OBESITY – ENVIRONMENTAL FACTORS AND/OR GENETIC INFLUENCE

Genomic Structure, Mutational Analysis and Promoter Function of The Human Uncoupling Protein-2/-3 (hUCP2/hUCP3) Genes

by

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To Hongmei, Yuchen and my parents
for
all happiness and care
they give to me

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OBESITY-Environmental Factors and/or genetic Influence

Genomic Structure, Mutational Analysis andPromoter Function of The Human Uncoupling Protein-2/-3 (hUCP2/hUCP3) Genes

Naxin Tu

Today overweight and obesity has been recognized as a disease, a rapidly growing threat to health in an increasing number of countries worldwide, which is prevalent in both developing and developed countries and affects children and adult alike. What causes obesity? In scientific terms, obesity occurs when a person's calorie intake exceeds the amount of energy he or she burns. What causes this imbalance between consuming and burning calories? Evidence suggests that obesity often has more than one cause. Genetic, environmental, psychological, and other factors all may play a part. Obesity tends to run in families, indicating that it has a genetic cause. However, family members share not only genes but also diet and lifestyle habits that may contribute to obesity. Still, growing evidence points to heredity as a strong determining factor of obesity. In many studies of adults who were adopted as children, researchers found that the subjects' adult weights were closer to their biological parents' weights than their adoptive parents'. The environment provided by the adoptive family apparently had less influence on the development of obesity than the person's genetic makeup.

The role of genetic factors in obesity development is currently the focus of much research. Among the major breakthroughs in obesity research during the past several years has been discovered that the adipocytes hormone leptin (the product of OB gene, Zhang *et al.*, 1994) and leptin receptor (Maffei *et al.*, 1995), melanin concentrating hormone (Gonzalez et al., 1997), the melanocortin 4 receptor (Yeo *et al.*, 1998), urocortin (Zhao *et al.*, 1998; Iino *et al.*, 1999), neuropeptide Y and its type 5 receptor (Pickavance *et al.*, 1999) and so on, all are involved in the control over the process of energy intake. In contrast, much less

is known about the molecular basis of for determination of energy expenditure. However, the discovery and characterization of mitochondrial inner membrane ions carrier proteins – uncoupling proteins –represents a major breakthrough towards understanding the molecular basis for energy expenditure, and therefore likely to have important implication for the cause and treatment of human obesity.

UCPs considered as prime candidate genes involved in the pathogenesis of obesity. Due to the fact of limited abundance of UCP1 containing brown adipose tissue is unlikely to be involved in wieght regulation in adult large size animal and human living in a thermoneutral environment. Identification of UCP2 and UCP3 homologues in rodents implied as a major breakthrough towards discovery of the molecular basis for the energy expenditure. Therefore, What's the case in human? What are the potential functional roles of human UCP2 and UCP3 in energy metabolism and body weight regulation? How the expression of human UCP2 and UCP3 regulated? What are the possible implications of human UCP2 and hUCP3 for the pathogenesis and treatment of human obesity? Genetic studies in humans provide a method to test hypotheses about the biological role of specific genes, therefore this study focuses on human UCP2 and UCP3 genes function and transcriptional regulation.

Methods

The studies entirely base on current molecular Biology and Genetics means. Elucidation of the structural organization of human UCP2 and UCP3 genes by molecular cloning, sequences determination and exon / intron mapping. Identification and characterization of genetic variants in the 3'-UTR of human UCP3 gene using Rapid Amplification cDNA Ends (RACE) and RT-PCR (Reverse transcription-Polymerase Chain Rection). Polymorphism analysis by Genotyping. Determination of functional properties of the 5'flanking and the promoter region of these two genes: elucidation the 5' Flanking Region of the hUCP2 gene by PCR-screening a human genomic library. Genome Walking of 5'-

Flanking Region of human UCP3 Gene. Promoter analysis human UCP –2 /-3 utilizing pCAT-3 reporter gene system, transient transfection, CAT ELISA and protein determination

Results

Uncoupling proteins (UCP) are members of the family of mitochondrial anion carriers, which creates a pathway that allows dissipation of the proton electrochemical gradient across the inner mitochondria membrane thereby release storied energy as heat, without coupling to any other energy consuming process, uncoupling fuel oxidation from the conversion of ADP to ATP. This implies a major role of UCPs in energy metabolism and thermogenesis, which when deregulated are key risk factors in the development of obesity and other eating disorders. From the three different human UCPs identified by gene cloning, the human UCP1 (hUCP1) gene was assigned to human chromosome 4 (4q31) (Cassard *et al.,* 1990). Both UCP2 and UCP3 were mapped in juxtaposition to regions of human chromosome 11 (11q13) (Pecqueur *et al.,* 1999) that have been linked to obesity and hyperinsulinaemia (Norman *et al.,* 1997; Bouchard *et al.* 1997). At the amino acid level hUCP2 has about 55% identity to hUCP1 while hUCP3 is 71% identical to hUCP2.

Genomic organization

The human UCP2 gene spans over 8.7 kb distributed on 8 exons. The localization of the exon/intron boundaries within the coding region matches precisely that of the hUCP1 gene and is almost conserved in the recently discovered hUCP3 gene as well. The high degree of homology at the nucleotide level and the conservation of the exon /intron boundaries among the three UCP genes suggests that they may have evolved from a common ancestor or are the result from gene duplication events.

Characterization the genomic structure of the human UCP3 gene implicates that hUCP3 gene spans about at least 7.5 kb distributed 7 exons and 6 introns, from which two

mRNA transcripts are generated, UCP3L and UCP3S, which encode long and short forms of the hUCP3 protein differing by the presence or absence of 37 amino acid residues at the C-terminus. Mapping the boundaries of hUCP3L and hUCP3S transcripts. The potential transcription initiation site of hUCP3 mRNA was mapped at position -186 of the 5'-UTR by 5'-RACE (based the first base of the translational start codon ATG). 3'-RACE showed that the short form of hUCP3 is generated by incomplete transcription caused by the presence of a cleavage and polyadenylation signal (AATAAA) in intron 6 terminating message elongation. Alternatively, the elongation continues until another AATAAA signal in exon 7 of hUCP3 gene once the mRNA synthesis passes through the first polyadenylation signal.

