incubated at 14°C overnight with 1 μl ($\sim$ 20 Weiss units[4]) T4 ligase (Promega) in 1$\times$ ligase buffer (50 mM Tris

[3] In general, it is useful to have approximately three to four times the molar amount of the insert compared to the vector for optimal formation of clones.

[4] One Weiss unit is defined as the amount of enzyme required to catalyze the exchange of 1nmol of ^{32}P from pyrophosphate into Nordit-adsorbable material in 20 minutes at 37°C [112]. 0.01 Weiss unit is that amount of enzyme required to catalyze the ligation of $\geqslant$ 95% of 1μg of λ *Hind*III DNA

[pH 7.4], 10 mM $MgCl_2$, 20 mM DTT, 5 μg/ml BSA, 1 mM ATP) in 20 μl volume.

3.4 Transformation of Bacteria

To identify and amplify large quantities of cloned DNA, the ligated vector has to be transformed into a bacterial host. Most methods for bacterial transformation are based on an observation of MANDEL and HIGA [113], who demonstrated that uptake of bacteriophage λ DNA is enhanced by treatment of bacterial cells with calcium chloride. Many variations in this basic technique have been described, all directed towards optimizing the efficiency of transformation for different bacterial strains. Most protocols yield 10^5 - 10^7 transformants per microgram of intact pBR322 DNA. Impressive though this efficiency is, it is important to realize, first, that only a very small proportion of the cells are competent to incorporate plasmid DNA in a stable fashion, and second, that only 1 DNA molecule in approximately 10,000 is successful at transformation. Once inside the bacterium, the plasmid DNA replicates and expresses the drug-resistance markers that allow the transformed cells to survive in the presence of an antibiotic.

The ability of bacteria to take up DNA is short-lived. After exposure to agents that enhance uptake, most strains of bacteria remain in a competent state for only 1–2 days.

3.4.1 Preparation of Competent Hosts

For the transformation of pUC plasmids, DH5α cells [114], an *E. coli* strain, were used. Non-competent cells[5] were plated on an agarose plate and incubated overnight. The next day, 50 ml LB medium were inoculated with one colony. The cells were grown with vigorous shaking at 37°C to a density of $\simeq 5 \cdot 10^7$ cells/ml. This usually takes 2-3 hours ($OD_{260} \sim 0.3$). The cell suspension is centrifugated at 3000 g for 5 minutes at 4°C. The pellet is resuspended in 20 ml of 0.1 M $CaCl_2$ and left on ice for 30 minutes. Then the suspension is centrifuged again at 3000 g for 5 minutes at 4°C. The cells are now resuspended in 2 ml $CaCl_2$ and the competent cells stored for 2-3 days at 4°C.

For maximum transformation efficiency, it is very important that the bacterial culture is in the logarithmic phase of growth and that cell density is low at the time of treatment with calcium chloride; and that the cells are maintained at 4°C for 12-24 hours. During this period, the efficiency of transformation increases fourfold to sixfold [115].

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fragments at 16°C in 20 minutes.

[5]The DH5α strain presents the following genotype: F^-, Φ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F), U169, λ^-

3.4.2 Transformation of Competent DH5α Cells

An aliquot of the ligation batch (see section 3.3.4 on page 40) was added to 200 µl competent cells, mixed and stored on ice for 30 minutes. Up to 40 ng of DNA can be used for each transformation reaction. The mixture was transferred to a water bath, preheated to 42°C, for 2 minutes. After addition of 1 ml LB medium, the cells were incubated at 37°C for one hour without shaking. This period allows the bacteria to recover and to begin to express antibiotic resistance.

The medium was now spread onto selective media (see section 2.4.3 on page 20), which were treated with 20 µl 100mM IPTG and 40 µl Xgal before. The cells were spread over the entire surface of the medium by moving a sterile, bent glass rod back and forth gently over the agar surface. The glass spreader was sterilized by dipping in 95% ethanol and then holding it into the flame of a bunsen burner.

The plates were incubated overnight at 37°C to allow plaque formation and colour indicator reaction[6]. Plaques were picked within the next 24 hours.

3.4.3 Isolation of the Plasmid after Alkaline Lysis

The plasmid "miniprep" method is useful for preparing partially purified plasmid DNA in small quantities from a number of transformants. It relies on an alkaline SDS lysis to free the plasmid DNA from the cell, leaving behind the *E. coli* chromosomal DNA with cell wall debris. The protocol described involves three basic steps: growth of bacteria and amplification of the plasmid; harvesting and lysis of the bacteria; and purification of the plasmid DNA.

These purification procedures exploit in one way or another the two major differences between *Escherichia coli* DNA and plasmid DNA:

1. The *E. coli* chromosome is much larger than the DNA of plasmids used as vectors.

2. The bulk of *E. coli* DNA extracted from cells is obtained as broken, linear molecules. By contrast, most plasmid DNA is extracted in a covalently closed, circular form.

The purification protocol therefore involves a differential precipitation step, in which the long strands of *E. coli* DNA, entangled in the remnants of lysed cells, are preferentially removed. Because each of the complementary strands of plasmid DNA is a covalently closed circle, the strands cannot be separated (without breaking one of them) by conditions such as exposure to mild alkali (up to pH 12.5), which break most of the hydrogen bonds of DNA. Closed circular molecules regain their native configuration when returned to neutral pH.*E. coli* remains in the denatured state. This method[7] provides enough purified plasmid DNA for sequencing.

[6]Plaques typically start to become apparent after 6 hours of incubation. The colour change due to metabolized Xgal is not apparent until 8-10 hours of incubation.

[7]This protocol is a modification of the method of BIRNBOIM and DOLY [116]

5 ml LB medium[8] were inoculated with a single bacterial colony. The tube was incubated at 37°C overnight with vigorous shaking. 4.5 ml of the culture were centrifuged for 20 minutes at 3500 rpm at 4°C. The remainder of the overnight culture was stored at 4°C. The medium was removed, leaving the bacteria pellet as dry as possible. The pellet was resuspended in 150 μl ice-cold Lysis buffer I (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0) and stored for 5 minutes at room temperature. After adding 300 μl freshly prepared Lysis buffer II (0.2 N NaOH, 1% SDS) and mixing by inversion, the mixture was incubated for 5 minutes on ice. Now 225 μl ice-cold 3M KAc/5M HAc (pH 6.0) were added and mixed gently. The tube was stored on ice for 5 minutes, precipitating the chromosomal bacteria DNA. After centrifugation for 15 minutes at 13000 rpm at 4°C, the supernatant was transferred to a fresh tube. Proteins were removed by vortexing with 400 μl phenol (see section 2.5.4 on page 23), adding 300 μl Sevac and centrifuging for 2 minutes at 13000 rpm.

500-600 μl of the aqueous layer were removed and mixed with 1 ml ethanol to precipitate the DNA (see section 2.5.5 on page 23). After incubating for 5 minutes at room temperature, the Eppendorf tube was centrifuged for 10 minutes at 13000 rpm at 4°C. The supernatant was removed, the pellet washed with 70% ethanol and recentrifuged. After vacuum drying, 50 μl of TE (pH 8.0) were added. After addition of 1.5 μl RNase A (10mg/ml), the mixture was incubated for 20 minutes at room temperature to remove RNA. The positive result of the miniprep was guaranteed by analytical digest with *Eco*RI of a 5 μl sample.

3.5 Sequencing

An important technique for increasing one's knowledge of gene structure is rapid, accurate DNA sequence analysis. The first method developed for rapid sequence analysis of cloned DNA fragments involved chemical modification and cleavage of specific nucleotides, followed by electrophoresis on high-resolution denaturing acrylamide gels [117, 118]. This chemical sequencing method was developed in 1977 by MAXAM and GILBERT.

An alternative method for rapid DNA sequencing, the dideoxy sequencing method, was used in this study [119, 120]. Once the clones have been generated and selected, the primer extension is performed to determine the sequence of interest. A synthetic oligonucleotide primer is annealed to the pUC DNA genome immediately adjacent to the cloned insert or to the insert itself. Four separate primer extension reactions employing *Taq* polymerase are subsequently initiated. Each reaction contains all four deoxynucleotide triphosphates (dNTPs), but one dNTP is labeled with ^{35}S to allow subsequent detection of elongated chains using autoradiography. In each of the four reactions, one dideoxynucleotide triphosphate (ddGTP, ddATP, ddTTP, or ddCTP) is added in low concentration. Thus, in each tube numerous primer extension reactions are taking place simultaneously; however, at any given

[8] containing 50 μg/ml ampicillin.

point of the extension, a small percentage will incorporate ddNTP at the end of a newly extended chain. For this small portion of reactions in the tube, the nascent chain cannot be further elongated because the 3'-hydroxyl group is absent. However, other elongation reactions will continue until a ddNTP is incorporated. Thus, in the four tubes, there will be a mix of all possible lengths of the sequence, all starting at the primer region and extending up to the dideoxynucleotide in each specific tube. These fragments will be radioactive due to the labeled nucleotide inserted at multiple points along its length. When the reaction products of the four tubes are run in adjacent lanes on a high-resolution sequencing gel, each fragment, from a few nucleotides in length to the maximal length completed by the primer extension, will be visualized as separate bands. The shortest fragments will run farthest down the gel.

Thus, for example, in the G tube with the dNTPs plus ddGTP, only those extended sequences ending with a G will be present, but all possible lengths of extended DNA ending in G will be in the tube. So, if C nucleotides exist in the DNA template inserted in the vector at positions 15, 17, 21, and 28 past the primer (position 1), the G tube will have fragments of dideoxynucleotide terminated at positions 15, 17, 21, and 28. Moreover, if six nucleotides of the template DNA contain the sequence 5'-ATTGCG-3', the newly synthesized primer-extended DNA fragment would carry the inverse complementary sequence that could be read 5'-CGCAAT-3'. The autoradiograph of the sequence gel would show a C signal, a G signal, a C signal, two A signals, and a T signal, as the gel is read from bottom to top.

This method is rapid, accurate, and simple, requiring less labor than chemical sequencing. In addition, less radioactive material and fewer hazardous chemicals are used in the dideoxy sequencing method. Autoradiographs of the gels can be read after one to two days, allowing the rapid collection of sequence data. Although only 200 to 400 bases are typically read from any given set of reactions, by using different starting primers (see table 3.1 on page 35) the whole gene can be accessed.

3.5.1 Alkali Denaturation of DNA Template

To prime efficiently, double-stranded plasmids must be converted to single-stranded form prior to sequencing. This is accomplished by alkali denaturation of supercoiled plasmid DNA.

The plasmid pellet left from section 3.4.3 on page 42 was resuspended in 18 μl water. 2 μl of a 2M NaOH, 2mM EDTA solution were added and incubated for five minutes at room temperature. The reaction was neutralized with 2 μl of 2M ammonium acetate (pH 4.6) and mixed. 75 μl of ethanol were added, mixed and precipitated for 10 minutes at –70°C. The reaction was now centrifuged for 10 minutes at top speed, the supernatant decanted, the pellet washed with 100 μl cold 70% ethanol and centrifuged for one minute. The supernatant was decanted and the pellet vacuum dried (Speed Vac). The remaining pellet can be stored at –20°C and is resuspended in 18 μl water for sequencing.

