Identification of tissue specific expression and polymorphic differences in neuropeptide Y receptors Y1 and Y5

Avery R. Soderman
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IDENTIFICATION OF TISSUE SPECIFIC EXPRESSION AND POLYMORPHIC DIFFERENCES IN NEUROPEPTIDE Y RECEPTORS Y1 AND Y5

by

Avery R. Soderman

A Thesis
Presented to the Graduate and Research Committee
of Lehigh University
in Candidacy for the Degree of
Master of Science
in
Molecular Biology

Lehigh University
November, 1999
IDENTIFICATION OF TISSUE SPECIFIC EXPRESSION AND POLYMORPHIC DIFFERENCES IN NEUROPEPTIDE Y RECEPTORS Y1 AND Y5

by

Avery R. Soderman

This thesis is accepted and approved in partial fulfillment of the requirements for the degree Master of Science in Molecular Biology.

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ABBREVIATION LIST

ACTH-Adrenocorticotropin Hormone

GPCR-G-Protein Coupled Receptor

MC1R-Melanocortin-1-Receptor

MC4R-Melanocortin-4-Receptor

MSH-Melanocyte Stimulating Hormone

NaOH-Sodium Hydroxide

NPY-Neuropeptide Y

NPY1R-Neuropeptide Y-1-Receptor

NPY5R-Neuropeptide Y-5-Receptor

PBS-Phosphate Buffered Saline

PCR-Polymerase Chain Reaction

POMC-Pro-opiomelanocortin

RACE-Rapid Amplification of cDNA Ends
ABSTRACT

Obesity is a serious health problem in Western society, affecting up to 35% of the adult population in the United States and is associated with cardiovascular disease, non-insulin dependent diabetes mellitus, and increased risk of many forms of cancer. Obesity is recognized as a disorder of energy balance, having genetic components, which develops when energy intake through feeding exceeds energy expenditure. Regulation of feeding occurs through the hypothalamic regulation of two main pathways, the catabolic or melanocortin pathway, and the anabolic or neuropeptide Y pathway. Evidence in rodent models shows that mutations in genes coding for neuropeptides and neuropeptide receptors in these pathways can cause obesity in rodents. Mutations in ortholog genes in humans may similarly be a cause of human obesity. Recent evidence has found that mutations in the melanocortin pathway in humans are either silent or occur at a frequency of less than 1%. The rate of mutations were examined in the genes coding for receptors involved in the anabolic pathway, specifically neuropeptide Y receptors Y1 and Y5. In addition, tissue specific expression patterns of the alternatively spliced forms of NPY Y1 and Y5 receptor mRNAs were determined. Three separate heterozygous mutations were identified in the coding regions of both receptor genes. These mutations, however, were either synonymous (silent) or rare, occurring at a frequency less than 1%, providing indirect evidence that obesity in humans is not caused by these genes. Tissue specific expression analysis showed that expression of the Y5 receptor gene is localized primarily to the brain whereas the expression of the Y1 receptor occurs in the brain as well as peripheral tissues and is regulated by three alternatively spliced 5’ exons.
Introduction

Obesity is a serious health problem in Western society, affecting up to 35% of the adult population in the United States. Obesity is associated with morbidity in cardiovascular disease, non-insulin dependent diabetes mellitus, osteoarthritis and increased risk of many forms of cancer. Once thought to be a social disease which could be treated with appropriate diet and exercise regimens, obesity is now recognized as a disorder of energy balance having genetic components, which develops when energy intake exceeds energy expenditure. Energy intake and energy expenditure are largely regulated by the central nervous system (CNS), particularly the hypothalamus (Strader, 1997). Afferent signals from the periphery communicate information about the metabolic and nutritional status to the CNS. The CNS responds to these afferent signals by altering the levels of several neurotransmitters, thereby generating efferent signals that alter appetite and energy expenditure.

Traditionally, control of energy intake has been the approach to the control of obesity. Over the past few years, however, the role of energy expenditure in the control of obesity has become increasingly apparent. Weight loss is difficult to maintain by control of energy intake alone, suggesting that body weight tends to stabilize at a physiological set point that is difficult to alter (Leibel, 1995). When energy intake alone is controlled, the body responds by controlling its energy expenditure in a compensatory direction. Therefore, successful treatment of obesity must regulate the mechanisms that control both energy intake and energy expenditure.
Leptin and the Leptin Receptors

Recent experiments in both mouse and human models have provided evidence that has helped to unravel several genetic components that regulate obesity. The cloning of the \textit{ob} gene and the demonstration that its protein hormone product, leptin, is secreted by adipocytes in the periphery and acts on the CNS to regulate body weight helped in the understanding of body weight control (Campfield, 1995). Further observations showed that leptin plays a central role in coordinating both food intake and satiety as well as regulation of energy expenditure (Woods, 1998). This hormone has both long term and short term affects on feeding. In the long term, this hormone conveys to the hypothalamus information about the size of energy stores and activates hypothalamic centers that regulate energy intake and expenditure (Flier, 1997). In addition, leptin affects several neuroendocrine mechanisms that regulate multiple hypothalamic-pituitary axes (Flier, 1997).

This hormone also acts in the short term, following periods of feeding and fasting, independent of energy stores. In humans, following periods of fasting, plasma leptin levels are decreased, much more than the amount of adipose tissue lost (Lonnqvist, 1995). Leptin and \textit{ob} gene mRNA levels during periods of fasting and feeding suggests that serum leptin is regulated by energy availability. This regulation is not immediate, however, and the adaptive response of leptin to energy availability is mediated through changes in serum insulin and fatty acids (Considine, 1996). Leptin levels reflect not only the amount of fat stored, but also energy imbalance; prolonged fasting decreases leptin levels, whereas overfeeding increases them (Tritos, 1997). The composition of the diet also controls circulating leptin levels. In addition, prolonged insulin infusions increase
leptin levels. Therefore, the control of feeding occurs through both long term and short term mechanisms that control leptin levels.

The mouse leptin gene consists of 3 exons and two introns and encodes a 4.5 Kb spliced mRNA (Zhang, 1994). Two different mutations in the mouse gene have been found to date, one of which abolishes the leptin gene transcription, the \textit{ob/ob} genotype, and the other which results in a truncated, inactive form of the protein (Zhang, 1994). Mice with this \textit{ob/ob} genotype are obese (Zhang, 1994). Later experiments showed that infusion of leptin into \textit{ob/ob} mice eliminated obesity (Halaas, 1995). This discovery raised the question as to whether obesity in humans could also be due to a deficiency in leptin production.

Human leptin is 84% homologous to the mouse protein. Recently, the first two individuals with mutated leptin genes resulting in inactive proteins have been discovered (Montague, 1997). However, an obese population DNA screening has failed to demonstrate a high incidence of mutations within the leptin gene, indicating that mutations in the leptin gene are a rare cause of obesity in humans (Considine, 1995 & Niki, 1996). It appears that obesity in humans may be a leptin-resistant rather than a leptin-deficient state (Considine, 1996).

One possibility for this resistance may be due to defective leptin receptors. In the mouse, six alternatively spliced forms of the leptin receptor are known (Gwo-Hwa, 1996). The two major splice variants are the short (Ob-R-S) and the long (Ob-R-L) forms (Gwo-Hwa, 1996). The short form is expressed in a variety of tissues, with the highest number of receptors found in the mouse choroid plexus and leptomeninges (Gwo-
Hwa, 1996). These receptors transport leptin into the cerebrospinal fluid, but not into the brain interstitial fluid which bathes the brain receptors (Lonqvist, 1999).

The second receptor form, the long form, is expressed at high levels in the hypothalamus (Gwo-Hwa, 1996). In order for leptin to reach the hypothalamus, it must be transported across the blood-brain barrier. This active transport across the blood-brain barrier occurs through a saturable, specific temperature dependent process that occurs via a dual process which includes binding and endocytosis (Golden, 1997). Because of the fact that the ratio of leptin concentrations between the serum and cerebrospinal fluid is much lower in obese mice, a decreased transportation efficiency across the blood-brain barrier could be one of the major factors that contribute to obesity (Schwartz, 1996).

In humans, however, a deficiency of the leptin receptor does not appear to be a cause of obesity. Although sequence mutations were discovered in the human leptin-receptor gene during population screening, most translated into silent mutations (Clement, 1998). There was, however a single base substitution that caused the change of a glutamine for an arginine. This mutation, however was found in both lean and obese individuals and most subjects were heterozygous for the mutation. Because most cases of human obesity are not associated with defective leptin or leptin-receptor genes, it is possible that a defect in the leptin signaling pathway may be the cause of obesity in humans.

Leptin circulates in the plasma in a free or bound form. Plasma leptin levels increase with increasing fat mass (Lonqvist, 1995). Leptin levels reflect not only the amount of adipocytes, but also energy imbalance. Prolonged fasting decreases leptin levels whereas overeating greatly increases them (Kolaczynski, 1996). In addition to
diet, adiposity, and energy balance, a number of intrinsic adipocyte factors as well as several cytokines may regulate leptin expression and circulating levels in humans (Mantzoros, 1998).