Mutational analysis

Mutational analysis of the hUCP2 gene in a cohort of 172 children of Caucasian origin revealed a polymorphism in exon 4 (C to T transition at position 164 of the cDNA resulting in the substitution of an alanine by a valine at codon 55) and an insertion polymorphism in exon 8 consisted of a 45 bp repeat located 150 bp downstream of the stop codon in the 3'-UTR. The allele frequencies were 0.63 and 0.37 for the alanine and valine encoded alleles, respectively, and 0.71 versus 0.29 for the insertion polymorphism. The allele frequencies of both polymorphisms were not significantly elevated in a subgroup of children characterized by low Resting Metabolic Rates (RMR). So far a direct correlation of the observed genotype with (RMR) and Body Mass Index (BMI) was not evident.

Promoter Analysis

To analyze promoter function and regulatory motifs involved in the transcriptional regulation of UCP2 gene expression, 3.3 kb of 5'flanking region of the human UCP2 gene have been cloned by PCR-screening a human genomic library. Utilizing 5'-RACE, the potential transcription initiation site of hUCP2 mRNA has been identified to localize at

position –364 of the 5'-UTR based the first base of the translational start codon ATG. Sequence analysis showed that the promoter region of hUCP2 lacks a classical TATA or CAAT box, however appeared GC-rich resulting in the presence of several Sp-1 motifs and Ap-1/-2 binding sites near the transcription initiation site. Functional characterization of hUCP2 promoter-CAT fusion constructs in transient expression assays showed that minimal promoter activity was observed within 65 bp upstream of the transcriptional start site (+1). 75 bp further upstream (from nt –141 to –66) a strong *cis*-acting regulatory element (or enhancer) was identified, which significantly enhanced basal promoter activity. The regulation of human UCP2 gene expression involves complex interactions among positive and negative regulatory elements distributed over a minimum of 3.3 kb of the promoter region.

To get insight into the mechanisms regulating human UCP3 expression, 5 kb of the 5'-flanking region of the hUCP3 gene were cloned and characterized by genome walking. The promoter region contains both TATA and CAAT boxes as well as consensus motifs for PPRE, TRE, CRE and muscle-specific factors like MyoD and MEF2 sites. Functional characterization of a 3 kb hUCP3 promoter fragment in multiple cell lines using a CAT-ELISA identified a *cis*-acting negative regulatory element between - 2983 and -982 while the region between -982 and -284 showed greatly increased basal promoter activity suggesting the presence of a strong enhancer element. Promoter activity was particularly enhanced in the murine skeletal muscle cell line C_2C_{12} reflecting the tissue-selective expression pattern of UCP3.

Future studies should be directed to clarify the potential significance these elements may have on regulation of hUCP2 / hUCP3 expression *in vitro* and *in vivo,* as well as to the identification of interactions between transcription factors and individual regulatory motifs localized in the 5' flanking region of hUCP3 and the entire 7 kb intergenic region between the UCP2 and UCP3 locus on human chromosome 11q13, in order to identify the underlying mechanisms orchestrating human UCP 2 and UCP3 genes transcription.

Conclusion

In conclusion, the discovery of the uncoupling proteins could be a breakthrough in understanding the complex mechanisms regulating energy expenditure and has given new stimuli for research in this field. The results so far strongly suggest a role for the UCPs in energy balance and obesity.

The genomic structure of the human UCP2 and UCP3 genes show great homology to the other known members of this family of mitochondrial carrier proteins; To get more definitive proof that UCPs are involved in regulating basal metabolic rates, and thus weight gain or loss,Analysis of the underlying mechanisms as regards regulation of UCP-2/-3 expression by promoter functional characterization in and cell culture determined regulatory motifs of tissue-specificity, hormone regulation and cis-/trans- acting elements in the upstream regions of UCP genes, which revealed the expression of UCPs is mainly controlled at the trancriptional level, and is positively regulated by the sympathetic nervous system CCAAT/enchancer-binding protein beta (C/EBP-b) plays a role as transcriptoinal activator of UCP genes, and peroxisome proliferator activated receptor(PPAR) gamma is also involved transcription regulation of UCPs via its ligands free fat acids, those basic studies will be on to find drugs that can combat obesity by tuning up the activity of the UCP proteins. If the level of uncoupling proteins could be slightly increased 1%-2%, then fat oxidation and thermogenesis would increased, and that could boost the resting metabolic rates of millions of people and whittle away their days of perpetual dieting.

Human uncoupling proteins have currently been in molecular basis of the research on energy expenditure, particularly nonshivering thermogenesis. Still, several Key questions are proposed and challenged in the near future, what are the molecular and physiological similarities and dissimilarities among UCPs? What are mechanisms of action? Are the other functions for UCP2 and UCP3? Finally, numerous different complex and diverse factors can

give rise to a positive energy balance, Human obesity is a result of the interaction between a number of these influences, rather than any single factors acting along.

Because obesity prevalence continues to increase sharply as people approach the new century, today the challenge to scientists and public health worker in this area has never been greater.

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Preface

An expert consultation on obesity was convened by the World Health Organization (WHO) in June 1997 with the aim to review current epidemiological information on obesity, and drawing up recommendations for developing public heath policies and programmes for improving the prevention and management of obesity which is emerging as a global public health problem. In the consultation, overweight and obesity has been recognized as a disease, a rapidly growing threat to health in an increasing number of countries worldwide, which is prevalent in both developing and developed countries and affects children and adult alike. In fact, overweight and obesity are now so common that they are replaced the more traditional public health concerns such as undernutrition and infectious diseases as some of most significant contributors to ill health. The health consequences of obesity are many and varied, ranging from an increased risk of premature death to several non-fatal but debilitating complaints that impact on immediate quality of life. The major health consequences associated with overweight and obesity are Noncommuniable Diseases (NCDs), Non-Insulin-Dependent Diabetes Mellitus (NIDDM), Coronary Heart Disease (CHD), Cardiovascular Diseases (CVD), hypertension, gallbladder disease, pyschosocial disturbances and certain types of cancer.