3.5.2 Sequencing Protocol

The enzymatic method is based on the ability of a DNA polymerase (*Taq* polymerase) to extend a primer, hybridized to the template to be sequenced, until a chain-terminating nucleotide is incorporated. Each sequence determination is carried out as a set of four separate reactions, each of which contains all four dNTPs supplemented with a limiting amount of one dideoxyribonucleoside triphosphate (ddNTP). Because the ddNTP lacks the necessary 3'-hydroxyl group required for chain elongation, the growing oligonucleotide is terminated selectively at G, A, T, or C, depending on the respective dideoxy analog in the reaction. The relative concentrations of dNTPs and ddNTPs can be adjusted to give a nested set of terminal chains several hundred bases in length. For a detailed summary of this reaction, see also section 3.5 on page 43.

Incorporating a radiolabel somewhere in the oligonucleotide chain permits the visualization of the sequencing products by autoradiography. Two basic radiolabeling protocols can be utilized to detect the reaction products. The incorporation labeling method, developed by TABOR and RICHARDSON [121], separates the sequencing reaction into a labeling step and an extension/termination step. In the first step, the primer is extended a short distance using limiting concentrations of the dNTPs and a single radiolabeled dNTP. In the second step, the *extended primers* are further extended in the presence of both dd- and dNTPs. Using the direct labeling method, a label is directly attached to the end of the primer [122–124]. The oligonucleotide is 5' end-labeled using T4 Polynucleotide Kinase and [γ-^{32}P]dATP. The subsequent extension/labeling reaction is not limiting for one of the dNTPs. For this study, the direct incorporation protocol with [α-^{35}S]dATP was used [125].

[α-^{35}S]dATP (see figure 3.4 on the next page) was preferred to [γ-^{32}P]dATP for several reasons. The strong particles emitted by ^{32}P create two problems. First, because of scattering, the bands on the autoradiograph are far larger than the bands of DNA in the gel. This affects the ability to read the sequence correctly and limits the number of nucleotides that can be read from a single gel. Second, the decay of ^{32}P causes radiolysis of the DNA in the sample. Sequencing reactions radiolabeled with ^{32}P can therefore be stored for only 1 or 2 days before the DNA is so badly damaged that it generates indecipherable sequencing gels. The use of [^{35}S]dATP [125] greatly alleviates both problems. Because of the weaker β particles produced by decay of ^{35}S, there is little or no loss of resolution between the gel and the autoradiograph. Furthermore, the lower energy produces less radiolysis, allowing sequencing reactions to be stored for up to 3 weeks at –20°C without noticeable loss of resolution. Though, longer exposure times of the autoradiograph are necessary.

The primer was annealed with the dsDNA plasmid template in a molar ratio of approximately 1:1. The 18 μl sample from the alkali denaturation step (see section 3.5.1 on the facing page) was splitted into two 9 μl volumes for two sequencing reactions with different primers. 2 μl primer[9] (approx. 2 pmol), 2 μl

[9] for data on the used primers, see table 3.1 on page 35

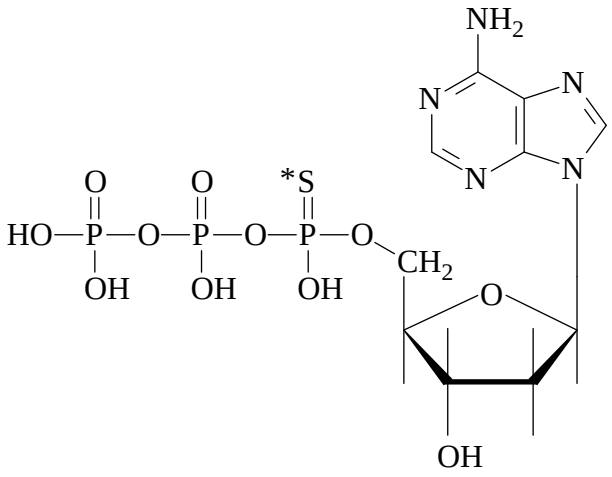

Figure 3.4: [^{35}S]-Deoxyadenosine 5'-[α-thio]triphosphate

extension/labeling mix, 5 μl *Taq* polymerase 5× buffer and water to 25 μl were added. This reaction was incubated at 37°C for 10 minutes.

2 μl of [α-^{35}S]dATP (1,000 Ci/mmol, approximately 10 μCi/μl, DuPont NEN) were added to the annealed primer/template mixture. 1.5 μl sequencing grade *Taq* polymerase (7.5 U) were added to the reaction and shortly centrifuged, and incubated at 37 °C for 5 minutes. The extension/labeling reaction was carried out at 37°C rather than 70°C to slow down the incorporation rate of *Taq* polymerase and thereby limit the number of bases incorporated in this step[10]. The incorporation of nucleotides is also limited by the limiting concentration of nucleotides present in the extension/labeling mix.

For each set of the sequencing reactions, four microcentrifuge tubes (0.45 ml) are labeled (A, C, G, and T) and 1 μl of the appropriate d/ddNTP mix is added to each tube. This nucleotide mix has a limiting amount of the specific base (e.g., dGTP), which is also included as dideoxy nucleotide (ddGTP). The loaded tubes are stored at 4°C until just before completion of the extension/labeling step. When the extension/labeling reaction is complete, 6 μl are aliquoted to each tube containing the d/ddNTP mix. They are briefly mixed by pipetting up and down, and spun to ensure that no liquid is left on the tube walls. The reaction is incubated at 70°C for 5 to 10 minutes, and stopped by adding 4 μl of stop solution (95% deionized formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) to each tube. [α-^{35}S]dATP labeled reactions can be stored at –20°C for 2-4 weeks.

[10]The number of bases can be further changed by shortening or extending this step.

3.5.3 Denaturing Polyacrylamide Gels

Both the amount and the accuracy of the nucleotide sequence depend on the quality of the polyacrylamide gels used to display the radiolabeled DNA fragments. Under optimal conditions, between 300 and 400 nucleotides can be obtained from a denaturing polyacrylamide gel. However, by electrophoresing aliquots of the sequencing reaction for different lengths of time, it is possible to read up to 500 nucleotides of sequence from a single gel.

The concentration of acrylamide used to prepare the gel depends on the size of the DNA fragments that are to be analyzed (see section 2.7.3 on page 34). Sequences lying between 25 and 400 nucleotides from the terminus of the primer can be read from gels containing 6% polyacrylamide.

A 0.4 mm thick, 8 M urea, polyacrylamide sequencing gel was used. The glass plates (34 $\times$ 40 cm) were cleaned with a detergent and absolute alcohol. The larger glass plate was layed flat on the bench and the two spacers arranged in place along the sides. Then the smaller (notched) plate is layed in position, resting on the spacers.

The plates were then clamped together using bulldog binder clips. A buffer gradient gel was made to slow down short fragments [125, 126]. The acrylamide solution was prepared by dissolving 38g of acyrylamide and 2g of N,N'-methylene-bisacrylamide in 100 ml water at 37°C.This solution was stored at 4°C in dark. The *Top* solution was composed of 25g urea, 7.5ml acrylamide solution, 2.5ml 10$\times$ TBE buffer and 21 ml of water. The *Bottom* solution was composed of 5g urea, 1.5 ml acrylamide solution, 2.5m l 10$\times$ TBE buffer, 2.5 ml of water and bromophenol blue[11]. To seal the bottom of the gel, 5 ml *top* solution were polymerized with 15 μl ammonium persulfate (25%) and 15 μl TEMED. This fast hardening gel was poured round the bottom of the plates and left for 20 minutes.

10 μl ammonium persulfate solution (25%) and 15 μl TEMED were added to the *bottom* fraction, 50 μl ammonium persulfate and 50 μl TEMED to the *top* fraction. They were mixed by swirling, and 11 ml of the *top* solution taken up in a 25 ml pipette carefully followed by 6 ml of the *bottom* solution. The solutions were allowed to gently mix by some air bubbles going up through the pipette. The pipette was then emptied into the gel plates[12]. The remaining plate volume was filled by the *top* solution. The gel has to be carefully examined for air bubbles. Bubbles in the upper portion of the gel can sometimes be removed by knocking.

The mold was then layed down at an angle so that the top of the mold rested on a support about 5 cm high. This reduced the hydrostatic pressure at the base of the mold and prevented leaks and bowing of the gel plates. Immediately the flat side of a shark's tooth comb was inserted approximately 0.5 cm into the gel solution. The comb was clamped in position and the gel allowed to polymerize overnight.

The shark's comb was removed and the slot washed with buffer. The gel mold

[11]The bromophenol blue serves as visible marker to check that the buffer gradient has formed correctly.

[12]To avoid producing air bubbles, the solution was poured in a continuous stream.

Table 3.3: Migration rates of marker dyes through denaturing polyacrylamide gels

% Polyacrylamide	Bromophenol Blue[a]	Xylene xyanol FF[a]
5	35	130
6	26	106
8	19	76
10	12	55
20	8	28

[a] The numbers are the approximate sizes of DNA (in nucleotides) with which the marker dyes will comigrate.

was attached to the electrophoresis apparatus together with a metal plate to ensure even diffusion of the heat produced during electrophoresis. Approximately 500 ml 1$\times$ TBE were filled to the upper and lower buffer reservoir. The shark's tooth comb was reinserted with its teeth just sticking into the loading surface of the gel. The slots were washed with buffer once again to remove any fragments of urea and polyacrylamide. The gel was loaded with 2 μl of loading solution to alternate wells. The gel was then run at 50 W constant power until the dye front ran a few centimeters into the gel[13].

In the meantime, the sequencing reactions were heat-denatured (see section 2.5.7 on page 24) for 5 minutes and stored on ice until all samples have been loaded onto the gel. 1.8 μl of each sequencing reaction was loaded in A C G T order. The gel was now run at constant power (40 W, $\sim$ 1800 V) for the time required to achieve optimal resolution of the sequence of interest (4 to 8 hours). The time required can be estimated by monitoring the migration of the marker dyes in the formamide/EDTA/xylene cyanol/bromophenol blue gel-loading buffer (see table 3.3).

At the end of the electrophoresis run, the gel mold was removed from the apparatus, and layed flat with the smaller plate uppermost. Using the end of a metal spatula, the plates of the mold were slowly pried apart. Now a piece of Whatman 3MM paper was placed on top of the gel. Applying gentle pressure the gel became firmly attached to the rough surface of the paper. Now the paper was slowly peeled from the glass plate. The gel was covered with plastic wrap and placed in a vacuum gel dryer. It was dried under vacuum at 80°C for 2 hours.

Afterwards, the gel was removed from the dryer and the wrap peeled off. An autoradiograph was established by exposing the gel to X-ray film (Kodak XAR-5) at –80°C for one to several days. The autoradiograph was developed and the sequence read.

Reading sequences from gels is not an easy task and skill is required. All gels were read and recorded at least twice. Discrepancies were resolved by further sequencing.

[13]Check for leaks between wells; dye should only be in alternate lanes and should not leak into adjacent lanes.

3.6 Results

All nucleotide locations are given according to the numbering scheme of Strosberg [77].

The amplified and cloned β_2 adrenergic receptor fragment (1242–2639) covered the entire coding region of the receptor, which starts at bp 1264 and ends at bp 2505 (see sequence on table 3.5 on page 51). Three additional sequence primers were used. The primers β1490, β1740 and β1890 resulted in readings starting around the positions 1560–1620, 1780–1820, and 2050–2090, respectively.