Leptin acts by activating specific leptin receptor isoforms which, as in the mouse, include a long and several short forms (Tartaglia, 1997). In humans, long leptin receptors are found in many areas of the brain, including the hypothalamus, cerebellum, and hippocampus (Steiner, 1996). The long leptin receptor isoform activates transcription and alters expression of many hypothalamic neuropeptides which are involved in two different types of feeding systems.

The first system is termed catabolic and is activated by high levels of leptin during positive energy balance. This system, exemplified by the melanocortin system, decreases food intake and increases energy expenditure to facilitate the loss of excess energy stores. The second system is termed anabolic and is activated by low levels of leptin during negative energy balance. This system, exemplified by the neuropeptide Y system, increases food intake and decreases energy expenditure to facilitate the regaining of lost energy stores. Activation of these neuropeptides starts a cascade of events which ultimately leads to modifications in energy intake and expenditure.

Catabolic Pathway of Feeding

In the first case, under obese conditions, the increased amount of adipose tissue corresponds to higher levels of peripheral leptin. Increased leptin causes the release of Melanocyte stimulating hormone (MSH) from the hypothalamus. MSH binds to the melanocortin-4 receptor in the hypothalamus and causes reduced food intake as well as
increased energy expenditure and sympathetic activity (Friedman, 1997). In other words, higher leptin levels lead to a state in which energy expenditure exceeds food intake.

In this case, when higher levels of leptin exist as a result of increased adipocytes, the melanocortin receptor pathway is activated and a decrease in food intake results (Woods, 1998). There are five known melanocortin receptors in the mouse. All of these receptors bind the peptide hormone class of melanocyte stimulating hormones (MSH). These peptide hormones are derived from pro-opiomelanocortin (POMC), a prohormone that is processed into three classes of hormones; the melanocortins, adrenocorticotropin hormone (ACTH), and various endorphins (Cone, 1996). Evidence for the role of the melanocortin pathway in feeding comes from mouse models.

The melanocortin-1-receptor (MC1R) has been shown to be involved in coat color whereas the Melanocortin-4-receptor (MC4R) has been shown to be involved with signal transduction in the hypothalamus (Satoh, 1998). Hypothalamic MC4R as well as the MC1R signaling is blocked in Agouti mutant mice by ectopic expression of the Agouti peptide (Lu, 1994). The Agouti peptide causes a yellow skin, obese phenotype. A MC4R knockout mouse was made to assess the role of MC4R in the regulation of weight homeostasis. These mice have identical phenotypes as the Agouti mice, develop age-onset obesity, with the exception of the coat color, verifying the separate roles of the MC-1 and MC-4 receptors (Huszar, 1997). Under normal conditions, binding of leptin to the Ob-R-L in the hypothalamus causes the production of POMC. POMC is then converted to melanocortin which binds to the MC4R. Binding of melanocortin to the MC4R causes a cascade of unknown events which reduces feeding.
Even though this pathway is poorly characterized in humans, it is believed that mutations in genes causing monogenetic forms of obesity in rodents are implicated in similar syndromes in humans (Montague, 1997). It is possible to think that mutations in the MC4R are responsible for obesity in humans. Factors downstream of the MC4R have not yet been identified. Recent research therefore has concentrated on determining whether mutations in the MC4R gene exist, and if so, at what frequency in the obese population. Several studies indicate that polymorphisms within the coding region of the MC4R receptor are unlikely to be a common cause of obesity in humans due to the low frequency of functionally significant mutations (Gu, 1999 & Hinney, 1999). Therefore, until factors downstream of the MC4R have been identified, it will be difficult to tie polymorphisms within the MC4R pathway to the development of obesity in humans. However, it is possible that polymorphisms within the coding regions of the NPY receptor genes, which are also known to be affected by leptin and have a role in feeding, may be the cause of early onset obesity in humans.

**Anabolic Pathway of Feeding**

In this pathway, during starvation, lower amounts of adipose tissue correspond to lower levels of leptin being released from this tissue as an afferent signal. Levels of leptin receptor mRNA and leptin binding are increased in the hypothalamus during fasting, principally in NPY neurons (Baskin, 1999). Leptin binding causes the activation of the Neuropeptide Y (NPY) pathway. Increased levels of NPY are released from the hypothalamus and NPY binds to both the NPY 1 and NPY5 receptors, causing a cascade of poorly understood events which increase food intake and decrease energy expenditure.
Neuropeptide Y, a 36 residue peptide, is known to participate in vascular tone, feeding, inhibition of seizures and in neuropeptide and anterior pituitary hormone secretion. NPY is expressed predominantly in the central nervous system and is one of the most abundant neuropeptides in the brain (Wahlestedt, 1993). Central administration of NPY exerts a powerful feeding stimulus and influences body energy homeostasis by affecting insulin secretion, hepatic glucose output and lipoprotein lipase activity (Kalra, 1997). In addition, mice deficient in NPY show a significant increase in susceptibility to seizures, suggesting that NPY may be an endogenous anticonvulsant. These diverse physiological actions are mediated through distinct NPY receptor subtypes (Yinghe, 1996). NPY receptors, like the MC4R and the leptin receptor, belong to the large superfamily of G-protein-coupled receptors (GPCRs).

GPCRs are a superfamily of seven transmembrane domain proteins. They are found in a wide range of organisms and are involved in the transmission of signals to the interior of the cell via interaction with a G-protein. All GPCRs are thought to operate through a similar molecular mechanism. The binding of an extracellular ligand to GPCRs causes poorly understood conformational changes. These conformational changes promote the association of the GPCR with distinct classes of heterotrimeric G-proteins. This association activates these G-proteins which are then free to interact with effector enzymes and ion channels (Wess, 1998).

Six subtypes of NPY receptor have been cloned, showing a large degree of sequence variation. These receptors have been studied most extensively in rodents where it was shown that ligands that preferentially bind NPY Y1 and Y5 receptors stimulate feeding whereas most Y1 and Y5 antagonists have been shown to inhibit food intake.
Oshea, 1997). In addition, pharmacological data suggest that NPY’s stimulatory effect on appetite is transduced by the NPY Y5 receptor (Oshea, 1997). The evidence suggests that NPY increases food intake by binding the Y1 and Y5 receptors.

Additional evidence for the role of NPY in feeding comes from rodent studies. In these animals it has been observed that leptin administration represses NPY production in the hypothalamus in response to fasting (Billington, 1991). Other evidence comes from the observation that chronic administration of NPY into the hypothalamus rapidly leads to obesity in rodents (Stanley, 1986). Finally, NPY signalling is elevated in the hypothalamus of leptin deficient mice (Stephens, 1995 & Schwartz, 1996). Because of these results, the NPY receptors are potential candidates for the control of obesity in humans.

The human NPY Y5 receptor has 88% homology to the rat NPY Y5 receptor. In humans, neurons of the hypothalamic arcuate nucleus exert both stimulatory and inhibitory effects on food intake and both NPY Y1 and Y5 mRNA are highly expressed at this site (Woldbye, 1998). These results, in addition to the data obtained from rat studies, make the NPY Y1 and Y5 receptors excellent candidates for drug development against obesity as well as excellent candidates for polymorphic analysis.

The human NPY Y1 and Y5 receptor are transcribed from a common promoter region in opposite directions (Herzog, 1997), (Fig.1). Both genes are alternatively spliced. To date, three alternatively spliced 5’ exons of Y1 (Herzog, 1997), and five alternatively spliced 5’ exons of Y5 have been discovered (Parker, 1999) which allow for the regulation of tissue specific expression. In all cases, these exons encode part of the 5’ untranslated region (Herzog, 1997 & Parker, 1999).
Overlapping gene structures of Neuropeptide Y1 and Y5 receptors

Figure 1. The human Neuropeptide Y1 and Y5 gene cluster as adapted from Herzog (1997). The coding region of both genes are shown as black vertical lines. Noncoding sequences are shown as blue boxes for Y5 and green boxes for Y1. Exon 1C of Y1 is part of the coding region of Y5 and is indicated by green and blue horizontal lines.
One of the alternatively spliced exons of Y1 (1C) is also contained in the coding region of Y5, and is transcribed from the opposite strand. In addition, both alternatively spliced Y5 exons (1A and 1B) are in close proximity to exon 1B of NPY Y1. Transcription of both genes from the same promoter region suggests the possibility that transcriptional activation of one will have an effect on the regulation of expression of the other. As both receptors are thought to play a role in the regulation of food intake through NPY, coordinating expression of these two genes will be important in controlling the effect of NPY. Transcription of Y1 exon 1C may have a direct inhibitory effect on the expression of Y5. This is supported by the fact that expression of the Y1 receptor is reported in the colon, kidney, adrenal gland, pancreas, placenta and brain whereas Y5 expression is reported only in the brain and testes (Ball, 1995).