Current knowledge of human obesity has progressed beyond former explanation by the single adverse behavior of inappropriate eating in the setting of attractive foods. The study of animal models of obesity, biochemical alterations in man and experimental animals, and the complex interactions of psychosocial and cultural factors that create susceptibility to human obesity indicate that this disease in man is complex and deeply rooted in biologic systems. Obesity research efforts therefore have been directed toward elucidation of biologic markers, factors regulating the regional distribution of fat, studies of energy regulation, and

studies utilizing the techniques of human genetics, molecular biology, anthropology, psychiatry, and the social sciences.

The etiology of obesity is multifactorial, and almost certainly under genetic and environmental as well as psychological influences. Although psychological factors may influence eating habits, most overweight people have no more psychological disturbance than normal weight. Numerous line of evidence suggest strong genetic influences on the development of obesity. Most convincing are genetic studies of adoptees and twins. In a study of approximately 4000 twins, a much closer correlation between body weights was found in monozygotic than dizygotic twins, and also, genetic factors accounted for approximately two thirds of the variation in weights. More recent studies of twins reared apart and the response of twins to overfeeding showed similar results. Studies of regional fat distribution in twins has also shown a significant (but not complete) genetic influence. In spite that family members share not only genes but also diet and lifestyle habits that may contribute to obesity, still, growing evidence points to heredity as a strong determining factor of obesity. In studies of adults who were adopted as children, researchers found that the subjects' adult weights were closer to their biological parents' weights than their adoptive parents'. The environment provided by the adoptive family apparently had less influence on the development of obesity than the person's genetic makeup.

From the research now available, a good number of genes seem to have the capacity to cause obesity or increase the likelihood of becoming obese. The uncoupling protein family members are currently focus of much research because of their potential function in mediation of mitochondrial proton leak, thermogenesis of thermoregulation, regulation of energy expenditure, protection against development of obesity, protection against damage by reactive oxygen speices.

The present studies include: elucidation of the structural organization of human UCP2 and UCP3 genes, identification and characterization of genetic variants, as well as

determination of functional properties of the 5'flanking and the promoter region of these two genes.

Major parts of this work have respectively been published in four publications and five abstracts presented at various international obesity meetings in Europe and the USA (see the list of publications and abstracts during the doctoral study).

Chapter 1. Introduction

1.1 Overview

In simple term, obesity is a consequence of an energy imbalance where energy intake gas exceeded energy expenditure over a considerable period. Numerous different complex and diverse factors can give rise to a positive energy balance, but it is the interaction between a number of these influences, rather than any single factor acting alone, that is thought to be responsible. Factors influencing the development of overweight and obesity include physiological regulation of body weight, individual / biological susceptibility, dietary and physical activity patterns, environment and societal influences and others (e.g. smoking, alcohol intake) (**Fig. 1-1**).

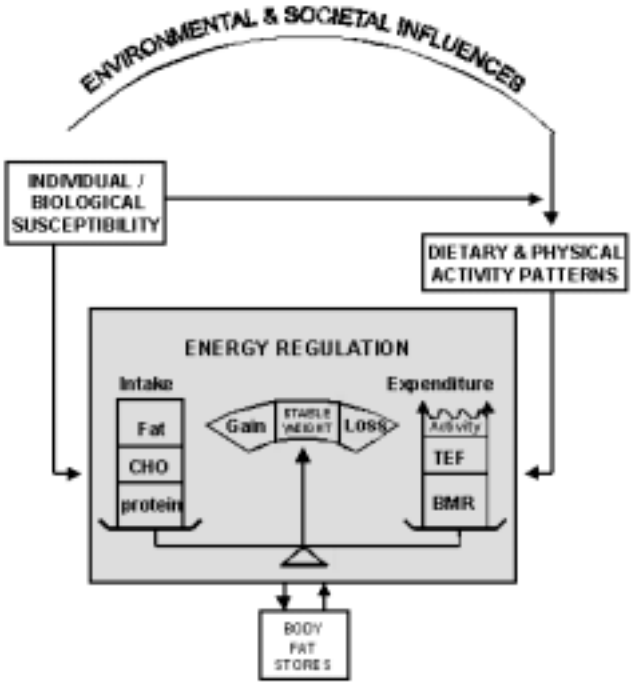


Fig. 1-1 The fundamental principles of energy balance and regulation. Positive energy balance occurs when energy intake is greater than energy expenditure and promotes weight

gain. Conversely, negative energy balance promotes a decrease in body fat stores and weight loss. Body weight is regulated by a series of physiological processes which have the capacity to maintain weight within a relatively narrow range (stable weight). It is thought that the body exerts a stronger defense against undernutrition and weight loss than it does against over-consumption and weight gain. TEF, thermic effect of food; BMR, basal metabolic rate; CHO, carbohydrate (Report of a WHO Consultation on Obesity).

Societal and cognitive factors can influence the control of body weight to certain extent, but it is a series of physiological processes that are primarily responsible for body weight regulation. The physiological mechanisms responsible for body weight regulation are incompletely understood. However, there is increasing evidence of a range of mechanisms within the intestine **(Fig. 1-2)**.

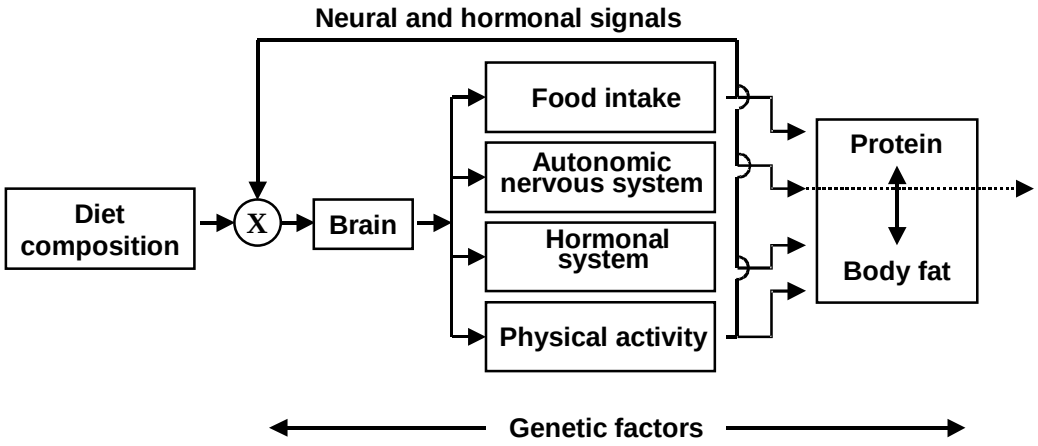


Fig. 1-2 Physiological processes involved in body weight regulation. A model of the interaction between different mechanisms which affect energy and body weight regulation within individuals. The brain integrates an array of afferent signals (nutrient, metabolic, hormonal and neuronal) and responds by inducing changes in food intake, autonomic nervous system activity, hormonal responses or in spontaneous physical activity. The different

components then directly or indirectly determine the partition of fat and protein (Report of a WHO Consultation on Obesity).