3.6.1 Mutations of the β_2-Adrenergic Receptor Gene

The structure gene of nine subjects was scrutinized. Three candidates had developed essential hypertension. They were compared to three non-hypertensive subjects of the same family. Another comparison was made between a known salt sensitive subject and two salt resistant ones [18].

One single base substitution leading to an amino acid change was found at position 1309 (amino acid 16), changing glycine to arginine (see figure 3.5 on the following page). In the β_2-adrenoceptor, amino acid 16 is located in the extracellular amino terminus, next to a glycosylation site (see figure 1.7 on page 15). The other mutations found were silent ones, possibly altering the mRNA stability. Especially the mutation at position 1515 (see figure 3.6 on the following page), encodes for an amino acid in one of the highly conserved regions of the human β_2 adrenergic receptor (membrane spanning domain II). The amino acid of the mutation at position 1786 is situated at the edge of membrane spanning domain III and extracellular loop II (see figure 3.7 on the next page), whereas the mutations at position 2316 and 2502 (see figure 3.8 on the following page) are located at the C-terminus of the receptor.

The found base substitutions were sequenced from 10 to 20 different clones for each subject, which made it possible to reveal heterozygous subjects for the mutations at 1309, 2316 and 2502. This led to the formulation of four different alleles for the human β_2-adrenergic receptor, as outlined in table 3.4. The β_2-adrenoceptor sequence, protein translation and found mutations are summarized in table 3.5 on page 51.

	base position				
	1309	1515	1786	2316	2502
wild type	**G**GA → Gly	CT**A**	**A**GG	GG**C**	CT**A**
allele I	**A**GA → Arg	CT**G**	**C**GG	GG**G**	CT**G**
allele II	**A**GA → Arg	CT**G**	**C**GG	GG**G**	CT**A**
allele III	**A**GA → Arg	CT**G**	**C**GG	GG**C**	CT**A**

Table 3.4: Alleles of the human β_2 adrenergic receptor

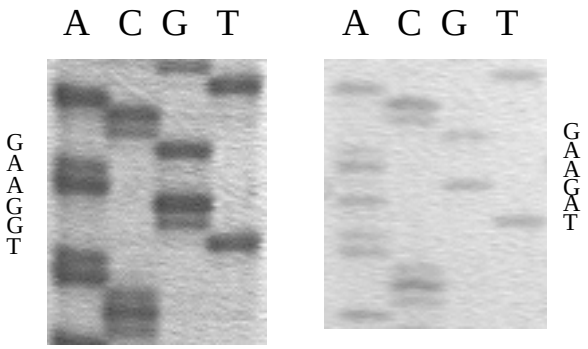

Figure 3.5: The amino acid changing mutation found at position 1309 on the left side compared to the wild type (right picture)

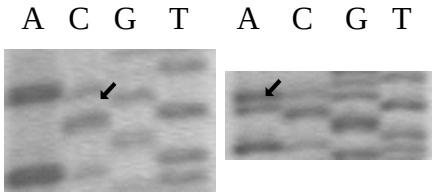

Figure 3.6: The point mutation at position 1515 (left side) compared to the published wild type (right side)

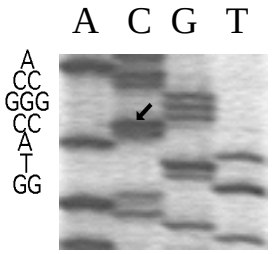

Figure 3.7: The point mutation at position 1786 of the human β_2-adrenergic receptor

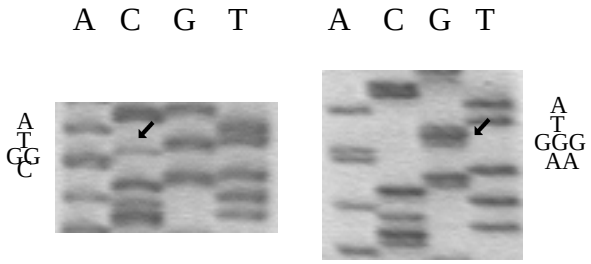

Figure 3.8: The point mutation at position 2316 of the human β_2-adrenergic receptor

Table 3.5: Nucleotide sequence of the human β_2-adrenergic receptor gene

```
        .          .          .
 1240  CGCTTACCTGCCAGACTGCGCGCC ATG GGG CAA CCC   1275
                                Met Gly Gln Pro   4

                                              A
GGG AAC GGC AGC GCC TTC TTG CTG GCA CCC AAT GGA   1311
Gly Asn Gly Ser Ala Phe Leu Leu Ala Pro Asn Gly   16
                                              Arg

AGC CAT GCG CCG GAC CAC GAC GTC ACG CAG CAA AGG   1347
Ser His Ala Pro Asp His Asp Val Thr Gln Gln Arg   28

GAC GAG GTG TGG GTG GTG GGC ATG GGC ATC GTC ATG   1383
Asp Glu Val Trp Val Val Gly Met Gly Ile Val Met   40

TCT CTC ATC GTC CTG GCC ATC GTG TTT GGC AAT GTG   1419
Ser Leu Ile Val Leu Ala Ile Val Phe Gly Asn Val   52

CTG GTC ATC ACA GCC ATT GCC AAG TTC GAG CGT CTG   1455
Leu Val Ile Thr Ala Ile Ala Lys Phe Glu Arg Leu   64

CAG ACG GTC ACC AAC TAC TTC ATC ACT TCA CTG GCC   1491
Gln Thr Val Thr Asn Tyr Phe Ile Thr Ser Leu Ala   76

                               G
TGT GCT GAT CTG GTC ATG GGC CTA GCA GTG GTG CCC   1527
Cys Ala Asp Leu Val Met Gly Leu Ala Val Val Pro   88

TTT GGG GCC GCC CAT ATT CTT ATG AAA ATG TGG ACT   1563
Phe Gly Ala Ala His Ile Leu Met Lys Met Trp Thr   100

TTT GGC AAC TTC TGG TGC GAG TTT TGG ACT TCC ATT   1599
Phe Gly Asn Phe Trp Cys Glu Phe Trp Thr Ser Ile   112

GAT GTG CTG TGC GTC ACG GCC AGC ATT GAG ACC CTG   1635
Asp Val Leu Cys Val Thr Ala Ser Ile Glu Thr Leu   124

TGC GTG ATC GCA GTG GAT CGC TAC TTT GCC ATT ACT   1671
Cys Val Ile Ala Val Asp Arg Tyr Phe Ala Ile Thr   136

TCA CCT TTC AAG TAC CAG AGC CTG CTG ACC AAG AAT   1707
Ser Pro Phe Lys Tyr Gln Ser Leu Leu Thr Lys Asn   148
```

Nucleotide sequence of the human β_2-adrenergic receptor gene (cont.)

```
AAG GCC CGG GTG ATC ATT CTG ATG GTG TGG ATT GTG   1743
Lys Ala Arg Val Ile Ile Leu Met Val Trp Ile Val   160

TCA GGC CTT ACC TCC TTC TTG CCC ATT CAG ATG CAC   1779
Ser Gly Leu Thr Ser Phe Leu Pro Ile Gln Met His   172

        C
TGG TAC AGG GCC ACC CAC CAG GAA GCC ATC AAC TGC   1815
Trp Tyr Arg Ala Thr His Gln Glu Ala Ile Asn Cys   184

TAT GCC AAT GAG ACC TGC TGT GAC TTC TTC ACG AAC   1851
Tyr Ala Asn Glu Thr Cys Cys Asp Phe Phe Thr Asn   196

CAA GCC TAT GCC ATT GCC TCT TCC ATC GTG TCC TTC   1887
Gln Ala Tyr Ala Ile Ala Ser Ser Ile Val Ser Phe   208

TAC GTT CCC CTG GTG ATC ATG GTC TTC GTC TAC TCC   1923
Tyr Val Pro Leu Val Ile Met Val Phe Val Tyr Ser   220

AGG GTC TTT CAG GAG GCC AAA AGG CAG CTC CAG AAG   1959
Arg Val Phe Gln Glu Ala Lys Arg Gln Leu Gln Lys   232

ATT GAC AAA TCT GAG GGC CGC TTC CAT GTC CAG AAC   1995
Ile Asp Lys Ser Glu Gly Arg Phe His Val Gln Asn   244

CTT AGC CAG GTG GAG CAG GAT GGG CGG ACG GGG CAT   2031
Leu Ser Gln Val Glu Gln Asp Gly Arg Thr Gly His   256

GGA CTC CGC AGA TCT TCC AAG TTC TGC TTG AAG GAG   2067
Gly Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu   268

CAC AAA GCC CTC AAG ACG TTA GGC ATC ATC ATG GGC   2103
His Lys Ala Leu Lys Thr Leu Gly Ile Ile Met Gly   280

ACT TTC ACC CTC TGC TGG CTG CCC TTC TTC ATC GTT   2139
Thr Phe Thr Leu Cys Trp Leu Pro Phe Phe Ile Val   292

AAC ATT GTG CAT GTG ATC CAG GAT AAC CTC ATC CGT   2175
Asn Ile Val His Val Ile Gln Asp Asn Leu Ile Arg   304

AAG GAA GTT TAC ATC CTC CTA AAT TGG ATA GGC TAT   2211
Lys Glu Val Tyr Ile Leu Leu Asn Trp Ile Gly Tyr   316
```

```
GTC AAT TCT GGT TTC AAT CCC CTT ATC TAC TGC CGG   2247
Val Asn Ser Gly Phe Asn Pro Leu Ile Tyr Cys Arg   328

AGC CCA GAT TTC AGG ATT GCC TTC CAG GAG CTT CTG   2283
Ser Pro Asp Phe Arg Ile Ala Phe Gln Glu Leu Leu   340

                                           G
TGC CTG CGC AGG TCT TCT TTG AAG GCC TAT GGC AAT   2319
Cys Leu Arg Arg Ser Ser Leu Lys Ala Tyr Gly Asn   352

GGC TAC TCC AGC AAC GGC AAC ACA GGG GAG CAG AGT   2355
Gly Tyr Ser Ser Asn Gly Asn Thr Gly Glu Gln Ser   364

GGA TAT CAC GTG GAA CAG GAG AAA GAA AAT AAA CTG   2391
Gly Tyr His Val Glu Gln Glu Lys Glu Asn Lys Leu   376

CTG TGT GAA GAC CTC CCA GGC ACG GAA GAC TTT GTG   2427
Leu Cys Glu Asp Leu Pro Gly Thr Glu Asp Phe Val   388

GGC CAT CAA GGT ACT GTG CCT AGC GAT AAC ATT GAT   2463
Gly His Gln Gly Thr Val Pro Ser Asp Asn Ile Asp   400

TCA CAA GGG AGG AAT TGT AGT ACA AAT GAC TCA CTG   2499
Ser Gln Gly Arg Asn Cys Ser Thr Asn Asp Ser Leu   412

  G
CTA TAA AGC AGT TTT TCT ACT TTT AAA GAC CCC CCC   2535
Leu End Ser Ser Phe Ser Thr Phe Lys Asp Pro Pro   424

CCG CCC AAC AGA ACA CTA AAC AGA CTA TTT AAC TTG   2571
Pro Pro Asn Arg Thr Leu Asn Arg Leu Phe Asn Leu   436
                                       .         .
AGG GTA ATA AAC TTA GAA TAA AATTGTAAAATTGTATAGA   2611
Arg Val Ile Asn Leu Glu End                      448
         .         .         .         .
GATATGCAGAAGGAAGGGCATCCTTCTGCCTTTTTTATT  2650
```

Nucleotide and deduced amino acid sequence of the human β_2-adrenergic receptor gene (cont.). Nucleotide numbers are to the right of the sequence. Amino acid numbers are below the nucleotide numbers. Found mutations are denoted above the nucleotide sequence. Amino acids are represented using the three-letter code.