Differences in tissue expression of the alternatively spliced exons of NPY Y1 have been studied in artery and kidney cells as well as a neuroblastoma cell line (SK-N-MC). RT-PCR analysis of the RNA from these tissues, using primers specific for the alternatively spliced exons, shows differential expression of the isoforms (Ewing, 1998). However, the analysis was limited to these three tissues. Other experiments studied the differences in tissue expression of the alternatively spliced exons of NPY (Parker et al., 1999). These experiments, however, had limited success. These researchers looked in several brain regions including the hippocampus, hypothalamus, thalamus and cortex using RT-PCR analysis. This analysis, however, could not specifically distinguish between the five different isoforms. In addition, these researchers found that their subcloning and sequencing results contradicted their RT-PCR results.
Research problem

At this time very little is known about the tissue specific expression of the coding regions of both the Y1 and Y5 receptor genes as well as the alternatively spliced exons of the Y1 receptor. In addition, a polymorphism analysis of the coding regions of both genes is needed to determine whether a high incidence of mutations are observed in the population and if so, whether these mutations predispose an individual to obesity. In order to study the occurrence of mutations in the two receptor genes, genomic DNA will be isolated from the whole blood from patients suffering from schizophrenia and unstable angina. A polymorphism analysis of the neuropeptide Y Y1 and Y5 receptor genes from each patient DNA sample will be performed by first PCR amplifying the genes and then performing direct sequencing from the PCR products. This sequence will then be analyzed for polymorphisms using several alignment programs. The tissue specific expression of the two receptors will be studied by first performing a multiple tissue dot blot on the coding regions of the two receptor genes and then a multiple tissue northern analysis on both the coding regions of the two receptors and the alternatively spliced 5’ exons of neuropeptide Y Y1 receptor gene.
Materials and Methods

Isolation of Genomic DNA from Blood

A collaboration between Merck & Co. and various hospitals around the United States was set up in order for Merck to obtain blood samples from patients suffering from both unstable angina and schizophrenia. Samples were shipped to Merck overnight after being drawn and lymphoid cells were isolated immediately. In most cases, the lymphoid cells were isolated and frozen at \(-70^\circ C\) within 24 hours after the blood was drawn.

Approximately 9 milliliters of blood was transferred to a 50 milliliter falcon tube. An equal volume of Phosphate Buffered Saline (PBS), pH 7.4 was added to the tube and gently mixed by inversion. In a separate 50 milliliter falcon tube the blood was slowly and carefully layered onto 15 milliliters of Ficoll-Plaque Plus (Pharmacia Biotech). The ficoll creates a gradient when centrifuged that separates the lymph and white blood cells from the red blood cells. The sample was spun at 1200 rpm for 30 minutes at room temperature in a Beckman GS-6 centrifuge. The lymphoid cells at the ficoll/media interface were collected and placed into a clean 50 ml falcon tube containing 10 milliliters PBS (pH 7.4). The tube was mixed gently and centrifuged for 10 minutes at 2000 rpm in a Beckman GS-6 centrifuge. The supernatant was discarded and the pellet containing lymphoid cells was resuspended in 1 ml PBS (pH 7.4) and aliquotted into 3 separate 1.5 ml eppendorf tubes. The eppendorf tubes were spun at 2000 rpm for 10 min in a bench-top centrifuge and the supernatant is discarded. These samples were then stored at \(-70^\circ C\) until they are needed for DNA isolation.

For the isolation of the genomic DNA from the lymphoid cells, 2 milliliters of lysis buffer (10mM Tris-HCl pH 7.5, 400mM NaCl, 2mM EDTA) was placed in a 15
milliliter falcon conical tube. The lymphoid cells which were frozen at -70°C are placed on ice to thaw. These cells were then added to the lysis buffer. Immediately, 330 microliters of Proteinase K (10mg/ml) and 133 microliters 10% SDS was added. The tube was inverted several times gently and incubated overnight (16-18 hours) at 50°C. This tube was then allowed to cool to room temperature and 667 microliters saturated NaCl (Approximately 6M) was added. The tube was inverted several times and spun at 3,000 rpm for 30 min in a Beckman GS-6 centrifuge. The supernatant was transferred to a clean 15 ml Falcon tube using a pipette-man P1000. Two volumes of 100% EtOH were added and the sample was spun at 3,000 rpm for 30 minutes at 4°C using a refrigerated Beckman GS-6 centrifuge. The supernatant was poured off and the sample was air dried for 20 minutes and resuspended in 400 microliters of TE (10 mM Tris, pH 8.0, 1 mM EDTA), quantitated and stored at 4°C for use in PCR.

**Design and synthesis of PCR primers**

PCR primers (Table 1) were designed to amplify the coding regions of both the NPY Y1 and Y5 receptor genes (Figures 2 and 3). In addition, a tail was added to the primers to perform direct sequencing off the PCR product. These primers were synthesized on an Applied Biosystems 394 DNA synthesizer at 0.2 micromolar scale with the trityl group automatically removed. After synthesis, the primers were heated at 65°C for one hour and then transferred to two eppendorf tubes in which they were dried down at high speed in a table-top speed-vac. After being dried down completely, the primers were resuspended in 100 microliters of water, quantitated and diluted to a concentration of 20 pmol. These primers were then used to set up a PCR reaction using genomic DNA isolated from the whole blood.
<table>
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<td></td>
</tr>
<tr>
<td>Y1-1B 5'-GCT ATG ACC ATG ATT ACG CCA TGT CTA TTA TTT GGT CTC C</td>
<td></td>
</tr>
<tr>
<td>Y1-2A 5'-GTT GTA AAA CGA CGG CCA GTC TCA GAC TTG CTT GTC GCC A</td>
<td></td>
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<tr>
<td>Y1-2B 5'-GCT ATG ACC ATG ATT ACG CCC CAT CAT GTT GTT TCT CCT TTT TAG</td>
<td></td>
</tr>
<tr>
<td>Y1-3A 5'-GTT GTA AAA CGA CGG CCA GTT TCC ATT TTT ACC TTC TTT ACA CAG</td>
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<tr>
<td>Y1-3B 5'-GCT ATG ACC ATG ATT ACG CCT GGG AGA ACA GGT AAT CAA AGT ATG</td>
<td></td>
</tr>
<tr>
<td>Y5-1A 5'-GTT GTA AAA CGA CGG CCA GTT GTA ATG TTT TTT TGG TGG CTG</td>
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<tr>
<td>Y5-3B 5'-GCT ATG ACC ATG ATT ACG CCT GAC CCC AGC ATT TGT TCT T</td>
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</table>

**Table 1.** PCR primers used to amplify the coding regions of the Neuropeptide Y Y1 and Y5 receptor genes. Underlined sequence represents the primer tail which will be used for direct sequencing. These underlined bases represent sequencing priming sites.
Figure 2. Black arrows indicate PCR primers designed to amplify the coding region of the NPY Y1 receptor. Overlapping PCR products ensure complete coverage. Exon 2 and Exon 3 are the blue boxes shown on the genomic DNA. Red horizontal lines indicate PCR product location. Red vertical lines are stop codons and the green P's indicate start codons as the genomic DNA is translated into all three frames. Exon 2 is in frame three and Exon three is in frame 1.
Figure 3. Black arrows indicate PCR primers designed to amplify the coding region of the NPY Y5 receptor. Overlapping PCR products ensure complete coverage. Exon 2 is the blue box shown on the genomic DNA. Red horizontal lines indicate PCR product location. Red vertical lines are stop codons and the green P's indicate start codons as the genomic DNA is translated into all three frames. Exon 2 is in frame 1.
Amplification of the Y1 and Y5 receptor genes

The three primer sets for each of the two receptor genes were used to amplify the coding regions by PCR. The PCR reaction included 50 ng of genomic DNA, 5.0 ul of 10X PCR buffer (Perkin Elmer) (100mM Tris-HCl pH 8.3, 500mM KCl, 15mM MgCl₂, 0.01% gelatin), 4.0 ul 2.5mM dNTP's (Gibco), 1.0 ul of each 20 pmol primer, 0.25 ul of TaqGold (5u/ul) (Perkin Elmer), and water to 50 ul. Reactions were set up at room temperature and then placed in a MJ Research PTC-225 thermocycler for cycling. Cycling conditions for all PCR reactions were 94°C for 12 minutes, and then 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 90 seconds. The last parameter was 1 cycle at 68°C for 5 minutes. PCR reactions were set up on 32 patients at a time in a Perkin Elmer cycling plate (Robbins). In total, over 200 patient samples were used for amplification and analysis (Table 2).

PCR reaction purification

All PCR reactions were immediately purified on a Qiagen Biorobot 9600, using a modified Qiagen protocol. Each set of 32 PCR reactions were purified separately. Four eight-strip Qiaquick strips were placed into Qiavac manifold. The 50 ul PCR reaction was combined with 250 ul buffer PB™ and added to the Qiaquick wells. A vacuum was then applied to the manifold until all the liquid has passed through the column. The vacuum is then switched off and the wells were washed with 1 ml of buffer PE™ two times. In each case the vacuum was applied until all PE passes through the column. After the second wash the vacuum was applied for an additional 5 minutes to dry the membrane. The vacuum was turned off and the manifold and columns were dried with a
**GENDER AND ETHNICITY BACKGROUND ON PATIENTS USED IN SNP ANALYSIS**

<table>
<thead>
<tr>
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<th>Patient Information</th>
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<td>B) Black</td>
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<td>C) Asian</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>2) Control (Non-Schizophrenic)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** The gender and ethnicity background for the tactics patients as well as the control vs. schizophrenic breakdown for the schizophrenia patient set. Tactic patients all suffer from unstable angina.
paper towel to ensure all ethanol has been removed from the strips. The manifold and strips were then placed over collection tubes and the DNA was eluted from the columns using 80 ul buffer EB(10mM Tris-Cl, pH 8.5) and collected in the collection tubes. This DNA was then run on an agarose gel to verify the presence of a produce and used for direct sequencing using Bodipy sequencing chemistry.