Macronutrient-related	Energy expenditure	Hormonal
• Adipose tissue lipolysis • Adipose tissue and muscle LPL activity • Muscle composition and oxidative potential • Free fatty acid and ß-receptor activities in adipose tissue • Capacities for fat and carbohydrate oxidation (respiratory quotient) • Dietary fat preferences • Appetite regulation	• Metabolic rate • Thermogenic response to food • Nutrient partitioning • Propensity for spontaneous physical activity	• Insulin sensitivity • Growth hormone status • Leptin action

Tab. 1-1 Some factors involved in the development of obesity thought to be genetically modulated

Epidemiological, genetic and molecular studies suggest that there are people who are more susceptible than others to becoming overweight and obese, in other words, who have an

inherited susceptibility to be in positive energy balance. These observation have been made in populations all over the world, indicating that susceptible individuals can be found across a wide range of lifestyle and environment conditions. Genetic factors are mainly responsible for such difference in the sensitivity of individuals to gain fat when chronically exposed to a positive energy balance coming from studies in both animals and humans. The genetic effect associated with the risk of obesity appears to be of the multigenic type, the genes exerting their influence on body mass and body fat as a result of DNA sequence variation either in the coding region or in the segments that affect gene expression. The number of genes and other markers that have been associated or linked with human obesity phenotypes is increasing vera rapidly and now approaches 200 (Perusse *et al*, 1998). Obesity is truely a complex multifactorial phenotype with a genetic component that includes both polygenic and major gene effects (**Tab. 1-1**).

The role of genetic factors in obesity development is currently the focus of much research. Among the major breakthroughs in obesity research during the past several years has been discovered that the adipocytes hormone leptin (the product of OB gene, Zhang *et al.*, 1994) and leptin receptor (Maffei *et al.*, 1995), melanin concentrating hormone (Gonzalez et al., 1997), the melanocortin 4 receptor (Yeo *et al.*, 1998), urocortin (Zhao *et al.*, 1998; Iino *et al.*, 1999), neuropeptide Y and its type 5 receptor (Pickavance *et al.*, 1999) and so on, are involved in the control over the process of energy intake. In contrast, much less is known about the molecular basis of for determination of energy expenditure. However, the discovery and characterization of mitochondrial inner membrane ions carrier proteins – uncoupling proteins – represents a major breakthrough towards understanding the molecular basis for energy expenditure, and therefore likely to have important implication for the cause and treatment of human obesity.

1.2 Some mechanisms

Obesity occurs when a person's calorie intake exceeds the amount of energy he or she burns. So obesity is an excess of body fat frequently resulting in a significant impairment of health. The excess fat accumulation is associated with increased fat cell and fat cell numbers. Regulation of energy balance is complex, but many aspects have begun to yield to investigation. Promising leads are: effects of the central and autonomic nervous systems and the endocrine system; Adipose tissue cellularity (in tissue culture) and metabolism; the role of various components of thermogenesis in the overall control of energy balance; control of food intake (e.g. endogenous opioids); satiety factors (e.g. gut hormones). In general, the regulation of body weight involved coordination of intake and expenditure of calories. Energy intake is simply defined as the mass of calories that enter the body, control over this process involves multiple neural circuits with specific neuropeptides, neurotransmitters and their cognate receptors, such as leptin, melanin concentrating hormone and so on.

The processes involved in energy expenditure of an organism can be divided into two categories: adeonsine triphosphate (ATP) consuming processes and non-ATP consuming processes. The first is ATP consuming processes, the obligatory energy costs of all cellular functions and physiological processes. These processes include muscle contraction, protein turnover (12% to 25% of RMR in human), Na^+/ K^+-pump (20%), Ca^{2+} pump (4% to 6%), and substrate cycles (8%). In these processes, the energy is provided by ATP. This molecule can release energy by donating 1 and 2 phosphate groups , leaving adenosine diphosphate (ADP) or adenosine monophosphate (AMP), respectively. ATP has to be continuously resynthesized from ADP by the processes of oxidative phosphorylation. During the oxidation of substrates (fat, carbohydrate, and protein), the cofactors NADH and a reduced form of flavin adenine dinucleotide are formed in mitochondrial matrix. At the level of the inner mitochondrial membrane, reduced nicotinamide adenine dinucleotide (NADH) and a reduced form of flavin adenine dinucleotide are converted to nicotinamide adenine dinucleotide (NAD^+), flavin

adenine dinucleotide, and H^+. According to the chemiosmotic hypothesis of Mitchel, the protons are then transported to the cytosolic side of the mitochondrial membrane by a series of reactions. This eventually generates a proton gradient across the membrane, which causes protons to flow back over the inner mitochondrial membrane. The energy generated is used by ATPase to transform ADP into ATP. Therefore, the processes of substrate oxidation are coupled to the formation of ATP. When cells perform minimal work and have low levels of ADP, the rate of proton entry via ATP synthase is limited. The proton gradient increases, electron transport slows. The resting metabolic rate (RMR) represents the basal energy requirements of the body and constitutes 60% to 70% of total energy expenditure. Under the resting conditions and without change the energy storage, all of the energy expenditure is lost as heat, because no external work is performed. Reduced RMR values have been shown to be a predictive factor for the development of human obesity.