Chapter 4

A Screening System for the Human β_2 Adrenergic Receptor

As a consequence to the study outlined in the previous chapter, it was necessary to establish a fast method for detecting the described mutations of the β_2-adrenoceptor. The receptor gene has proved to show an increased polymorphism, as the sequence analysis of only nine subjects revealed four different alleles. We hoped to be able to associate one or more of the found point mutations (see section 3.6 on page 49) with an established phenotype. Therefore, in an extension of the previous study, a scanning method for the detection of these mutations was developed, and the mutation pattern of persons with known β_2 receptor density was determined.

4.1 Methods of Detection of Single Point Mutations

Due to their subtle nature, the point mutations are the most difficult to detect of all the genetic alterations. Nevertheless, there are quite a few techniques capable of recognizing the presence of single base changes in mammalian genes. A diagram of the methods currently utilized for detection and characterization of single base substitutions, deletions, and insertions is shown in figure 4.1 on the following page. In the first row (A) are listed methods for detection of the presence of the mutation. In (B) are the methods that permit characterization of the molecular nature of the mutation. While detection of the presence of a mutation might be sufficient for some diagnostic and prognostic purposes, characterization of the single base substitutions is also important for several reasons.

The RNase A mismatch cleavage method is based on the ability of pancreatic ribonuclease to recognize and cleave single base mismatches in RNA:RNA [127] and RNA:DNA heteroduplexes [128]. The method is performed by hybridization of the target sequence to a labeled complementary riboprobe, digestion with RNase A, and analysis of the resistant products by polyacrylamide electrophoresis in denaturing gels. Mutations are detected and localized by the presence and size of the

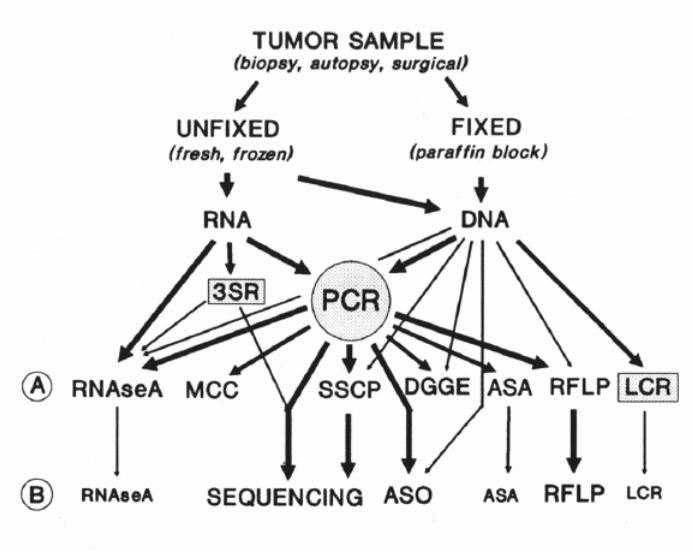

Figure 4.1: Methods for the detection (A) and characterization (B) of point mutations

RNA fragments generated by cleavage at the mismatches.

The single strand conformation polymorphism (SSCP) is another method developed by HAYASHI, SEKIYA, and colleagues [129, 130]. It is based on the differences in the secondary structure of single-strand DNA molecules differing in a single nucleotide, which also is frequently reflected in an alteration of their electrophoretic mobility in nondenaturing gel electrophoresis.

In denaturing gradient gel electrophoresis (DGGE), the double stranded DNA is subjected to electrophoresis in gel that has an increasing concentration of denaturant along the length of the gel [131]. The fragment melts while traveling through the gel. The melting proceeds in segments, called melting domains, because of the cooperative nature of the denaturation of the double-stranded DNA [132]. When a domain melts, the fragment assumes a branched structure that causes significant retardation of movement. Thus, the position of the fragment in the gel after a certain time of electrophoresis is determined by the history of melting of the fragment that is altered if the sequence is different. The principle of separation in DGGE is such that sequence changes in the melting domain of highest stability cannot be detected, because the fragment no longer has a branched structure when the last domain melts. If, however, a stretch of sequence that serves as an extremely stable domain is attached to one side of the fragment, then mutations at any sites within certain types of sequence context can be detected by DGGE [133]. This extra sequence of extremely high stability can be conveniently attached to the target sequence of PCR by using one primer that has 40 nucleotides of an artificial GC-rich sequence (GC-clamp) extending at its 5'-end. With the use of this

clamp, DGGE may be able to detect nearly all possible mutations in any given sequences [134, 135].

Hybridization with radioactively labeled allelic specific oligonucleotides (ASO) also has been applied to the detection of specific point mutations [136]. The method is based on the differences in the melting temperature of short DNA fragments differing by a single nucleotide. Single point mutations have been also detected by the creation or destruction of restriction fragment length polymorphisms (RFLP).

We successfully established a DGGE protocol for scanning three of the found mutations. Anyway, this method didn't result in the required specificity and therefore a second technique was searched for. As the found point mutations altered three restriction sites of the human β_2-adrenoceptor gene, the method of choice was the restriction fragment length polymorphism (RFLP).

4.2 Detection of Point Mutations by RFLP of PCR Amplified DNA Sequences

In contrast with the scanning detection methods (RNase A, DGGE, SSCP) that cannot characterize the mutations, the PCR/RFLP approach combines detection and characterization capabilities. The generation or destruction of restriction sites allows the rapid detection of point mutations after the genomic sequences are amplified by the PCR. In the former case, the mutation is cleaved by the specific restriction endonuclease, while the wild-type sequence is not. Gel electrophoresis easily identifies the mutations, since they generate smaller DNA fragments [137]. The situation is reversed when the mutation destroys the restriction site previously present in the wild-type sequence. In this case the mutations are evidenced by the presence of undigested DNA fragments [138, 139].

Although many point mutations do not create or abolish a restriction site and therefore cannot be detected by this straightforward approach, the use of PCR primers containing mismatches relative to the target sequences can circumvent this limitation. The DNA fragments incorporate the sequence of the primers that can be designed to create a new RFLP [137, 139, 140]. The main advantage of this approach is that it does not require the use of radioactive isotopes and it is more amenable therefore to analyses in clinical settings.

4.2.1 Restriction Map of the β_2 Adrenergic Receptor Codon

By using the GCG (Genetics Computer Group, Wisconsin) `map` programme[1], the codon was mapped to all known restriction endonucleases. This mapping was done for the published sequence [77] as well as for one including the found base substitutions (see section 3.6 on page 49). The comparison of the two generated

[1]Version 8.0-OpenVMS, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711, installed on OpenVMS AXP

maps revealed that three mutations (see table 3.4 on page 49) altered accompanying restriction sites: bp 1515, 1786, and 2316. As the nucleotide exchange by mutation 1309 did not alter the restriction maps, but was suspected to have significant influence to the receptor, a new restriction site was introduced by generating a mismatch of the 5' oligonucleotide primer. The altered restriction sites are shown in tables 4.1 on the next page to 4.3 on page 61.

4.2.2 Amplified Fragments

This strategy led to the amplification of three different segments of the receptor codon. Fragment I, extending from position 1242 to position 1919, included the found point mutations at position 1515 and 1786. Fragment II, from position 1721 to position 2639, contained the single base substitution at position 2316. The location of these fragments was chosen due to the distribution of the restriction sites of the restriction endonucleases in question (see section 4.2.3).

The third fragment, reaching from position 1284 to position 1466, included the amino acid changing point mutation of position 1309 (GGA $\rightarrow$ AGA, see section 3.6 on page 49). As this mutation does not alter any restriction site, a *Bfa*I site was introduced by altering the last but one nucleotide of the 5' oligonucleotide primer of the fragment. By this means the nucleotide change at position 1309 resulted in the creation of a restriction site for the AGA allele (coding arginine). The introduction of the *Bfa*I site was constructed because it required the change of one nucleotide only. The used PCR primers and size of the amplified fragments are shown in table 4.4 on page 62.

4.2.3 Selecting the Used Restriction Endonucleases

The restriction endonuclease used for the third fragment resulted from the mismatch introduced into the 5' oligonucleotide primer as shown below:

```
1284
                                                  *
   C AGC GCC TTC TTG CTG GCA CCC ACT
5'-C AGC GCC TTC TTG CTG GCA CCC AAT GGA AGC CA
                                     Gly
5'-C AGC GCC TTC TTG CTG GCA CCC AAT AGA AGC CA
                                     Arg
                                   C▼T AG
                                      BfaI
```

This construct leads to an uncut fragment with a length of 183 bp for the glycine allele (GGA) and to two fragments, 159 and 24 bp in length, for the arginine allele. Therefore, in a 2.5% agarose gel, you can expect a 183 bp band for a homozygous GGA amplificate, the cut 159 bp band for a homozygous AGA amplificate, and both bands in heterozygous amplificates (see figure 4.3 on page 68).

```
                                B
                                s
                NS H            p
                laCa          N T1
                auveB    B  B lBs2
                I9iIf    s  a amp8
                I6JIa    l  n IgR6
                IIIII    I  I VIII
                 / /           /
     TCTGGTCATGGGCCTAGCAGTGGTGCCCTTT
1500 +---------+---------+---------+ 1530
     AGACCAGTACCCGGATCGTCACCACGGGAAA
```

```
                                B
                                s
                NS EH           p
                laCcaS        N T1
                auvoec   B  B lBs2
                I9iRIr   s  a amp8
                I6JIIF   l  n IgR6
                IIIIII   I  I VIII
                 / ///         /
     TCTGGTCATGGGCCTGGCAGTGGTGCCCTTT
1500 +---------+---------+---------+ 1530
     AGACCAGTACCCGGACCGTCACCACGGGAAA
```

Table 4.1: Restriction sites of the wild type (above) compared to the 1515 mutant (below)

```
                  S  H
          C        T a Ca
          v      BRs u ve
          i      ssp 9 iI
          R      raR 6 JI
          I      III I II
                  //    /
     TCAGATGCACTGGTACAGGGCCACCCACCAG
1770 +---------+---------+---------+ 1800
     AGTCTACGTGACCATGTCCCGGTGGGTGGTC


                     HS
          C       N    CaaST
          v    B BlKMNRveucs
          i    a sapscsiI9rp
          R    n rInpiaJI6FR
          I    I IVIIIIIIII
                  / /// ////
     TCAGATGCACTGGTACCGGGCCACCCACCAG
1770 +---------+---------+---------+ 1800
     AGTCTACGTGACCATGGCCCGGTGGGTGGTC
```

Table 4.2: Restriction sites of the wild type (above) compared to the 1786 mutant (below)

```
              H
            C  a         CBC
            vHBeS        vsj
            iapIt        ire
            JemIu        JDP
            IIIII        III
             / //          /
     CTTTGAAGGCCTATGGCAATGGCTACTCCAG
2300 +---------+---------+---------+ 2330
     GAAACTTCCGGATACCGTTACCGATGAGGTC


              H
            C  a         CC
            vHBeS        vj
            iapIt        ie
            JemIu        JP
            IIIII        II
             / //
     CTTTGAAGGCCTATGGGAATGGCTACTCCAG
2300 +---------+---------+---------+ 2330
     GAAACTTCCGGATACCCTTACCGATGAGGTC
```

Table 4.3: Restriction sites of the wild type (above) compared to the 2316 mutant (below)

The first fragment includes changed restriction sites for the mutations at position 1515 and 1786. The point mutation located at position 1515 destroys a *Bfa*I restriction site. The mutation at position 1786 introduces five new restriction sites (see table 4.2 on page 60). *Kpn*I is the only rare cutting one as the complete β_2 adrenergic receptor structure lacks this specific site type.