**PCR verification using agarose gels**

A 100ml solution of 1% agarose was made by adding 1 gram agarose (GibcoBRL), 10 ml of 10X TBE(8.9 M Tris, 8.9 M Borate, 0.02 M EDTA) and 2.5 ul of EtBr(10mg/ml). Before adding the EtBr, the rest of the components were microwaved on high for 3 minutes and allowed to cool to approximately 60° C before the EtBr was added and poured into a gel casting tray with two twenty well combs. 5 ul of the PCR product was combined with 2 ul of loading buffer(40 % sucrose, 0.25 mg/ml Bromophenol Blue, 0.25 mg/ml Zylene cyanol) and loaded onto the 1% agarose gel and run for 1 hour at 150 volts. All 32 patients were run with a negative PCR control(no DNA) and a phiX 174 marker(GibcoBRL). A picture of this gel containing PCR products from 32 patients was then taken for documentation (Figure 4).

**Bodipy Primer synthesis**

Primers were synthesized for sequencing off the purified PCR products. These primers were synthesized on an Applied Biosystems 394 DNA synthesizer at 1.0 micromolar scale with the trityl group left on. The final synthesis step included the addition of either a C3 or a C6 amino modifier which would allow for the addition of a Bodipy Dye (Figure 5 and Table 3). After synthesis, the C3 primers were heated at 37° C
Figure 4. PCR products from patients 1-32 run on a 1% agarose gel with a PhiX 174 marker. Gel A represents patient amplification using primer set Y1-1A and Y1-1B. Gel B represents patient amplification using primer set Y5-1A and Y5-1B. Marker (M) and Negative control (B) are marked along with the 32 patients.
Agarose gel verification of PCR products

Figure 4. PCR products from patients 1-32 run on a 1% agarose gel with a PhiX 174 marker. Gel A represents patient amplification using primer set Y1-1A and Y1-1B. Gel B represents patient amplification using primer set Y5-1A and Y5-1B. Marker (M) and Negative control(B) are marked along with the 32 patients.
Bodipy Dye Chemistry

Figure 5. Chemical structures of the four Bodipy dyes for automated DNA sequencing. All structures are obtained from Science Vol. 271 Pg 1420.
### SEQUENCING PRIMERS USED FOR BODIPY SEQUENCING

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<tr>
<th>PRIMER DESIGNATION</th>
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<tr>
<td>MRL 41</td>
<td>C6-TTG TAA AAC GAC GGC CAG T</td>
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<tr>
<td>MRL 42</td>
<td>C3-TTG TAA AAC GAC GGC CAG T</td>
</tr>
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<td>MRL 88</td>
<td>C6-TAT GAC CAT GAT TAC GCC</td>
</tr>
<tr>
<td>MRL 89</td>
<td>C3-TAT GAC CAT GAT TAC GCC</td>
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</table>

**Table 3.** Sequencing primers used to sequence directly from the PCR products. One of four Bodipy dyes are added to this C3 or C6 amino modifier.
for 24 hours and the C6 primers were heated at 65° C for one hour and then 20 ul of 0.5M NaOH was added to each primer and each primer was transferred to two eppendorf tubes in which they were dried down at high speed in a table-top speed-vac. After being dried down completely, the primers were resuspended in 160 microliters of water and transferred to a 2 ml eppendorf tube. To each tube, 640 ul of glacial acetic acid was added, mixed and incubated at room temperature for 30 minutes. Each sample was split into two tubes and to each tube, 200 ul of 10 N NaOH and 1240 ul of 100% EtOH was added. The samples were vortexed and centrifuged at 14,000 rpm for 5 minutes. The supernatant was decanted and to the C6 primers, 300 ul of 0.25 M NaHCO₃ was added to each tube and the sample was split into three tubes. 5 ul of the respective Bodipy dye (PE Applied Biosystems) was added to one of the three tubes (503/512, 523/547, 564/570). For C3 primers, 200 ul of 0.25 M NaHCO₃ was added to each tube. The tubes were combined and 5 ul of Bodipy 581/591 dye was added. The dyes were incubated for 16 hours at room temperature in the dark. For C6 primers, 20.5 ul of 3M sodium acetate (pH 5.2) and 451 ul of 100% EtOH was added. For C3 primers, 40.5 ul of sodium acetate (pH 5.2) and 851 ul of 100% EtOH was added. The tubes were centrifuged for 15 minutes at 14,000 rpm, the supernatant was poured off and the pellets were washed three times with 70% EtOH for 2 minutes at 14,000 rpm. The C6 primers were dissolved in 20 ul of 10mM TEAA and C3 primers were dissolved in 30 ul of 10mM Triethylamine Acetate, pH 7.0 (TEAA). The primers were then purified using a Beckman Biosys 510 HPLC and an Applied Biosystems 250x4.6mm Aquapore RP-300 7 micron column (Figures 6-13). After purification, the primers were diluted to 0.4 pmol/ul using water.

**Bodipy Sequencing**
HPLC trace of MRL 41 (503/512) purification

Figure 6. HPLC trace of MRL 41 (503/512) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 41 (523/547) purification

Figure 7. HPLC trace of MRL 41 (523/547) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 41 (564/570) purification

Figure 8. HPLC trace of MRL 41 (564/570) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 42 (581/591) purification

Figure 9. HPLC trace of MRL 42 (581/591) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 88 (503/512) purification

Figure 10. HPLC trace of MRL 88 (503/512) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 88 (523/547) purification

Figure 11. HPLC trace of MRL 88 (523/547) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 88 (564/570) purification

Figure 12. HPLC trace of MRL 88 (564/570) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
HPLC trace of MRL 89 (581/591) purification

Figure 13. HPLC trace of MRL 89 (581/591) purification. Elution off of the HPLC column was measured at a wavelength of 260. The arrows indicate the primer peak that was collected.
Approximately 200ng of purified PCR product was used in Taq FS sequencing reactions using custom primers labeled with Bodipy dyes. Sequencing of a PCR product included both a forward and a reverse reaction, each of which is set up separately. Reactions were set up on a Beckman Biomek 2000 robot using Dye-Primer chemistry, where each di-deoxynucleotide reaction was set up in a separate reaction (Chadwick, 1996). Each reaction was set up in a volume of 8 ul. This included 2 ul of PCR DNA(100ng/ul), 1 ul of the appropriate Dye Primer (0.4 pmol/ul), 1ul of the appropriate di-deoxynucleotide mix (0.5 mM each of the 3 deoxynucleotides and 1.5uM of the di-deoxynucleotide with the exception of ddATP which was 0.75 uM) and 2 ul of dilute Taq FS buffer (20 mM Tris-HCl, pH 9.0, 0.50 mM MgCl_, 4.2 units Taq FS). Reactions were then centrifuged briefly and placed in a MJ Research PTC-225 thermocycler for cycling. Cycling conditions were 20 cycles at 94° C for 10 seconds, 60° C for 20 seconds, and 70° C for one minute. Following cycling, all four reactions(A,C,G,T) were pooled and precipitated using 80 ul of ice cold 100% EtOH and spun at 3000 rpm for 30 minutes at 4° C in a bench-top Beckman GS-6R centrifuge. The EtOH was removed and the samples were dried for 10 minutes on low heat in a speed-vac. Samples were then resuspended in 3 ul 98% formamide, 10 mM EDTA (Fisher Scientific). After resuspension, these samples were heated at 68° C for 3 minutes before loading onto a sequencing gel.

**Sequencing Gels**

All Bodipy samples were run on a 48 centimeter 4.5% polyacrylamide gel which was made from 36% Urea, 4.5 % Long Ranger (FMC Bioproducts), 1X TBE(.89 M Tris,.89 M Borate, .002 M EDTA), .05% Ammonium PerSulfate(Biorad) and .0007% TEMED (Biorad) for 10 hours on an Applied Biosystems 377 DNA Sequencer.
Sequence Analysis

The forward and reverse reads of sequenced PCR products were assembled using Sequencher assembly program (Ewing, 1998). In addition, all sequencing reads on the three PCR products for each patient were assembled using this program (Figure 14). After this was done, the sequence chromatogram was analyzed by eye for any possible mistakes. After this was done the consensus sequence of the patients for both the Y1 and Y5 receptor genes were compared to find any single nucleotide polymorphisms (SNPs) (Figure 15-16).

Northern Analysis Probe Synthesis

Probes to perform a northern analysis on the coding regions of Neuropeptide Y Y1 and Y5 were selected by comparing the three PCR products covering both the Y1 and Y5 receptor genes against the National Center for Biotechnology Information (NCBI) nucleotide and protein databases. The comparisons were done in all six frames and the PCR product from Y1-1A and Y1-1B was selected as a probe for the coding region of Y1 and the Y5-1A and Y5-1B PCR product was selected as a probe against Y5. In both cases, only the corresponding gene contained these specific sequences.