The second category is Non-ATP consuming processes, not all energy is coupled to ATP use. There are at least two processes that contribute to energy expenditure and thus heat production without the involvement of ATP. 1, Non-mitochondrial oxygen consumption: There are a number of processes in mammals that use oxygen outside the mitochondria (e.g. peroxisomal fatty acid oxidation). The total contribution of nonmitochondrial oxygen consumption has been estimated at ~10% of Resting Metabolic Rate (RMR). 2, Mitochondrial proton leak (uncoupled mitochondrial respiration): the formation of ATP from ADP requires a proton gradient across the mitochondrial membrane. The flow of proton from the cytosolic side of the membrane into the mitochondrial matrix provides the energy to transform ADP to ATP. However, when inner mitochondrial membrane proton conductance is increased, insufficient proton gradient is generated and oxygen consumption is uncoupled from ATP production. Such proton leak dissipating energy as heat. It has been calculated that the overall contribution of proton leak to RMR is ~ 20% in humans (Schrauwen *et al.,* 1999), the question is where and how this mitochondrial uncoupled respiration occurs.

Mammals have two types of adipose tissue. White adipose tissue that consists of lipid storing adipocytes, and brown adipose tissue that composed of multilocular lipid storing adipocytes and also contains abundant mitochondria. The functions of the two types of adipose tissue are different. The primary function of white adipose tissue is energy storage , and human obesity is characterized by an increase in the amount of white adipose tissue. Adipose tissues has a very active metabolism resulting in thermogenesis. It has been shown that brown adipose tissue can account for up to 40% of the two–fold increase in RMR of rats infused with norepinephrine or exposed to cold. Food intake can also increase the thermogenic activity of the brown adipose tissue, thereby contributing to diet-induced thermogenesis. Mitochondria of brown adipose tissue are exceptionally permeable to protons, and this could lead to leakage (Nicholls *et al.*, 1984).

Possible ways for the mitochondrial proton leak are membrane proteins, the phospholipid bilayer, and protein / phospholipid interfaces. In 1978, it was demonstrated that a mitochondrial inner membrane protein, called thermogenin or uncoupling protein (UCP1), was responsible for the mitochondrial proton leak (Nicholls *et al.*, 1978), which act as H^+ or (OH^-) ions translocators in a carrier like fashion, and can also function as anion (such as Cl^-) transporters. UCP creates a pathway that allows dissipation of the proton electrochemical gradient across the inner mitochondrial membrane, without coupling to any other energy consuming process, uncoupling fuel oxidation from the conversion of ADP to ATP (**Fig. 1-3**) thereby releasing stored energy as heat. In other words, uncoupling protein dissociates the reactions that break down food or fat from those that produce the body's chemical energy, in effect, it punch holes in the energy-production pipeline, raising the body's resting metabolic rate. Since the lost chemical energy is dissipated as heat, UCP helps hibernators and other cold-adapted animals maintain their core body temperature in frigid weather.

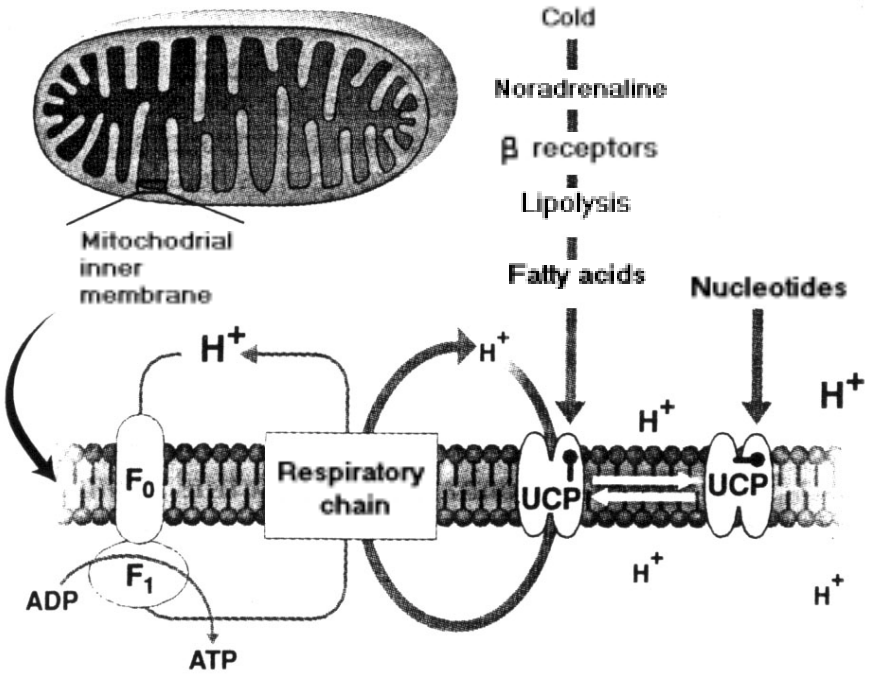


Fig. 1-3 Uncoupling protein (UCPs) let hydrogen ions pass through the inner mitochondrial membrane. Thereby abolishing the hydrogen ion gradient needed to drive ATP synthesis.

1.3 History of Uncoupling Proteins Research

The phenomenon of nonshivering thermogenesis (NST) was first understood in the mid-1950s as whole body thermogenetic response to the noradrenaline that was much increased by acclimation of rats to cold and allowed them to maintain their body temperature without shivering. It was already realized then that could not be permanent property of the tissue, but must subject be to being switch on and off *in vivo.* Although the work of Robert Smith established the thermogenetic function of brown adipose tissue in the mid-1960s, this tissue was believed to be too small to account the phenomenon of the NST.

It was only in the late 1970s that the location of NST in the BAT was established, and the participation of a 32 kda mitochondrial membrane protein in this process was described independently by biochemist David Nicholls at the University of Dundee in the UK and Daniel Ricquier at the National Center for Scientific Research in Pairs. In the meantime, studies of BAT mitochondria and brown adipocytes, particularly by Lindberg's group and by Nicholls, established many of the properties of the stimulated thermogenesis in the BAT mitochondria (mediated in adipocytes by noradrenaline, inhibited by GDP and stimulated by fatty acid in mitochondria) (Himms *et al.,* 1999). Purification of the 32 kDa protein by Klingenberg (Klingenberg *et al.*, 1988), *in vitro* translation, and sequencing of the protein rapidly followed. It was 1985 the rat gene for the protein now known as uncoupling protein 1 (UCP1) was cloned by Ricquier's group and by Kozak and showed to be uniquely expressed in brown adipocytes. Humans have brown adipose tissue, which disappears shortly after birth.