The single base change at position 2316 (see section 3.6 on page 49) destroys a *Bsr*DI site. The used fragment was selected to include another *Bsr*DI cleavage site for reference. The used restriction endonucleases and the resistant fragments are summarized in table 4.5 on the next page.

4.3 PCR of the RFPL fragments

4.3.1 Used Protocol

The polymerase chain reaction for all three fragments was done in a 25 μl volume using 0.2 μg of genomic DNA extracted from whole blood (see section 2.5.1 on page 21). In addition to the sample DNA, the mixture contained 2.5 μl tenfold *Taq* polymerase buffer (Promega), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (Pharmacia) and 1 unit *Taq* polymerase (Promega). Primer (see table 4.4) were used at a final concentration of 0.4 to 0.8 μM each. For the third fragment, the reaction volume was doubled (50 μl) when polymorphism analysis was performed on agarose gels.

The amplification was performed in 0.2 ml tube-strips in a compact silver microblock of an Omnigene thermal cycler (Hybaid Ltd., England). The block was equipped with a heated lid. This heating plate heats the air temperature at the top of each reaction mixture to a temperature that is permanently higher than the sample

Fragment	Primer	
I	Forward primer	5'-CTT ACC TGC CAG ACT TGC G-3'
	Reverse primer	5'-CCA TGA TCA CCA GGG GAA CG-3'
II	Forward primer	5'-TCA TTC TGA TGG TGT GGA TT-3'
	Reverse primer	5'-CAG AAG CAT GCC CTT CCT TCT GC-3'
III	Forward primer	5'-AGC GCC TTC TTG CTG GCA CCC ACT-3'
	Reverse primer	5'-GGT GAC CGT CTG CAG ACG CTC GAA C-3'

Forward primer indicates the primer for the sense direction of the DNA strand.

Fragment	Size (bp)	from–to	Annealing Temp.
I	669	1242–1919	52°C
II	919	1721–2639	48°C
III	183	1284–1466	57°C

Table 4.4: Nucleotide sequence of DNA primers used for PCR amplification for RFLP of the β_2 adrenoceptor codon (above) and their characteristics (below)

Nucleotide	Restriction enzyme	Recognition sequence	Source
1309	*Bfa*I	5'...C▼TAG...3'	*Bacteroides fragilis*
1515	*Bfa*I	5'...C▼TAG...3'	*Bacteroides fragilis*
1786	*Kpn*I	5'...GGTAC▼C...3'	*Klebsiella pneumonia*
2316	*Bsr*DI	5'...GCAATGNN▼...3'	*Bacillus stearothermophilus*

Nucleotide	Restriction enzyme	Fragments cut	Fragments uncut	Codon cut/uncut
1309	*Bfa*I	159, 24	183	AGA/GGA
1515	*Bfa*I	397, 272	669	CTA/CTG
1786	*Kpn*I	544, 125	669	CGG/AGG
2316	*Bsr*DI	460, 317, 142	777, 142	GGC/GGG

Table 4.5: RFLP fragments and used restriction endonucleases

temperature (120°C). This elevated air temperature relative to the sample temperature minimizes evaporation so that there is no condensation of the reaction mixture as the reaction mixture is repeatedly heated and cooled. This technique eliminates the necessity of an oil overlay. The sample temperatures were controlled based on a remote thermistor probe mounted in an appropriate tube in the block. Tube control means, the actual sample temperatures are held at the programmed temperature for the programmed time, whereas with block control, there will inevitably be a lag between the block reaching target temperature and the samples reaching target temperature. Thus the used incubation times have been reduced dramatically.

For cycle protocol, an initial denaturation step (95°C for 1.5 minutes) was followed by an annealing step (35 s), extension step (72°C) starting at one minute with an one second time increment, and denaturing step (94°C) for 20 s. The annealing temperature was 48–57°C. After 25 cycles, a final extension step (72°C) for 5 minutes was added.

4.3.2 PCR Optimization

The key to successful PCRs lies in the design of appropriate primers. The used primers were arranged to have about the same annealing temperatures and no significant secondary structures. The chosen primers also show one hybridization site only throughout the entire gene. It was also taken care of that oligonucleotide primers did not display significant homology either internally or to one another. The used primer concentrations were determined empirically, as an increased primer concentration may lead to the generation of artifacts (see also section 2.6.2 on page 26).

The incubation times for the three temperatures corresponding to the three steps in a cycle of amplification – denaturation, annealing, and extension, were short-

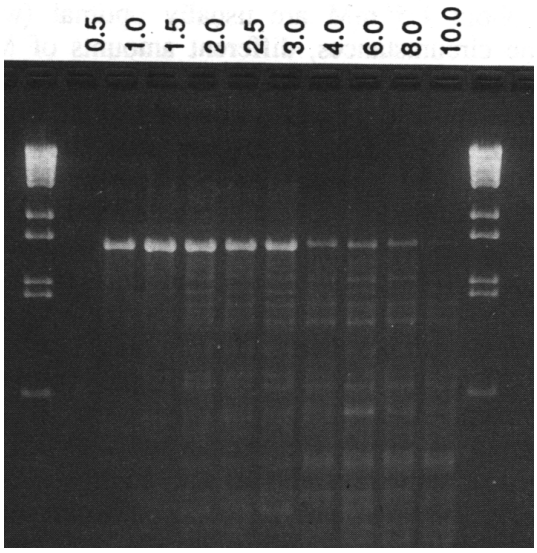

Figure 4.2: Effect of Mg^{2+} concentration on PCR specifity and yield. Amplifications were performed as described except that $MgCl_2$ was varied from 0.5 to 10 mM.

ened. The amplification was performed in a 0.2 ml microblock Omnigene thermal cycler (Hybaid Ltd., England) using sample temperature control and heated lids. Therefore the usual one minute steps could be cut back to 30 seconds and less.

As the concentration of Mg^{2+} ions significantly alters the specificity and yield of amplification products (see also section 2.6.1 on page 26), each new primer pair was routinely tested under a variety of magnesium concentrations prior to routine use (see figure 4.2). For all three primer pairs, the standard Mg^{2+} concentration of 1.5 mM could be retained.

4.4 RFLP Protocol

The result of the polymerase chain reaction was tested by agarose gel electrophoresis of a 4 μl sample. The positive samples were ethanol precipitated (see section 2.5.5 on page 23) and resuspended in 10 to 20 μl of water for one to two restriction digests, respectively. The total digest reaction volume was 20 μl, enzyme buffer (tenfold) and BSA were added following the manufacturers recommendations.

The digests for differentiation of the point mutation at position 1309 and 1515 were both incubated at 37°C with 5 units of *Bfa*I (New England Biolabs) added. The digest for the polymorphism at position 1786 was incubated at 37°C with 6 units of *Kpn*I (AGS) added, whereas the digest for differentiation of the polymorphism at position 2316 was incubated at 65°C with 8 units of *Bsr*DI (New England Biolabs). All assays were incubated for 5 to 10 hours.

4.5 Analysis of RFLPs

Two different techniques were used for analyzing the restriction fragment length polymorphisms. As the length of the analyzed fragments varied between 125 and 919 base pairs, agarose gels turned out to be a difficult means for resolving all fragments. For different fragments, different agarose concentrations were used (1.5 to 3%), but the differentiation between the uncut fragment II (919 bp) and cut fragment (717 bp) remained difficult. The differentiation between cut and uncut fragment III (24 base pairs) was resolvable by using high concentration agarose gels (3%). Anyhow, the yield of a 200 base pair fragment during ethanol precipitation is low (see section 2.5.5 on page 23), so the amplification volume for PCR had to be doubled. Another problem arising during establishing the protocol was the loss of evidence after photographing the agarose gels.

DNA separations are most frequently performed in large sequencing polyacrylamide gels or in agarose gels in presence of the fluorescent dye ethidium bromide (see section 2.7.1 on page 33). The agarose method exhibits a low resolution, relatively low sensitivity and requires a photograph for a storable pherogram. The method employing long sequences gels has the disadvantage of radioactive labelling and is time consuming. Because of these drawbacks, another protocol was introduced for clinical applications.

Thin, washed, and dried polyacrylamide gels with sample wells (CleanGels, Pharmacia), bound to a polyester backing film, which were rehydrated to 0.5 mm thick gels with a specially designed buffer, were used. They were applied together with a highly sensitive silver staining (see section 4.6 on the following page). These pherograms can be easily dried for evaluation and filing of the results.

The discontinuous polyacrylamide gels[2] (stacking gel T=5%, C=3% and separating gel T=10%, C=2%) were rehydrated with 25 ml of a 112 mM Tris-acetate buffer (acetic acid to pH 6.4), containing 0.001% bromophenol blue and 0.001% Orange G, placed on a rotating shaker for 60 minutes to assure even rehydration. The excess buffer was removed with clean filter paper, sample wells dried and the buffer wiped of the gel surface. Electrophoresis was performed using a Multiphor II Electrophoresis Unit (Pharmacia), connected to a MultiTemp Thermostatic Circulator (Pharmacia), set to a temperature of 15°C. A very thin layer of kerosene was applied onto the cooling plate in order to ensure good cooling contact. The gel was placed on to the center of the cooling plate, the side containing the wells oriented towards the cathode. Two electrode wicks were soaked with 22 ml of an electrode buffer (0.20 mM Tris, 0.2 mM Tricin, 0.55% SDS, pH 8.3 with acetic acid [141]) each. One electrode strip was placed onto the edge of the gel so that there was a distance of 4 mm between the edge of the strip and the sample wells. Another strip was placed onto the other edge of the gel so that the strip overlapped the gel by 5 mm. Air bubbles were smoothed out by sliding bent-tipped forceps along the edges

[2] Its separation range is 100 to 1500 base pairs with a resolution down to 8 bp in the 100 to 200 base pair range.

of the strips lying in contact with the gel. 7 μl sample per well were applied. No sample preparation was performed apart from ethanol precipitation. The running conditions for a whole gel are given below.

200 V_{max},	20 mA_{max},	10 W_{max},	20 min
375 V_{max},	30 mA_{max},	20 W_{max},	50 min
450 V_{max},	30 mA_{max},	20 W_{max},	10–20 min

Table 4.6: Running conditions for CleanGels

Electrophoresis was performed until the bromophenol blue front reached the anode.