The PCR reaction to generate the probe included 50 ng of genomic DNA, 5.0 ul of 10X PCR buffer (Perkin Elmer) (100mM Tris-HCl pH 8.3, 500mM KCl, 15mM MgCl₂, 0.01% gelatin), 4.0 ul 2.5mM dNTP’s (Gibco), 1.0 ul of each 20 pmol primer, 0.25 ul of TaqGold (5u/ul) (Perkin Elmer), and water to 50 ul. Reactions were set up at room temperature and then placed in a MJ Research PTC-225 thermocycler for cycling. Cycling conditions for all PCR reactions were 94° C for 12 minutes, and then 35 cycles at
Figure 14. Alignment of the forward and reverse sequencing reads for all three PCR products covering the coding region of Neuropeptide Y Y5 receptor of patient np528a. Alignment was done in Sequencher.
**Figure 15.** Alignment of forward and reverse sequence reads covering the NPY Y1 receptor coding region. Green arrows correspond to forward reads and red arrows correspond to reverse reads.
Figure 16. Alignment of the consensus sequences of the Neuropeptide Y Y1 receptor gene from 32 patients. The blue horizontal line indicates a position where patient 3397 has a nucleotide variation from the other patients.
**Consensus Alignment-NPY Y5**

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**Figure 17.** Alignment of the consensus sequences of the Neuropeptide Y Y5 receptor gene from 32 patients. The blue horizontal line indicates a position where patient 528A has a nucleotide variation from the other patients.
94° C for 30 seconds, 60° C for 30 seconds, and 68° C for 90 seconds. The last parameter was 1 cycle at 68° C for 5 minutes. PCR reactions were set up on 32 patients at a time in a Perkin Elmer cycling plate (Robbins). PCR products were purified on the Qiagen robot as explained earlier and then run on a 1% agarose gel as explained earlier.

The PCR product was then subcloned into Invitrogen's pCR-Blunt II-TOPO vector (Figure 17) using their Zero Blunt TOPO PCR cloning kit. In this cloning reaction, 4 ul of PCR product (200ng) is mixed with 1 ul of pCR-Blunt II-TOPO vector and incubated for five minutes at room temperature. After incubating the reaction, 2 ul is transformed into Invitrogen’s TOP10 competent cells (F' mrcA mrr-hsdRMS-mcrBC phi80lacZ' M15 lacX74 recA1 deoR araD139 ara-leu7697 galU galK rpsL (StrR) endA1 nupG). This was done by adding 2 ul 0.5 M Beta-mercaptoethanol to the vial of competent cells. After 10 minutes, 2 ul of the TOPO cloning reaction was added on ice to the competent cells. The reaction was incubated for 30 minutes on ice and then placed at 42° C for 30 seconds and back on ice for 2 minutes. After incubating on ice for 2 minutes, 250 ul of room temperature SOC (2% Tryptone, 0.5% yeast extract, 0.06% NaCl, 0.02% KCl, 0.2% MgCl2, 0.25% MgSO4) was added to the tube and the tube was then shaken for 1 hour at 37° C. After incubating for 1 hour, 50 ul of the reaction was spread onto a LB plate with 1.5% Agar (Gibco) and 50ug/ml Kanamycin (Sigma). Plates were incubated overnight at 37° C. Single colonies were picked and placed into a 5ml culture of LB with 50 ug/ml Kanamycin and grown overnight while shaking at 37° C. Cultures were then centrifuged for 10 minutes at 3,000rpm in a Beckman J6-MI centrifuge. The supernatant was poured off and the DNA was extracted and purified from the pellets using the Qiagen robot and the mini-prep protocol.
Figure 18. PCR products were cloned into Invitrogen's pCR-Blunt II-TOPO vector for amplification. Inserts were later removed using the EcoRI restriction sites.
The bacterial pellet is resuspended in 250 ul of buffer P1 (.606% Tris Base, .372% Na₂EDTA, pH8.0 and .1mg/ml Rnase A). 250 ul of buffer P2 (0.8% NaOH, 1% SDS) is added to the tube and the tube is gently mixed by inversion six times. The reaction is incubated for five minutes at room temperature. After incubating for five minutes, 350 ul of buffer N3 (29.4% potassium acetate, pH5.5) is added to the sample and the reaction is mixed by inversion and transferred into a Qia gen turbofilter plate. A vacuum is applied to the QIA prep plate until all the samples have passed through. The QIA prep plate is then washed with 0.9ml buffer PE (unknown salts, 80% EtOH) several times. The DNA is eluted using 125 ul water.

One ug of the sample is then digested using EcoRI restriction enzyme to isolate the purified insert and the insert is gel extracted for use in the northern analysis. A 50 ul reaction is set up using 1.0 ug of DNA, 5 ul of Promega buffer H (450mM Tris-HCl, 50 mM MgCl₂, 250mM NaCl, pH7.5), and 3 ul of EcoRI (12u/ul). The reaction was incubated for three hours at 37° C and then placed on ice.

A 100ml solution of 1% agarose is made by adding 1 gram agarose (GibcoBRL), 10 ml of 10X TBE (8.9 M Tris, 8.9 M Borate, 0.02 M EDTA) and 2.5 ul of EtBr (10mg/ml). Before adding the EtBr, the rest of the components are microwaved on high for 3 minutes and allowed to cool to approximately 60° C before the EtBr is added and poured into a gel casting tray with two twenty well combs. All 50 ul of the digest is combined with 10 ul of loading buffer (40 % sucrose, 0.25 mg/ml Bromophenol Blue, 0.25 mg/ml Zylene cyanol) and loaded onto the 1% agarose gel and run for 1 hour at 150 volts.
The band of interest was cut out of the gel using a clean razor and placed into an eppendorf tube. The gel slice was weighed and 3 volumes of buffer QG™ to 1 volume of gel slice was added. The tube was incubated for 10 minutes at 50°C. One gel volume of 100% isopropanol was added and the tube is mixed by inversion. The sample was applied to a QIAquick column and the column was spun at 14,000rpm for 1 minute. The flow through was discarded and the column was washed with 0.75 ml buffer PE™ and spun for 1 minute at 14,000rpm. The column was placed in a clean centrifuge tube and 50 ul of water was added. The column is allowed to sit for 2 minutes and then spun at 14,000rpm for 2 minutes. This DNA was then sequence verified using the Bodipy sequence protocol previously described.

This verified and purified PCR product was then used for random primer labeling using Amersham's Rediprime II kit. The PCR product was diluted to 50 ng in 45 ul TE (10 mM Tris, pH 8.0, 1 mM EDTA) and denatured at 95°C for 5 minutes. The sample was then snap cooled on ice and added to the Rediprime 2 reaction tube which contained a buffered solution of dATP, dGTP, dTTP, endonuclease free klenow enzyme and random primers in a dried, stabilized form. 5 ul of Redivue (³²P) dCTP is added to the reaction tube and the tube is mixed and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 5ul of 0.2M EDTA. The sample was then denatured at 95°C for 5 minutes. The sample was then snap cooled on ice and purified using a Microspin G-25 column from Pharmacia Biotech.

A Microspin G-25 column was prepared by resuspending the resin by gentle vortexing. The cap was loosened and the column is spun at 735 x g for 1 minute. The column was then placed in a clean eppendorf tube and the labeled reaction was added to
the resin. The column was spun at 735 x g for 2 minutes and the column was then discarded. The flow through is the labeled probe. 1 ul of this probe was used to measure counts per million (CPM). After determining the CPM of each probe, a hybridization was set up on a multiple tissue dot blot (Figure 18) and several multiple tissue brain blots (Figure 19).

Probes for the alternatively spliced 5'-UTR's of neuropeptide Y1 were isolated and labeled differently. Probes were designed using the sequence published by Ball et al. in 1995. Oligonucleotides 80 bases in length were designed and synthesized as the antisense of the three alternatively spliced exons of neuropeptide Y Y1 receptor. These were synthesized and gel purified by Gibco and shipped lyophilized. Primers were resuspended to 100 ng/ul and end labeled using Promega's DNA 5' end-labeling system phosphorylation reaction. In this reaction, 100 ng of oligonucleotide is combined in a 50 ul reaction with 5 ul of T4 PNK 10X buffer, 7 ul of gamma-32P ATP (3,000 Ci/mmole), 1 ul of T4 polynucleotide kinase (10u/ul). This reaction was incubated for 30 minutes at 37° C. The reaction is stopped by the addition of 5ul of 0.2M EDTA. The sample is then denatured at 95° C for 5 minutes. The sample was then snap cooled on ice and purified using a Microspin G-25 column from Pharmacia Biotech.

A Microspin G-25 column was prepared by resuspending the resin by gentle vortexing. The cap was loosened and the column is spun at 735 x g for 1 minute. The column was then placed in a clean eppendorf tube and the labeled reaction was added to the resin. The column was spun at 735 x g for 2 minutes and the column was then discarded.
Clontech’s RNA Dot Blot

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**Figure 19.** Clontech’s RNA Dot Blot used for tissue specific expression analysis of the coding regions of Neuropeptide Y1 and Y5.
Clontech's Multiple Tissue Northern Blots

**Figure 20.** Brain regions included in Clontech's Multiple Tissue Northern Blots that were used for analysis with the Neuropeptide Y1 and Y5 receptor probes.
The flow through was the labeled probe. 1 ul of this was used to measure counts per million (CPM). After determining the CPM of each probe, a hybridization was set up on a multiple tissue dot blot (Figure 18) and several multiple tissue brain blots (Figure 19).