Three more mammalian UCPs have now been cloned so far, their potential function for example mediation of mitochondrial proton leak, thermogenesis of thermoregulation, regulation of energy expenditure, protection against development of obesity, protection against damage by reactive oxygen species and so on. Uncoupling proteins not only provide a better understanding of obesity, but also might be good targets for obesity therapy (especially as they appear to act mainly in fat and muscle, whereas many other weight-regulatory molecules seem to work mainly in the brain.), simply by slightly increasing the level uncoupling – by 1% or 2%, to increase fat oxidation and thermogenesis (or RMR) (Gura, 1998).

Therefore, the uncoupling proteins represent a significant breakthrough towards understanding the molecular basis for energy expenditure somehow has revolutionized obesity research.

1.4 Human Uncoupling Protein 1 (hUCP1)

Bouillaud (1988) first succeeded in detection a specific 1.8 kb mRNA corresponding to the rat UCP1 in human adipose tissue obtained from patients with pheochromocytoma. Cassard showed that the human UCP1 gene spans 13 kb distributed on 6 exons, it contains a transcribed region that covers 9 kb. Human UCP1 gene was assigned to chromosome 4q31. Human UCP1 has 305 amino acids and a molecular weight of 32,786. It has no N-terminal targeting sequence and is 79% homologous to rat UCP1 (306 aa) both at nucleotide and amino acid levels. They found that the primary structure of UCP1 is similar to that of ADP/ATP translocator of skeletal muscle. A TATA box was found in 2 kb of the 5' flanking region of hUCP1, neither CCAAT sequence nor Sp-1 binding motif were detected. hUCP1 is 79 % homologous to mouse UCP1(302 aa) in anino acid level (Cassard *et al.,* 1990). Whereas mouse UCP1 gene was mapped in murine chromosome 8 (Jacobson *et al.,* 1985).

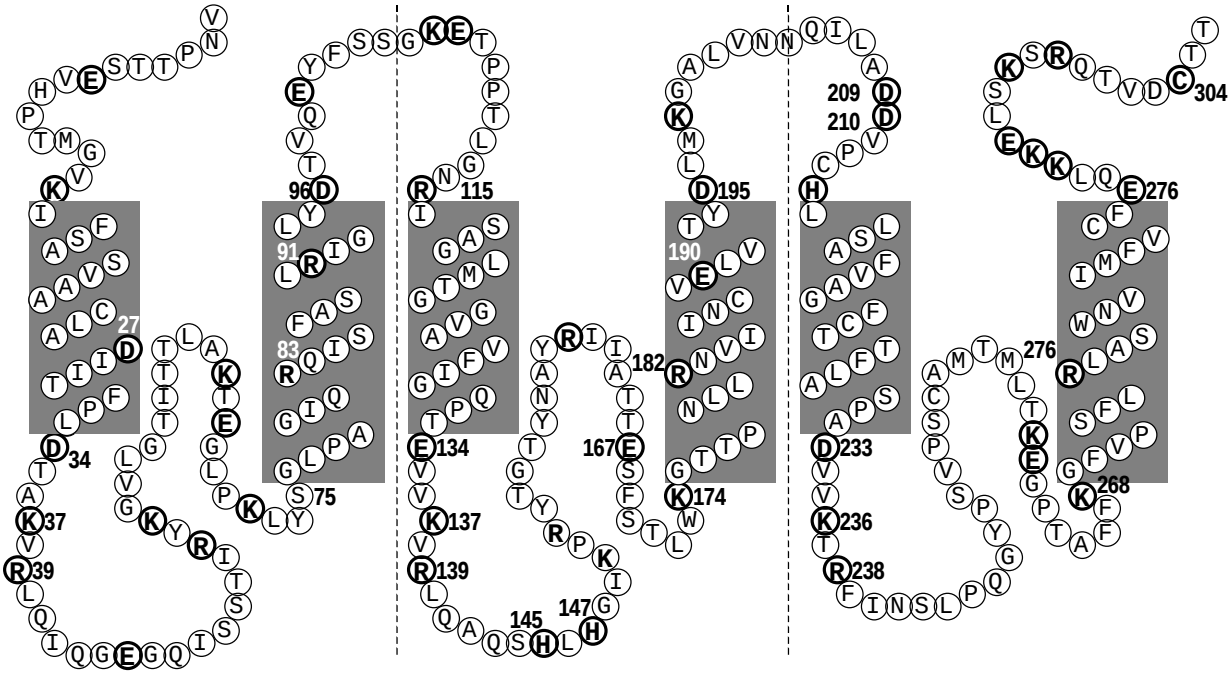


Fig. 1-4 A folding diagram of uncoupling protein 1 (UCP1). The transmembrane organization relies on topological studies and on assigning the transmembrane terminals to charged

residues. Three intermembrane loops are assumed to exist between the thre helix pairs, based on thee accessibility from the cytosol to membrane impermeant probes (Klingenberg *et al.*, 1990).

Genetic variation in brown fat specific mitochondrial UCP 1 expression and brown adipocytes morphology, have provide models to test the hypothesis that nonshivering thermogenesis is associated with the regulation of body weight. Genetic manipulation using transgenic animals and gene targeting has resulted in mice with overexpression of UCP1; these animals consistly show that overexpression UCP1 reduced adiposity. However, less agreement is found in models that reduce nonshivering thermogenesis. In contrast, inactivation of the UCP1 gene by gene targeting does not increase adiposity when compared to control animals (Kozak *et al.*, 1999).