4.6 Silver Staining of DNA in Polyacrylamide Gels

Highly sensitive detection of nucleic acids in the nanogram range has been achieved by the specific chemical reduction of silver ions. The methods for silver-staining nucleic acids employ either a histologically derived procedure that uses ammoniacal solutions of silver [142–145], or a photochemically derived reaction in which silver binds to nucleic acid bases and is then selectively reduced by chemical agents or light [146–149]. These silver staining protocols can be as sensitive as radioisotopic methods. However, they are complex and time consuming and require the preparation and handling of several solutions. In an attempt to simplify the routine use of silver stains to detect nucleic acids, BASSAM *et. al.* [150] have optimized the photochemically derived silver stain originally introduced by MERRIL *et. al.* [151] for protein staining and later applied to nucleic acids [146, 147, 149] which uses formaldehyde to selectively reduce silver ions to metallic silver under alkaline conditions.

Several steps were omitted and others modified. Image development was in the presence of sodium thiosulfate as suggested by BLUM *et. al.* [149] but instead using higher concentrations of formaldehyde. This procedure is highly sensitive, avoids unspecific background staining without loss of contrast, uses less silver and no oxidizing pretreatments, and stains complex mixtures of DNA resolved in polyacrylamide gels bound to polyester backing film. The originally suggested concentration of $AgNO_3$ (12 mM) can be halved provided the concentration of formaldehyde is increased at least two- to threefold during image development. Reduction of silver by formaldehyde is concentration dependent. Optimal band intensity occurs at about 0.00555% (by volume) formaldehyde. Thiosulfate dissolves insoluble silver ions from the gel surface which in turn decreases nonspecific staining [149]. A concentration of 4 μM thiosulfate is sufficient to reduce nonspecific background staining without noticeably affecting DNA image development.

Gels polymerized onto polyester backing films are handled easily during staining, and, when dried, produce a permanent record. However, the polyester film layer has the disadvantage of decreasing the surface area of the gel in contact with

the liquid, slowing diffusion of solutes in and out the gel matrix. This lengthens the time required for image development during staining which can substantially increase background staining (see figure 4.8 on page 70).

The limit of detection upon visual inspection of double-stranded DNA was approximately 1 pg/mm^2. This is about 1000 to 10,000 times more sensitive than ethidiumbromide staining (see section 2.7.1 on page 33).

4.6.1 Silver Staining Protocol

The following protocol was used based on the staining improvements of BASSAM [150]. The desilver and impregnation steps were modified.

Step	Time	Reagents
1. Fix	30 min	10% acetic acid (v/v)
2. Rinse	3×2 min	water (bidest.)
3. Impregnate	20–30 min	$AgNO_3$ (1 g/l)
		+ 100 μl 37% formaldehyde per 100 ml
4. Rinse	20 s	H_2O (bidest.)
	Thoroughly wash the gel surface and back of the gel	
5. Develop	2–5 min	2.5% $NaCO_3$
	(visual control)	+ 100 μl 37% formaldehyde per 100 ml
		+ $Na_2S_2O_3 \cdot 5H_2O$ (2 mg/liter)
6. Stop	10 min	2% (g/v) glycine
		+ 0.5% EDTA disodium salt solution
7. Impregnate	10 min	5% (v/v) glycerol
8. Dry	overnight	room temperature

Table 4.7: Procedure for silver staining of nucleic acids

4.7 Results

Four of the sequenced mutations (1309, 1515, 1786 and 2316, see section 3.6 on page 49) were scanned by RFLP using agarose as well as polyacrylamide gels. Most of the samples used were scrutinized in previous studies, and a phenotype (β_2 receptor density) was available. RFLP results for both, agarose minigels with ethidiumbromide staining, as well as polyacrylamide gels with silver staining, are given below. The marker used for agarose gels was Biomarker Low (BioVentures, Inc.), showing bands at 100, 200, 300, 400, 500, 525, 700 and 1000 base pairs. The marker used for non-denaturing polyacrylamide gels (CleanGels) was the 100bp ladder (Pharmacia). Note, that in non-denaturing gels the mobilities of DNA fragments are also dependent on the base sequence (content of A and T). This can be easily seen in figures 4.7 on page 70 and 4.8 on page 70.

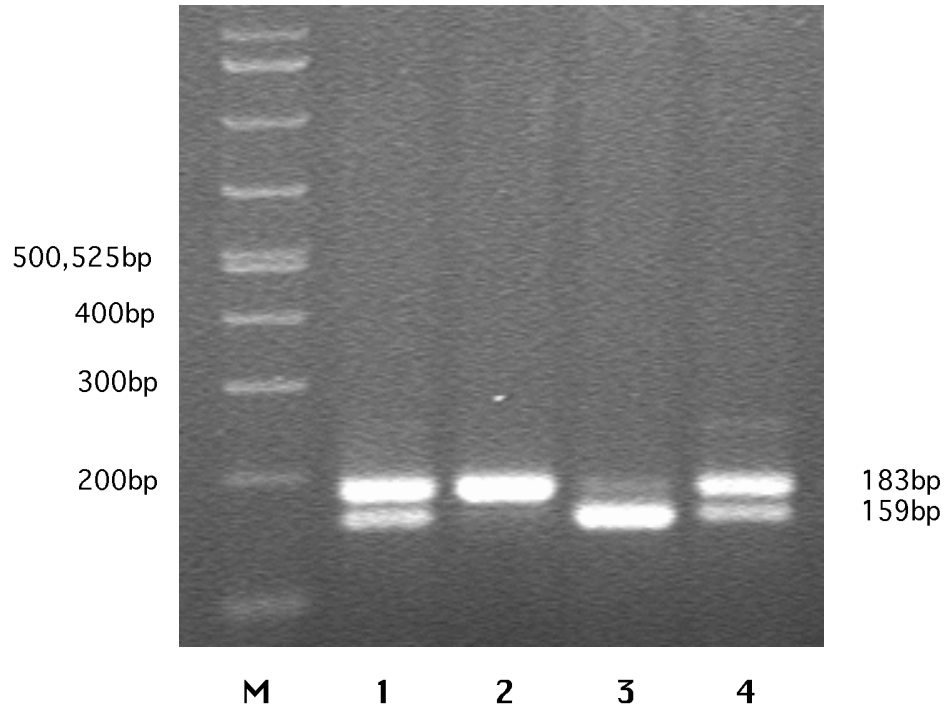

Figure 4.3: RFLP for position 1309 (agarose gel) Lane M: Marker; Lanes 1, 4: heterozygous; Lane 2: homozygous G; Lane 3: homozygous A

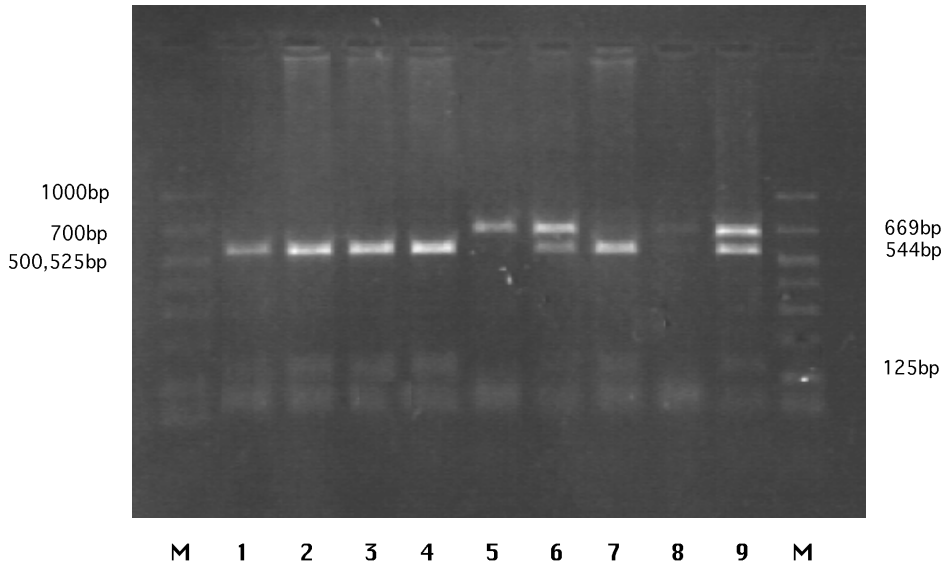

Figure 4.4: RFLP for position 1786 (agarose gel) Lane M: Marker; Lanes 1-4, 7: homozygous C; Lane 5: homozygous A; Lanes 6, 9: heterozygous

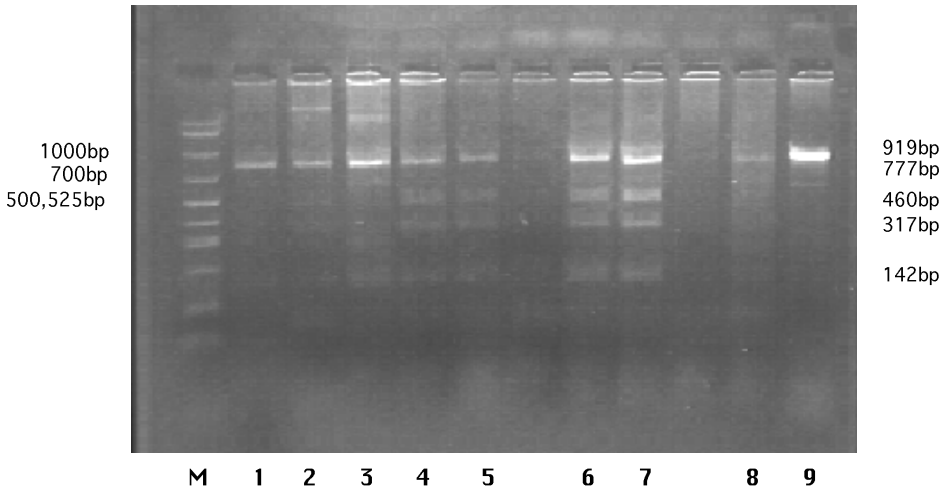

Figure 4.5: RFLP for position 2316 (agarose gel) Lane M: Marker; Lanes 1-3, 8: homozygous G; Lanes 4-7: heterozygous; Lane 9: uncut PCR product

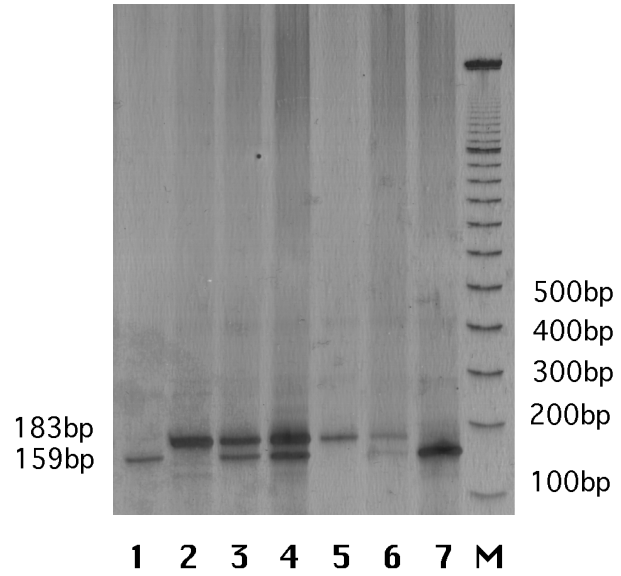

Figure 4.6: RFLP for position 1309 (PAGE) Lane M: Marker; Lanes 1, 7: Arg; Lanes 2, 5: Gly; Lanes 3, 4, 6: Arg/Gly