Hybridization of PCR Probes to Dot Blots

The blot was placed in a 250 ml hybridization bottle and 10 ml of 65°C ExpressHyb solution (Clontech) containing 100 ug denatured sheared salmon testes DNA (Sigma) was added to the bottle. The bottle was rotated in a 65°C incubator for 30 minutes. The radiolabeled and denatured probe was added to an ExpressHyb/salmon testes DNA solution and mixed. The 10 ml of pre-hybridization solution was poured out of the bottle and was replaced with the 5 ml of hybridization solution containing the probe which has been heated to 65°C. The probe was hybridized overnight at 65°C with continuous agitation. The hybridization solution was poured off and was replaced with 100 ml of wash solution 1 (0.3 M NaCl, 0.03 M Na₃Citrate, 1% SDS). The blot was washed for 20 minutes at 65°C with continuous agitation. This wash step was repeated four times. The blot was then washed for 20 minutes at 55°C with 100 ml pre-warmed wash solution 2 (0.015 M NaCl, 0.0015 M Na₃Citrate, 0.5% SDS). This wash procedure was repeated once. After washing, the blot was removed from the bottle and immediately wrapped in plastic wrap and exposed to Kodak’s X-OMAT film for 24 hours.

Hybridization of Oligonucleotide Probes to Dot Blots

The blot was placed in a 250 ml hybridization bottle and 10 ml of 37°C ExpressHyb solution (Clontech) containing 100 ug denatured sheared salmon testes DNA (Sigma) was added to the bottle. The bottle was rotated in a 37°C incubator for 30 minutes. The radiolabeled and denatured probe was added to an ExpressHyb/salmon testes DNA solution and mixed.
testes DNA solution and mixed. The 10 ml of pre-hybridization solution was poured out of the bottle and was replaced with the 5 ml of hybridization solution containing the probe which has been heated to 37° C. The probe was hybridized overnight at 37° C with continuous agitation. The hybridization solution was poured off and was replaced with 100 ml of wash solution 1 (0.3 M NaCl, 0.03 M Na3Citrate, 1% SDS). The blot was washed for 20 minutes at 25° C with continuous agitation. This wash step was repeated four times. The blot was then washed for 20 minutes at 25° C with 100 ml pre-warmed wash solution 2 (0.015 M NaCl, 0.0015 M Na3Citrate, 0.5% SDS). This wash procedure was repeated once. After washing, the blot was removed from the bottle and immediately wrapped in plastic wrap and exposed to Kodak's X-OMAT film for 24 hours.

Hybridization of PCR Probes to Multiple Tissue Northern Blot

The blot was placed in a 250 ml hybridization bottle and 10 ml of 68° C ExpressHyb solution (Clontech) was added to the bottle. The bottle was rotated in a 68° C incubator for 30 minutes. The radiolabeled and denatured probe was added to 5 ml of ExpressHyb solution and mixed. The 10 ml of pre-hybridization solution was poured out of the bottle and was replaced with the 5 ml of hybridization solution containing the probe which has been heated to 68° C. The probe was hybridized overnight at 68° C with continuous agitation. The hybridization solution was poured out and the blot was washed with 100 ml of wash solution 1(0.3M NaCl, 0.03M Sodium Citrate pH 7.0, 0.05% SDS) for 20 minutes at room temperature. This wash step was repeated twice. The blot was then washed with 100 ml of wash solution 2 (0.015M NaCl, 0.0015M sodium citrate pH 7.0, 0.1%SDS) at 50° C for 20 minutes. This wash step was repeated once. The wash
solution was poured off and the blot was immediately wrapped in plastic wrap and exposed to Kodak's X-OMAT film for 5 days.

**Hybridization of Oligonucleotide Probes to Multiple Tissue Northern Blot**

The blot was placed in a 250 ml hybridization bottle and 10 ml of 37° C ExpressHyb solution (Clontech) was added to the bottle. The bottle was rotated in a 37° C incubator for 30 minutes. The radiolabeled and denatured probe was added to 5 ml of ExpressHyb solution and mixed. The 10 ml of pre-hybridization solution was poured out of the bottle and was replaced with the 5 ml of hybridization solution containing the probe which has been heated to 37° C. The probe was hybridized overnight at 37° C with continuous agitation. The hybridization solution was poured out and the blot was washed with 100 ml of wash solution 1 (0.3M NaCl, 0.03M Sodium Citrate pH 7.0, 0.05% SDS) for 20 minutes at room temperature. This wash step was repeated twice. The blot was then washed with 100 ml of wash solution 2 (0.015M NaCl, 0.0015M sodium citrate pH 7.0, 0.1% SDS) at room temperature for 20 minutes. This wash step was repeated once. The wash solution was poured off and the blot was immediately wrapped in plastic wrap and exposed to Kodak's X-OMAT film for 5 days.
RESULTS

Sequencing of the NPY Y1 Receptor Gene

DNA from 204 patients was isolated and the neuropeptide Y Y1 receptor gene was amplified using PCR and the primers listed in Table 1. Three PCR products were generated which gave complete coverage of the coding region of the Y1 receptor gene (figure 2). A forward and a reverse sequencing reaction was set up on each PCR product to provide redundant coverage of the sequence for the entire coding region of the gene. These sequencing reactions were imported into and aligned in a computer alignment program called Sequencher (Figure 15). The sequencing chromatograms were then analyzed by eye to determine the presence of any heterozygous polymorphisms. In addition the consensus sequence of each individual’s DNA was compared to the sequence of all other patients to determine the presence of homozygous polymorphisms (Figure 16). Within the coding region of neuropeptide Y Y1 receptor gene of the 204 patients screened, there were three single nucleotide polymorphisms (SNPs) found as well as one SNP found 14 bp upstream of the start methionine (Figure 21-24 and Table 4). Table 4 shows that mutation 1 is a silent mutation within transmembrane domain 4 and mutation 2 is a valine to a methionine substitution in transmembrane domain 5, each found in only one patient. Mutation 3 however is a mutation found in nearly one percent of the patients and is located in the final intracellular region towards the carboxy-terminus of the protein. This mutation is a lysine to a threonine substitution. In addition, mutation 4 which is located 14 bp upstream of the start codon, was found to have a frequency of
Neuropeptide Y Y1 Receptor Gene Mutation #1

Figure 21. Sequencher alignment and chromatogram trace of patient 2870. Y indicates a heterozygous mutation of a cytidine and a thymidine.
Neuropeptide Y Y1 Receptor Gene Mutation #2

**Figure 22.** Sequencher alignment and chromatogram trace of patient 3397. R indicates the presence of a heterozygous mutation between an adenosine and guanosine nucleotide.
Neuropeptide Y Y1 Receptor Gene Mutation #3

Figure 23. Sequencher alignment and chromatogram trace of the NPY Y1 receptor gene for patient 114A. M represents a heterozygous mutation between an adenosine and a cytidine.
Neuropeptide Y Y1 Receptor Gene Mutation #4

Figure 24. Sequencher alignment and chromatogram trace of patient 989A. Y indicates a heterozygous mutation of a cytidine and thymidine. This mutation is found 14 bp upstream of the start codon.
### Table 4. NPY Y1 receptor gene mutations. Mutation 1-3 are located in the coding region of the receptor. Mutation 4 is located 14 bp upstream of the start methionine.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Amino Acid Position of 384</th>
<th>Nucleotide codon change (From/To)</th>
<th>Amino Acid change (From/To)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>169 (Transmembrane Region 4)</td>
<td>TCC/TCT</td>
<td>Serine/Serine</td>
<td>2/204=0.49%</td>
</tr>
<tr>
<td>2</td>
<td>217 (Transmembrane Region 5)</td>
<td>GTG/ATG</td>
<td>Valine/Methionine</td>
<td>1/204=0.24%</td>
</tr>
<tr>
<td>3</td>
<td>374 (Final Intracellular Domain)</td>
<td>AAA/ACA</td>
<td>Lysine/Threonine</td>
<td>4/204=0.98%</td>
</tr>
<tr>
<td>4</td>
<td>-14bp upstream of start AUG</td>
<td>CAACC/CAATC</td>
<td></td>
<td>12/204=2.94%</td>
</tr>
</tbody>
</table>
nearly three percent in the patients screened. The three coding mutations as they are located in the protein can be seen in figure 25.