Martin Klingenberg presented a detailed analysis of structure function relationships of UCP1, which revealed a number of charged residues involved in either H^+ transport, Cl^- transport, nucleotide binding and pH sensing for regulating nuclear binding. A few residues are specifically involved in only one function, particularly E190 and H214 which are pH sensors for nucleotide binding. Also H145 and H147 appear essential for H^+ transport only. Some residues are strongly involved in H^+ transport, such as D233 and E195, but they are also important for Cl^- transport. Thus they are not only H^+ transport group but rather important for the common translocation channel. This identification of the amino acid residues responsible for the translocatoion of protons, for the binding of the GDP, and for the influence of pH on this binding provides a framwork for the comparing the sequence of UCP2, and UCP3 with the sequence of UCP1 to see whether these various functional sits are conserved in these proteins. For example, targeted mutation of two histidine residues important for proton translocation in UCP1 results in a protein that does not uncouple. That these histidine residues

are absent in UCP2 and that only one is present in UCP3 suggests a different function for these proteins (Klingenberg *et al.,* 1998). (**Fig. 1-3**)

1.5 Human Uncoupling Protein 2 (hUCP2)

Human UCP1 is expressed exclusively in human perirenal brown adipose tissue, which is very scarce in adult humans; however, in neonate, brown adipose tissue is present, later in life, the white adipose tissue is by far more than brown adipose tissue (under prolonged periods of cold exposure, some brown adipose tissue may remain in adult humans). UCP1 therefore is unlikely to be involved in weight regulation in human and adult large-size animals living in a thermoneutral environment because there is little brown adipose tissue present in adults.

However measurements in human and other animal cells show that from 25% to 35% of oxygen consumed in metabolizing food is being used to compensate for mitochondrial proton leak, thus only the novel members of UCP family may provide an explanation. Fleury *et al*. (1997) and Gimeno *et al* (1997) discovered a homolog of human UCP1, designated UCP2 (hUCP2), which has 59% amino acid identity to hUCP1 and consists of 309 amino acids with molecular weight of 33 kDa. Not only several protein motifs were found to be conserved in hUCP2, including three mitochondrial carrier protein motifs and amino acids essential for ATP binding, suggesting that hUCP2 has also a function role as a mitochondrial uncoupler. But also the decrease in the mitochondrial potential was measured in yeast after introducing hUCP2 in a vector, the results showed that hUCP2 influenced mitochondrial activity and could partially uncouple respiration from ATP synthesis. The tissue distribution of human UCP2 gene expression (with 1.7 kb mRNA) is markedly different from UCP1, UCP2 mRNA is present in skeletal muscle, lung, heart and kidney as well as in tissue of the immune system.

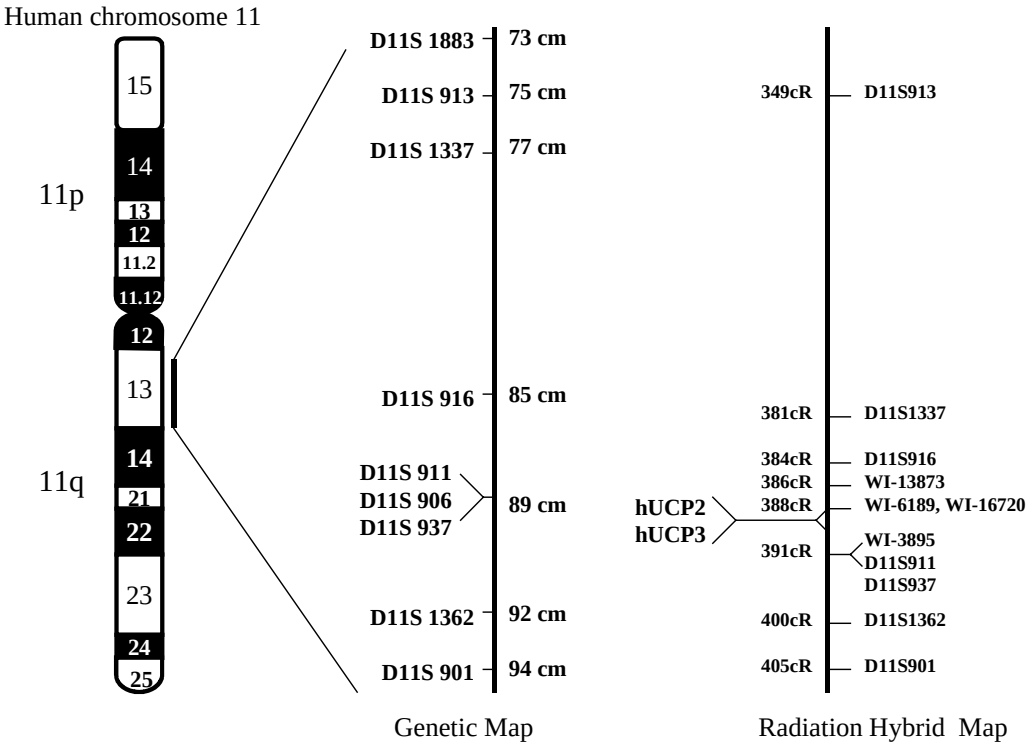


Fig. 1-5 Chromosomal localization of human UCP2 and UCP3 genes. On the right is shown the Whitehead Institute Center for Genome Research radiation hybrid map with human UCP2 and UCP3 positioned relative to framework markers (Solanes *et al.*, 1997).

The hUCP2 gene was mapped to human 11q13 by using 2 independent sequence tagged sites derived from human UCP2 clones. The mouse homolog UCP2 (308 aa, which is 95% identical to human UCP2) mapped the to murine chromosome 7, tightly linked to the 'tubby' mutation, in an area of homology of synteny to 11q13. human UCP2 is 95 % homologous to rat UCP2 (308 aa) in amino acid level. Furthermore, the chromosomal mapping of UCP2 was coincident with quantitative trait loci (QTLs) for obesity in at least 3 independent mouse models, one congenic strain, and human insulin-dependent diabetes locus-4 (Fleury *et al*. 1997). Bouchard *et al*. (1997) studied the linkage relationships between 3 microsatellite markers that encompass the UCP2 gene location on 11q13 with resting

metabolic rate (RMR), body mass index, percentage body fat, and fat mass in 640 individuals from 155 pedigrees in the Quebec family study. Suggestive evidence of linkage led them to conclude that the 3 markers encompassing the UCP2 locus and spanning a 5-cM region on 11q13 are linked to resting energy expenditure in adult humans (Bouchard *et al.,* 1997). Kaisaki *et al* (1998) localized UCP2 gene to a region linked to glucose intolerance and adiposity in Goto-Kaikizaki type 2 diabetic rat. The evidence is more than enough to warrant a search for DNA sequence variation in the gene itself.