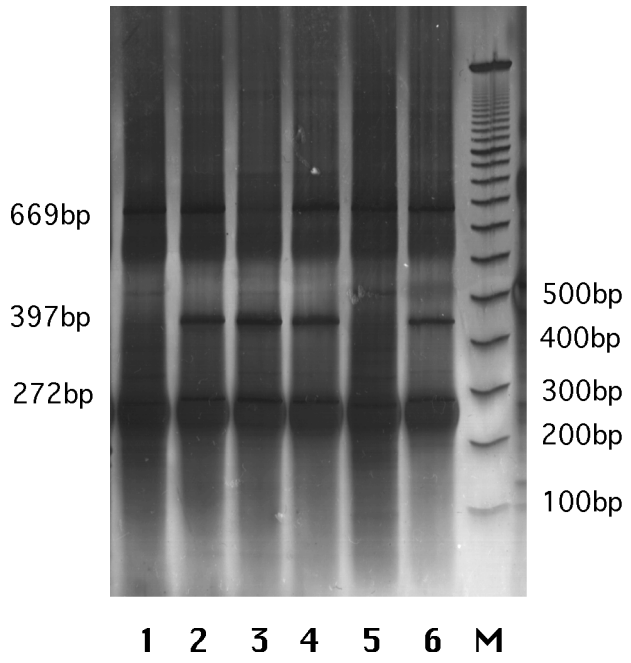

Figure 4.7: RFLP for position 1515 (PAGE) Lane M: Marker; Lanes 1, 5: GG; Lane 3: AA; Lanes 2, 4, 6: A/G

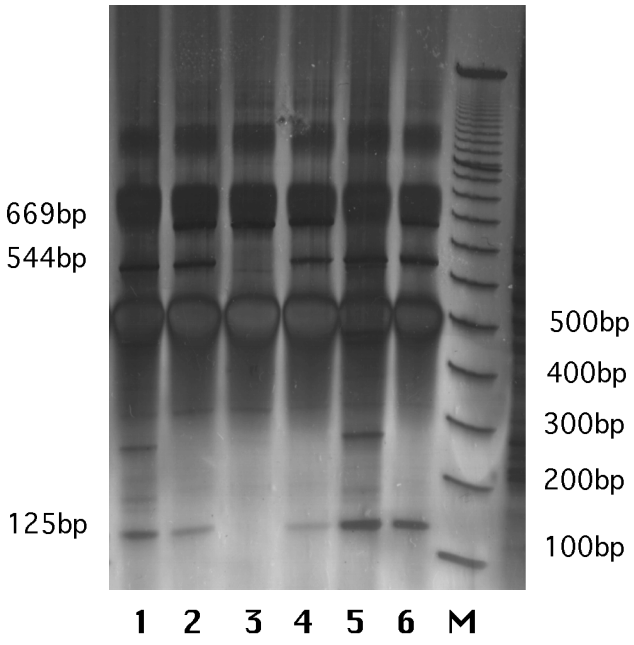

Figure 4.8: RFLP for position 1786 (PAGE) Lane M: Marker; Lanes 1, 5: CC; Lane 3: AA; Lanes 2, 4, 6: A/C

4.7.1 Human β_2 Adrenergic Receptor Allele Frequencies and Haplotypes

All mutations observed coincided with the Hardy-Weinberg equilibrium. Haplotype frequencies were determined on the basis of the results obtained from 37 unrelated subjects. The results are displayed in table 4.8.

Nucleotide				Frequency
1309	1515	1786	2316	
G	G	C	G	$\sim$ 36%
A	G	C	G	$\sim$ 27%
G	A	A	G	$\sim$ 12%
G	A	A	C	$\sim$ 10%
G	G	C	C	$\sim$ 10%
A	G	C	C	$\sim$ 5%

Table 4.8: Alleles of the human β_2 adrenergic receptor

The degree of polymorphism of a marker may be assessed by the proportion of individuals in the population who are heterozygous for that marker. In other words, the probability, that a random individual is heterozygous is used as a measure of the degree of polymorphism. This probability may be estimated in two principal ways: The first measure is the amount of heterozygosity observed $\hat{h}$ and is simply the proportion of heterozygous individuals observed in the sample [152]. In human genetics, a more precise estimate is used. It rests on the assumption that the genotypes are in Hardy-Weinberg equilibrium. This expected heterozygosity is defined as $H = 1 - \sum p_i^2$, where the sum is taken over all alleles, with p_i denoting the frequency of the i-th allele. The maximum likelihood estimate of h is given by $\hat{H}_m = 1 - \sum(\hat{p}_i)^2$ and is slightly biased. An unbiased estimate is $\hat{H}_u = \hat{H}_m n/(n-1)$, where n is the number of alleles observer in a sample [153]. The estimate $\hat{H}_u$ is preferable over $\hat{H}_m$ because it is unbiased and has a smaller mean square error than $\hat{H}_m$.

An older measure of heterozygosity is the Polymorphism Information Content (PIC) value [154], which is defined as

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

where a is the number of alleles at the given locus. For family data PIC may be somewhat more appropriate, whereas the heterozygosity is more general.

Heterozygosity and the calculated Polymorphism Information Content (PIC) are shown in table 4.9 on the next page.

Mutation	Allele	Number	*PIC*	$\hat{H}_u$
	A/A	10		
1309	A/G	21	0.5436	0.6376 ±0.0346
	G/G	22		
	G/G	15		
1515	A/G	6	0.5511	0.6909 ±0.0861
	A/A	2		
	A/A	3		
1786	A/C	14	0.4327	0.5216 ±0.0587
	C/C	38		
	C/C	4		
2316	C/G	11	0.4264	0.5270 ±0.0581
	G/G	21		

Table 4.9: PIC and $\hat{H}_u$ of the scanned mutations

4.7.2 β_2 Adrenoceptor Alleles in Relation to Adrenoceptor Expression in Cultured Skin Fibroblasts

β_2 adrenoceptor density ranged from 5 to 298 fmol/mg (median 101 fmol/mg). The $C^{2316} \rightarrow G^{2316}$ nucleotide exchange did have no impact on β_2 adrenergic receptor density. There was a tendency towards lower β_2 adrenoceptor densities in fibroblasts derived from subjects with AA^{1309} as compared to GG^{1309} genotypes. The frequency of A^{1786} in fibroblast cultures with adrenoceptor densities < 100 fmol/mg was 0.125 (0.049–0.268, 95% confidence interval) as compared to the group with β_2 adrenoceptor densities above the median, which showed a A^{1786} frequency of 0.278 (0.142–0.452, 95% confidence interval, p=0.09).

Chapter 5

Discussion

Hypertension is a leading cause of human cardiovascular morbidity and mortality, with a prevalence rate of 25%–30% in the adult Caucasian population in the USA [155] and in Europe. The primary determinants of essential hypertension, which represents 95% of the hypertensive population [156], have not been elucidated despite intensive research. Studies of large populations, of twins, and of adoptive siblings, provide evidence for a strong genetic component in the regulation of blood pressure [157]. On the basis of these observations molecular determinants which contribute to the pathogenesis of hypertension have been searched for. There is evidence suggesting that 30% of the variation of blood pressure may be genetically determined [158].

Epidemiologic studies indicate a continuous distribution of blood pressure in the population, and the genetic disorder appears to be polygenic and therefore does not follow simple Mendelian patterns of inheritance [159]. It is likely that several genes interact with environmental stimuli to produce high blood pressure in susceptible persons [158].

It has been shown recently that the locus of the angiotensinogen (AGT) gene, which encodes a key component of the renin-angiotensin system, is linked to essential hypertension both in Caucasians and in African Caribbeans [10, 12]. This finding represents a major advance in the search for genes associated with essential hypertension. The exact mutations within the AGT gene influencing disease susceptibility remain to be discovered.

Among those environmental factors possibly contributing to the development of essential hypertension salt intake appears to be of prominent importance [160]. There is convincing evidence, that in populations with a high salt intake the prevalence of essential hypertension is increased [7]. The mechanisms by which salt causes hypertension are not completely understood. Of particular interest is the interaction of the sympathetic nervous system and salt intake. The sympathetic nervous system plays a key role in the regulation of blood pressure. It acts via neurotransmitters (epinephrine, nor-epinephrine) on adrenergic receptors. The cellular equipment with various receptor subtypes defines the cellular response. In

vascular smooth muscle cells, the main determinants of the peripheral resistance and hence the blood pressure, vasoconstriction is mediated by α_1 and α_2 receptors and vasodilatation by β_2 receptors. It has been shown recently, that the blood pressure response to salt is at least in part explained by changes of the densities of these adrenoceptor subtypes: a high salt intake causes a rise of α_2 adrenoceptors (and promotes therefore vasoconstriction) and a fall of β_2 receptors (resulting in a blunted vasodilation) [161]. A subpopulation of normotensives is especially prone to blood pressure rise when exposed to a high salt intake (this group is coined salt sensitive). On contrast, a substantial proportion of the population (about 60-70%) are salt resistant, i.e. they demonstrate no rise of blood pressure when consuming a high salt diet. In longitudinal studies it has been shown that salt sensitivity in normotensives may be a precursor state of essential hypertension [162]. A genetic basis of salt sensitivity has been suggested recently when it appeared that in cell culture skin fibroblasts from salt sensitive subjects express less than half the number of β_2 adrenoceptors as compared to salt resistant subjects [163]. This finding prompted us to study the genetics of the β_2 adrenoceptor in greater detail.

The etiological heterogeneity and multifactorial determination that characterize diseases as common as hypertension expose the limitations of the classical genetic arsenal. Definition of phenotype, model of inheritance, optimal familial structures, and candidate gene impose critical strategic choices [164, 165]. Therefore the establishing of a direct phenotype, the β_2-adrenoceptor density, was tried for easier interpretation of the results.

Almost 50 years after AHLQUIST [19] first uncovered evidence of the heterogeneity of adrenergic receptors, the number of receptor subtypes is still unclear, although nine subtypes are well documented (three subtypes each of α_1-, α_2-, and β-adrenergic receptors). Each type preferentially links to members of a subfamily of G proteins: α_1 to G_q, α_2 to G_i, and β to G_s, and in turn to effector molecules to which those G proteins link (G_q to phospholipase C_β, G_i and G_s to adenylyl cyclase). The number of activated G proteins exceeds the number of corresponding receptors and effectors; thus, the activation of G proteins amplifies signaling by adrenergic receptors. Receptor desensitization, mediated in part by G-protein-receptor kinases and β-arrestins, is involved in decreasing the ability of agonists to activate adrenergic receptors. Alterations in adrenergic receptors have a role in many clinical settings. Recent data suggest a role for the increased expression of G-protein-receptor kinases in hearts of patients with congestive heart failure and for genetic polymorphisms in β_3-adrenergic receptors in patients with obesity. Studies using molecular and biochemical techniques are likely to provide additional new and unexpected insights into the role of adrenergic receptors in both normal physiologic function and disease.