**Sequencing of the NPY Y5 Receptor Gene**

DNA from 203 patients was isolated and the neuropeptide Y Y5 receptor gene was amplified using PCR and the primers listed in Table 1. PCR amplification of the Y5 receptor gene was done similarly to the Y1 gene. A forward and a reverse sequencing reaction was set up on each PCR product to provide redundant coverage of the sequence for the entire coding region of the gene. Sequences were analyzed for heterozygous mutations using the computer software package previously described. In addition, the consensus sequence of each individual's DNA was compared to the sequence of all other patients to determine the presence of homozygous polymorphisms (Figure 17). Within the coding region of neuropeptide Y Y5 receptor gene of the 203 patients screened, there were three single nucleotide polymorphisms (SNPs) found (Figures 26-28 and Table 5). All SNPs are heterozygous mutations. Mutations 1 and 2 were found in only one patient of the 203 screened. Mutation 1 is a arginine to a cysteine substitution and mutation 2 is a silent mutation. Mutation 3 which is also a silent mutation was found in 29 patients all of which are heterozygous for the SNP. The three coding mutations as they are located in the protein can be seen in figure 29.
Figure 25. NPY Y1 receptor protein structure. Red arrows indicate the position of mutations discovered in sequencing.
Figure 26. Sequencher alignment and chromatogram trace for patient 528A. Y represents a heterozygous mutation of a cytidine and a thymidine.
Neuropeptide Y Y5 Receptor Gene Mutation #2

Figure 27. Sequencher alignment and chromatogram trace for patient 421A. Y represents a heterozygous mutation of a cytidine and a thymidine.
Neuropeptide Y Y5 Receptor Gene Mutation #3

Figure 28. Sequencher alignment and chromatogram trace of patient PL90. R indicates the presence of a heterozygous mutation between an adenosine and guanosine nucleotide.
### NEUROPEPTIDE Y Y5 RECEPTOR GENE SINGLE NUCLEOTIDE POLYMORPHISMS

<table>
<thead>
<tr>
<th>Mutation</th>
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<th>Nucleotide codon change (From/To)</th>
<th>Amino Acid change (From/To)</th>
<th>Frequency</th>
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<tr>
<td>1</td>
<td>132 (Transmembrane Region 3)</td>
<td>CGT/TGT</td>
<td>Arginine/Cysteine</td>
<td>1/203=0.24%</td>
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<tr>
<td>2</td>
<td>217 (Extracellular loop 2)</td>
<td>TAT/TAC</td>
<td>Tyrosine/Tyrosine</td>
<td>1/203=0.24%</td>
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<tr>
<td>3</td>
<td>426 (Final Intracellular Domain)</td>
<td>GGG/GGA</td>
<td>Glycine/Glycine</td>
<td>29/203=7.14%</td>
</tr>
</tbody>
</table>

**Table 5.** NPY Y5 gene polymorphisms and their frequency in the tested Population. All mutations are within the coding region of the receptor gene.
Figure 29. NPY Y5 receptor protein structure. Red arrows indicate positions of mutations.
Tissue Specific Expression of NPY Y1 Coding mRNA

A 500 bp DNA PCR product from within the coding region of the NPY Y1 receptor gene was labeled using a random primer labeling system and used as a probe to perform a northern analysis on a multiple tissue Dot Blot. $5 \times 10^6$ counts per ml was used to probe the blot. Dot Blot analysis showed expression of the coding region of NPY Y1 in various brain regions including the amygdala, caudate nucleus, cerebral cortex, frontal lobe, occipital lobe, putamen temporal lobe and subthalamic nucleus. The greatest amount of expression was seen in the peripheral tissues and included the aorta, uterus, pancreas, pituitary gland, adrenal gland, thyroid gland, mammary gland, kidney liver and spleen. In addition high expression was seen in fetal brain, kidney and spleen. (Figure 30)

Because NPY Y1 receptor expression was seen in the brain regions, a multiple tissue northern analysis was done using two separate brain specific blots to look for size differences and additional brain region expression. NPY Y1 receptor is a 2.7 Kb transcript that is seen in the cerebral cortex, medulla, occipital pole, frontal lobe, temporal lobe, putamen, amygdala, caudate nucleus, hippocampus, thalamus and to a lesser degree in the cerebellum and the spinal cord (Figure 31).

Tissue Specific Expression of the three alternatively spliced 5’ exons of NPY Y1

Complementary oligonucleotides were designed against the three alternatively spliced exons of NPY Y1 receptor mRNA. These probes were end-labeled and hybridized to two different brain region multiple tissue northern blots. Each of the three alternatively spliced exons (1A, 1B and 1C) was hybridized separately to the two multiple tissue brain blots. Each transcript is 2.7 Kb and the three 5’ exons are found to
RNA Dot Blot with Neuropeptide Y1

<table>
<thead>
<tr>
<th>Whole Brain</th>
<th>Amygdala</th>
<th>Caudate Nucleus</th>
<th>Cerebellum</th>
<th>Cerebral Cortex</th>
<th>Frontal Lobe</th>
<th>Hippocampus</th>
<th>Medulla Oblongata</th>
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<tr>
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<td>Aorta</td>
<td>Skeletal Muscle</td>
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<td>Small Intestine</td>
<td>Spleen</td>
<td>Thymus</td>
<td>Peripheral Lymphocyte</td>
<td>Lymph Node</td>
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<td>Trachea</td>
<td>Placenta</td>
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<td></td>
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<td></td>
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<tr>
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<td>Fetal Heart</td>
<td>Fetal Kidney</td>
<td>Fetal Liver</td>
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<tr>
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<td>Yeast tRNA 100ng</td>
<td>E. Coli rRNA 100ng</td>
<td>E. Coli DNA 100ng</td>
<td>Poly r(A) 100ng</td>
<td>Human Cot1 DNA 100ng</td>
<td>Human DNA 100ng</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 30.** RNA Dot Blot of the tissue specific expression of the Neuropeptide Y Y1 coding region
RNA Dot Blot with Neuropeptide Y1

<table>
<thead>
<tr>
<th>Whole Brain</th>
<th>Amygdala</th>
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<tr>
<td>Testis</td>
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<td>Adrenal Gland</td>
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<tr>
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<td>Thymus</td>
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<td>Placenta</td>
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<td>Fetal Spleen</td>
<td>Fetal Thymus</td>
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<td>Yeast rRNA 100ng</td>
<td>E. Coli rRNA 100ng</td>
<td>E. Coli DNA 100ng</td>
<td>Poly r(A) 100ng</td>
<td>Human Cot1 DNA 100ng</td>
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<td>Human DNA 500ng</td>
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</table>

**Figure 30.** RNA Dot Blot of the tissue specific expression of the Neuropeptide Y Y1 coding region
Figure 31. Brain region multiple tissue northerns of the coding region of neuropeptide Y Y1. The transcript is 2.7 Kb in size.
MTN with Neuropeptide Y1

**Figure 31.** Brain region multiple tissue northerns of the coding region of neuropeptide Y Y1. The transcript is 2.7 Kb in size.
be alternatively expressed. Exon 1A is expressed in all regions of the brain studied with
the highest expression found in the medulla and thalamus (Figure 32). Exon 1B
expression was not found in any regions of the brain using several hybridization and
wash conditions. Exon 1C, which is contained within the coding region of the NPY Y5
receptor gene and transcribed from the opposite strand, is expressed in the medulla,
spinal cord, amygdala, caudate nucleus, hippocampus, substantia nigra and the thalamus.
(Figure 33)

**Tissue Specific Expression of NPY Y5 Coding mRNA**

A 500 bp DNA PCR product from within the coding region of the NPY Y5
receptor gene was labeled and used as a probe similarly to the aY1 probe. Dot blot
analysis showed expression of the coding region of NPY Y5 in various brain regions
including the amygdala, caudate nucleus, cerebral cortex, frontal lobe, hippocampus,
occipital lobe, putamen, temporal lobe, thalamus, subthalamic nucleus and spinal cord.
The Y5 receptor was also expressed in peripheral tissues including the aorta, testes,
adrenal gland, mammary gland, kidney and spleen. The receptor mRNA was also
expressed in fetal brain and spleen. (Figure 34)

Because NPY Y5 receptor expression was seen in the brain regions, a multiple
tissue northern analysis was done using two separate brain specific blots to look for size
differences and additional brain region expression. NPY Y5 receptor is a 2.6 Kb
transcript that is seen in the cerebral cortex, medulla, frontal lobe, temporal lobe,
amygdala, whole brain, substantia nigra with the highest expression found in the occipital
pole, putamen and the caudate nucleus. (Figure 35).
Multiple Tissue Northern with Neuropeptide Y1 Exon 1A

Figure 32. Multiple Tissue Northern using Exon 1A of Neuropeptide Y Y1 receptor as the probe. Tissue specific expression of the exon can be seen in various regions of the brain.
Multiple Tissue Northern with Neuropeptide Y1 Exon 1A

Figure 32. Multiple Tissue Northern using Exon 1A of Neuropeptide Y1 receptor as the probe. Tissue specific expression of the exon can be seen in various regions of the brain.
Multiple Tissue Northern with Neuropeptide Y1 Exon 1C

Figure 33. Multiple Tissue Northern using Exon 1C of Neuropeptide Y Y1 receptor as the probe. Tissue specific expression of the exon can be seen in various regions of the brain.
Multiple Tissue Northern with Neuropeptide Y1 Exon 1C

Figure 33. Multiple Tissue Northern using Exon 1C of Neuropeptide Y Y1 receptor as the probe. Tissue specific expression of the exon can be seen in various regions of the brain.
RNA Dot Blot with Y5 Coding

**Figure 34.** RNA dot blot of NPY Y5 receptor coding region expression. Spots indicate positive expression of the receptor mRNA.
RNA Dot Blot with Y5 Coding

<table>
<thead>
<tr>
<th>Whole Brain</th>
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</table>

**Figure 34.** RNA dot blot of NPY Y5 receptor coding region expression. Spots indicate positive expression of the receptor mRNA.
MTN with Neuropeptide Y5

Figure 35. Brain region multiple tissue northerns of the coding region of neuropeptide Y Y5. The transcript is 2.7 Kb in size.
Discussion

Sequencing is the most accurate method available for screening for polymorphisms within genomic DNA. In addition, due to the production of uniform peak heights, Bodipy sequencing chemistry is the most accurate chemistry which can be used when looking for heterozygous mutations. Therefore, Bodipy sequencing chemistry was used as the tool to screen patients for single nucleotide polymorphisms within the coding regions of both the neuropeptide Y Y1 and neuropeptide Y Y5 receptor genes. Experiments have already demonstrated that these receptors are involved in the anabolic pathway of feeding and it has been suggested that mutations within these genes may result in obesity (Fan, 1997). It has also been demonstrated that mutations within the coding region of the melanocortin-4-receptor, involved in the catabolic pathway of feeding, are either silent or occur at a frequency less than 1%. As a result the neuropeptide receptors make good candidates for mutation analysis.

There is also preliminary evidence that alternatively spliced 5'-UTRs are involved in tissue specific expression of the Y1 receptor. In addition, tissue specific expression studies were needed to understand the roles of both the Y1 and the Y5 receptors. Northern analysis was used to study the expression patterns of the two receptors using a multiple tissue dot blot to study the expression in both central and peripheral tissues. Multiple tissue northerns were then used to study expression of the receptors within various regions of the brain.

Neuropeptide Y Y1 and Y5 Receptor Polymorphisms
Polymorphism analysis of the NPY Y1 and Y5 receptor genes showed three mutations within the coding regions of both receptors. All mutations occurred at a frequency less than one percent. (Table 4 and Table 5) Using patients positive for angina and schizophrenia provided a diverse gender and ethnic population for polymorphism analysis. The occurrence of mutations in the population suggests that mutations within the coding regions of the Y1 and Y5 receptor genes may be responsible for the genetic predisposition to develop obesity. Mutation 3 of the Y1 gene occurred at approximately 1% and was found within the carboxy-terminus of the protein, a region known to be important for G-Protein binding and phosphorylation. Mutation 2, although only at 0.25% in frequency was found within the fifth transmembrane domain, an area of the protein which may be involved with ligand binding. Only one coding mutation within the Y5 receptor gene was found. This cysteine/Arginine substitution occurred at a frequency of 0.2% and is located within the third transmembrane domain, an area of the protein which may be involved with ligand binding. At this time, little is known about the downstream elements in the anabolic pathway of feeding, and it is possible that mutations within the genes coding for these elements may contain additional mutations.

**Tissue specific expression of the Neuropeptide Y Y5 receptor gene**

Previous experiments showed that expression of the Y5 receptor gene was limited to the brain and the testes. RNA Dot Blot analysis using the coding region of the Y5 receptor gene as the probe showed expression mainly in the brain but also in several peripheral tissues. It was also hypothesized that transcription of neuropeptide Y1 Exon 1C would have a direct inhibitory affect on the transcription of neuropeptide Y5. In some cases expression of only Y5 or Y1 exon 1C was found. However, in some tissue types,
such as the medulla, spinal cord, amygdala, caudate nucleus and hippocampus, both were expressed. This suggests that both receptors can be expressed within the same tissue, however, they may be expressed in different cell types within these tissues.

**Tissue specific expression of the Neuropeptide Y Y1 receptor gene**

Previous experiments showed that expression of the Y1 receptor was found mainly in peripheral tissues. RNA Dot Blot analysis using the coding region of the Y1 receptor gene as the probe showed expression mainly in several peripheral tissues but also in many regions of the brain. Previous evidence suggested that the Y5 receptor was responsible for central binding of NPY whereas Y1 was the peripheral receptor for Y1. This new evidence suggests that dual expression of both receptors regulates NPY binding in both the CNS and the periphery and both receptors are important in controlling the effect of NPY.

**Tissue specific expression of the Neuropeptide Y Y1 receptor gene 5' UTRs**

Oligonucleotides were synthesized to use as probes to bind specifically to the three alternatively spliced 5' exons of the neuropeptide Y Y1 receptor gene. Exon 1A was found to be expressed throughout all brain regions studied. Exon 1B was not found in any of the brain regions studied. Several hybridization and wash conditions were used to identify the expression pattern of this exon without success. This may be due to the possibility that exon 1B is expressed only in peripheral tissue or that exon 1B is not an alternatively expressed exon of Y1 as suggested (Herzog, 1997). Exon 1C, which is contained within the coding region of Y5 and is transcribed from the opposite strand, was found to be alternatively expressed. However, as stated earlier, the expression of exon 1C of Y1 and Y5 occur in the same tissues.
Conclusion

Obesity in humans does not seem to be a monogenic disorder, as is the case in several rodent models. However, several mutations within the coding regions of the neuropeptide Y Y1 and Y5 receptor may be responsible for the genetic predisposition to develop obesity. Genetic obesity in humans appears to be caused by mutations within several genes of the feeding pathways, including the leptin, melanocortin-4-receptor, NPY Y1 and NPY Y5.

Northern analysis shows that NPY Y1 and Y5 are both expressed in several brain regions and that both are expressed in some of the same tissues. This suggests that coordinated expression of both the Y1 and Y5 receptor genes does not exist, at the tissue level, but rather dual expression of these receptors regulate NPY binding in both the CNS and the periphery and both receptors are important in controlling the effect of NPY. As a result, one would expect that a mutation causing altered function in either of the two receptors may cause a predisposition to obesity in humans.

Future Research

An in depth analysis of the expression of the alternatively spliced forms of the Y1 receptor gene is needed in peripheral tissues. This will provide evidence for the relevance of exon 1B. In addition, Rapid Amplification of cDNA Ends (RACE) experiments need to be performed to determine whether different 5’ exons of the Y5 receptor gene exist. If there are in fact alternatively spliced isoforms, a northern analysis should be done to determine their expression patterns. In addition, a SNP analysis of the 5’UTR’s of both receptors in needed to determine whether a mutation is present that might affect translation of the receptors. Finally, the common promoter region of the two
genes should be sequenced to identify putative regulatory elements and to determine whether other possible isoforms exist.
References


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Education:
Lehigh University: MBA Program
5/99-present
Expected date of completion is May 2001
Lehigh University: M.S. Molecular Biology
9/97-present
Expected date of completion is January 2000
Cumulative GPA: 3.5/4.0
University of Pennsylvania: Post-Baccalaureate Program
9/96-5/97
Bucknell University: B.S. Biochemistry
9/91-5/95

Experience:
Merck & Co., Inc. Biochemist 6/97-Present
Dept. of Human Genetics/Dept. of Virus and Cell Biology
Responsible for:
-SNP analysis of Neuropeptide Y, Y1 and Y5 receptors as well as
tissue expression analysis of the mRNA from both receptors. This
work included isolation of genomic DNA from whole blood,
sequencing both receptors from 200 patients, assembly and
analysis using multiple alignment programs as well as Dot Blot
and multiple tissue northerns.
-Supervision and training of several interns.
-Shotgun and concatinated library production which were used in
the identification of novel genes.
-Synthesis and HPLC purification of oligos and Bodipy primers for
sequencing, PCR and site-directed mutagenesis.

University of Pennsylvania: 6/95-6/97
Dept. of Medicine: Ed Holmes, Chairman of Medicine
-Performed molecular biology techniques in an alternative splicing
lab.
-Studied the alternative splicing of AMPD1 in the attempt to
associate the alternative spliced form to a disease state.
-Responsible for construction of splice intermediate clones and
transfecting these clones into several cell lines to check mRNA
processing.
**CollegePro Painters:** 1/94-3/95
District manager: In charge of hiring, payroll, advertising and customer relations for a painting company. I managed twenty-five painters divided into five work crews who produced $65,000 over the course of fifteen months.

**Relevant Course work:**
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- Financial Accounting
- Microeconomics
- Statistics
- *Molecular Genetics
- *Molecular Cell Biology I
- *Molecular Cell Biology II
- *Biochemistry I
- *Biochemistry II
- *Pathophysiological Chem.
- *Human Genetics and Reproduction
- *Developmental Biology
- *Thesis Research

**Computer Expertise:**
- Microsoft Excel, Access, PowerPoint, Word, Sequencher 3.0, Oligo, Canvas, Endnote. Familiar with Mac, IBM, Windows NT and Unix operating systems.

**Laboratory Expertise:**
- Northern's, Western's, HPLC Purification, DNA Isolation, RNA Isolation, Concatinated Library Production, Tissue Culture Techniques, Shotgun Library Production, PCR, RT-PCR, Sequencing, Aseptic Technique, Transfections, Transformations, Oligo Synthesis, Restriction Analysis, Cloning, Site-directed Mutagenesis

**Publications:**
END OF TITLE