Receptor biosynthesis, processing, and insertion in the plasma membrane are not well understood. The regulation of adrenergic receptors by receptor-specific agonists and antagonists has been actively studied for many years, whereas the mechanism by which a high salt diet influences adrenergic receptors is not known yet. Given the widespread expression of adrenergic receptors and their role in reg-

Clinical Condition	Type of Adrenergic Receptor	Alteration
Adrenergic-agonist treatment, pheochromocytoma	α,β	Desensitization and down-regulation of receptors
Antagonist withdrawal syndrome	β,?α	Supersensitization and up-regulation of receptors
Myocardial ischemia	β	Up-regulation and uncoupling of receptors
	α_1	Enhanced receptor coupling
Hypertension	α	Upregulation and altered coupling of receptors
	β	Down-regulation of receptors
Congestive heart failure	β_1	Down-regulation of receptors
	β_2	Receptor uncoupling
Morbid obesity	β_3	Polymorphism (? with decreased activity)

Table 5.1: Clinical Conditions associated with possible alterations in adrenergic receptors

ulating a wide variety of events, it is conceivable that alterations in these receptors have been suspected in many clinical settings (see table 5.1).

As salt sensitivity resembles a pre-hypertensive state, we believe that the human β_2 adrenergic receptor gene should be considered as a candidate gene involved in the development of essential hypertension. The aim of this thesis was to search for mutations of the β_2 adrenoceptor in several subjects and to develop a fast screening method in order to eventually determine the frequencies of alleles of the β_2 adrenergic receptor gene in the general population, in salt sensitives and salt resistants, and in hypertensive subjects. Recent measurements of β_2-adreneoceptor mRNA content in cultured human skin fibroblasts revealed no difference irrespective of the number of β_2-adrenoceptor expressed on the cell surface [15]. These findings are in line with data obtained in fat cells [166] and point towards the possibility of a modification of β_2 adrenoceptor density at a level beyond gene expression.

Mutational analysis of the β_2-adrenoceptor gene showed that N-glycosylation at Asn^6 and Asn^{15} is important for correct trafficking of the receptor molecule through the cell. Glycosylation deficient mutants showed in COS-7 cells a 50% decrease in the level of accumulation of β_2 adrenergic receptor on the cell surface [57]. The $G^{1309} \rightarrow$A codon change results in a $Gly^{16} \rightarrow$Arg primary structure change. The $Gly^{16} \rightarrow$Arg mutation resembles the *X* within the Asn *X* Ser/Thr glycosylation consensus sequence. The influence of the $Gly^{16} \rightarrow$Arg amino acid change on the glycosylation pattern of the β_2 adrenergic receptor protein is not known.

Change	Codon	Allele	β_2 adrenoceptor density	
			< 100 fmol/mg	> 100 fmol/mg
GGA→**A**GA	1309	G/A	7	8
		G/G	7	10
		A/A	3	2
AGG→**C**GG	1786	A/C	3	8
		A/A	1	1
		C/C	16	9
GG**C**→GG**G**	2316	C/G	5	5
		C/C	1	1
		G/G	10	10

Table 5.2: Alleles of the human β_2 adrenergic receptor gene in relation to β_2 adrenoceptor expression on cultured skin fibroblasts

The secondary structure of mRNA is known to influence translation efficacy. Silent mutations may have a significant impact on secondary mRNA structure. Whether the silent mutations described in this thesis are associated with alterations of secondary mRNA structure, mRNA stability, or translation efficiency remains to be clarified. The GCG program `mfold`[1] was used to model secondary mRNA structures obtained with two of the detected mutations (position 1309 and 1786); this analysis revealed altered secondary mRNA structures and energies.

Although the number of samples used in this study was low, the point mutations at positions 1309 and 1786 show a tendency towards higher (1309) or lower β_2 adrenoceptor densities (1786), respectively. This can be further demonstrated by plotting the actual β_2 adrenergic receptor density versus genotypes (see figure 5.1 on the next page). Due to the fact that this thesis resembles a pilot study only a small number of subjects was involved. This limitation will be overcome in the very near future by an international joint study with Mark CAULFIELD (St. Bartholomew's Hospital, London) using DNA from several hundreds of clinically well characterized subjects [10].

The fact that in this small association study no direct relationship of $\beta_2$adrenoceptor alleles and salt sensitivity or hypertension has been shown does not preclude the view that, on the basis of previous studies [163], the β_2 adrenergic receptor should be considered as one of the candidate genes for the development of essential hypertension. Clear answers to the question whether or not the β_2 adrenergic receptor is involved in the pathogenesis of essential hypertension can be expected from large scale linkage studies. Genetic polymorphism is a conditio sine qua non for linkage studies. This thesis work has provided evidence for a polymorphism of the human β_2-adrenoceptor gene. The six β_2-adrenoceptor alleles described so far provide sufficiently polymorphic genetic markers to embark on large scale family studies.

[1] `mfold` predicts optimal and suboptimal secondary structures for an RNA molecule using the most recent energy minimization method of Zuker [167–170].

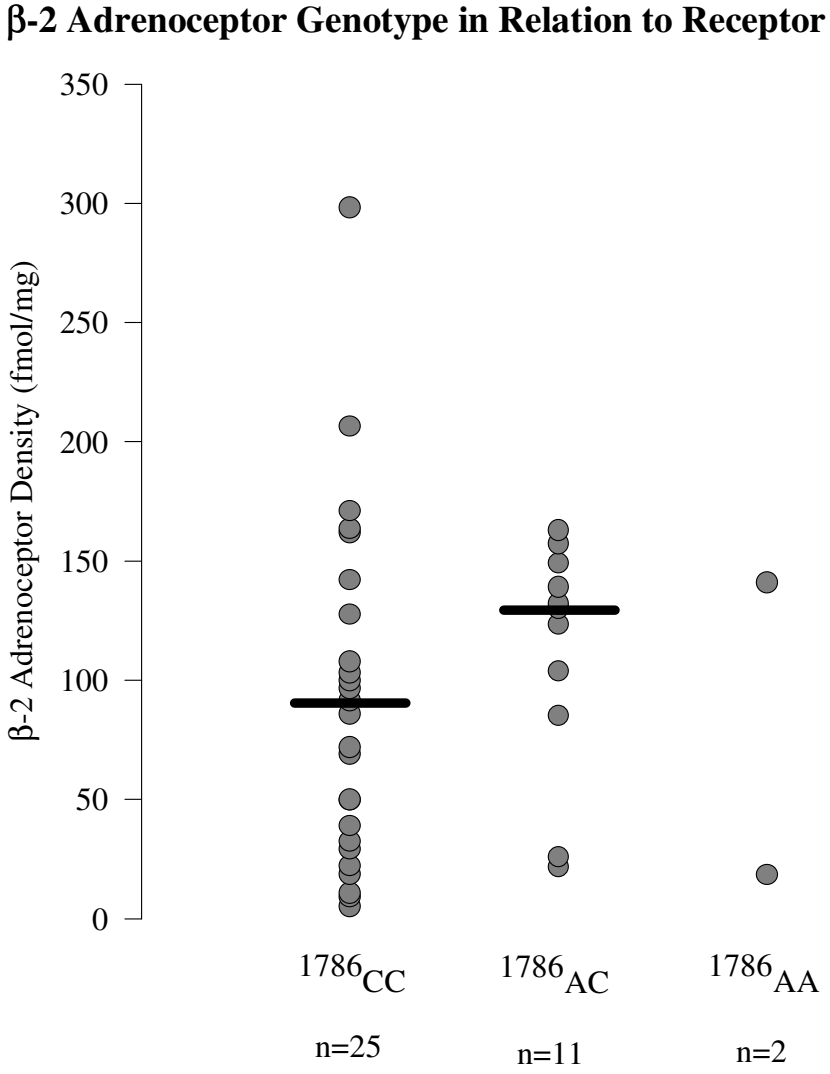

Figure 5.1: β_2 Adrenoceptor genotype at position 1786 in relation to receptor density

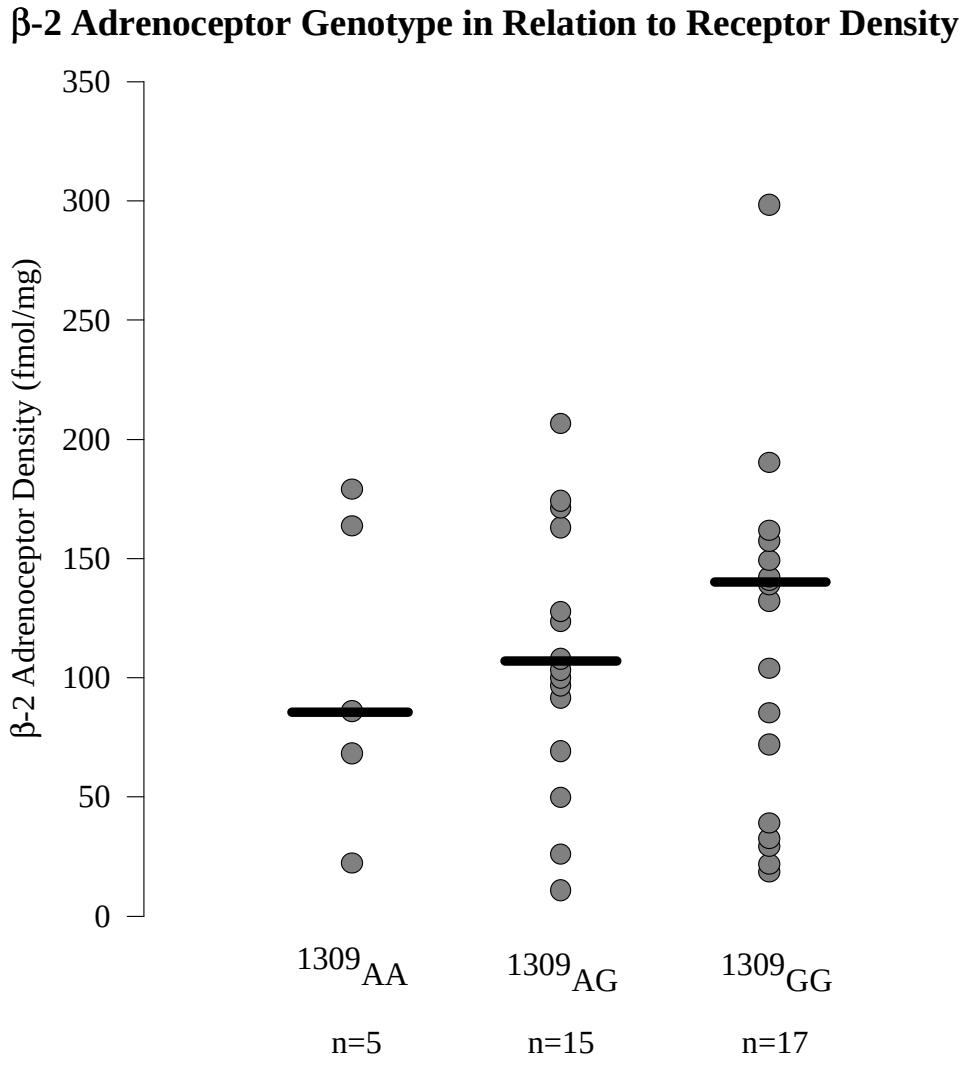

Figure 5.2: β_2 Adrenoceptor genotype at position 1309 in relation to receptor density

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