1.6 Human Uncoupling Protein 3 (hUCP3)

By the fact that skeletal muscle determines 40% of whole-body adrenaline-induced thermogenesis, Boss *et al*. (1997) searched for UCP homologs in skeletal muscle, and found three products similar to the muscle UCP1 product, with amino acid lengths of 309, 312 and 275. The 309 amino acid product obviously was UCP2 protein, the other two products were identical for the first 275 amino acids, suggesting that they are isoforms of the same protein. They have 57% and 73% amino acid identical to UCP1 and UCP2, respectively, and they were named UCP3 long and short forms (UCP3L and UCP3S). UCP3S only has five transmembrane domains and lacks the purine nucleotide binding region. Northern blotting revealed that the UCP3 gene was expressed as 2.3 kb mRNA predominantly in skeletal muscle and brown adipose tissue, and at low level in heart muscle. UCP3 gene expression in skeletal muscle is four–fold higher than that of the UCP2 gene. Furthermore the ratio of UCP3L to UCP3S form expression is 1:1.

The genomic structure of human UCP3 was first described by Solanes *et al.* (1997), it contains 7 exons spread over 8.5kb and is located on chromosome 11 (11q13), adjacent to UCP2. Human UCP3 is 86% identical to mouse UCP3 (307 aa) and 87% homologous to rat UCP3 (307 aa) at the amino acid level, hUCP3 expressed two mRNA transcripts, hUCP3L and hUCP3S differ only by the presenc or absence of 37 residues on the C terminus, these 37

residues are encoded by exon 7 which is missing from hUCP3S. The domain encoded by exon 7 is highly homologous to C-terminal residues found in UCP1 and UCP2, thus UCP3S is unique in lacking these residues, since this region is believed to participate in purine nucleotide-mediated inhibition of UCP1 activity, it is therefore suggested that UCP3S may have altered uncoupling activity. Of interest, a special mutation in the first base pair of the intron between exon 6 and 7 has been identified (Argyropoulos *et al.,* 1998). This mutation destroys the splice donor site, preventing UCP3L from being generated. Consequently, this allele can only generate UCP3S, it was reported that individuals heterozygous for this mutation have normal body weight. However, basal fatty acid oxidation was reduced by 50% and respiratory quotient was markedly increased in these individuals. These findings are consistent with regards to UCP3S has a decreased activity and that UCP3 plays an important role in free fatty acid metabolism. However, a more detailed analysis of the individuals with this mutation, as well as the determination of functional properties of UCP3S *vs* UCP3L protein is required to fully understand the significance of these observations.

1.7 Genetics of UCP2 and UCP3 in Energy Expenditure and Obesity

A direct comparison of UCP2 gene expression in obesity-resistant (A/J) mice and obesity-prone (B6) mice shows higher mRNA levels of UCP2 were increased in white adipose tissue by a high-fat diet in the A/J strain, but not in the B6 mice (Fleury *et al*., 1997). This result suggested that UCP2 plays a role in preventing obesity in A/J mice fed a high-fat diet, possibly by increasing energy expenditure. Another finding was provided by Enerbäck (1997). They showed that mice lacking UCP1 (UCP1 knock-out) did not become obese when fed a high-diet, but UCP2 was upregulated five-fold in brown aipose tissue of these mice, therefore for maintaining body weight, UCP2 possibly compensates for the absence of UCP1. These results suggest that UCP2 may be involved in the regulation of body weight.

Surwit *et al.* (1998) showed that the mouse UCP3 gene is localized 5' to the UCP2 gene and that the two genes are only 8kb apart. Solanes *et al.* (1997) demonstrated that P1 clones containing human genomic inserts contained both the UCP2 and UCP3 genes, both the hUCP2 and hUCP3 genes have been mapped to human chromosome 11q13. Studies of these two genes are intertwined because the two genes were separated by only 7 kb (Pecqueur *et al*., 1999). Linkage studies in family have suggested UCP2 and / or UCP3, or a closely linked gene, may influence resting metabolic rate (RMR). Chromosome 11q13 is in the proximity (-15cM) of a locus (11q21-q22), which was found to be linked to percent body fat in Pima Indians (Norman *et al.,* 1997). Bouchard *et al.* (1997) genotyped three markers in the vicinity of 11q13 in 640 individuals from 155 pedigrees from the Quebec Family study.

Millet *et al.* (1997) showed a positive correlation between UCP2 mRNA levels in adipose tissue and body mass index (BMI). However, no difference in the expression of UCP2 and UCP3 in skeletal muscle was found between subjects with obesity and lean subjects.. In contrast, Boss *et al.* (1999) recently showed a negative correlation between skeletal muscle UCP3 expression and BMI, and positive correlation between UCP3 mRNA levels and RMR in Pima Indians. Assuming that mRNA levels reflect UCP3 protein concentrations and activity, these data indicate that reduced skeletal muscle UCP3 may result in a reduced RMR. Because a low RMR is a predisposing factor for weight gain, it was expected that individuals with low UCP3 gene expression would have increased BMI. This is in accordance with negative correlation between BMI and UCP3 gene expression (Millet *et al.*, 1997). Positive results have also been shown for association of the splice variant with respiratory quotient in African Americans. A linkage between marker D11S911 and RMR was found, suggesting a role for UCP2 and /or UCP3 in energy metabolism (Bouchard *et al.*, 1997). In contrast, Elbein *et al.* (1997) did not find linkage between markers in the 11q13 region and BMI in 42 North European families with type 2 diabetic siblings. So far no studies have reported linkage or association of UCP2 or UCP3 with diabetes.

1.8 Regulation of UCP1 ,UCP2 and UCP3 Gene Expression.

Expression and activation of UCP1 is usually mediated by the sympathetic nervous system and is directly controlled by norepinephrine. This mechanism is part of the adaptive response to cold temperatures. It also regulates energy balance. Manipulation of thermogenesis could be an effective strategy against obesity (Lowell *et al.*, 1993).

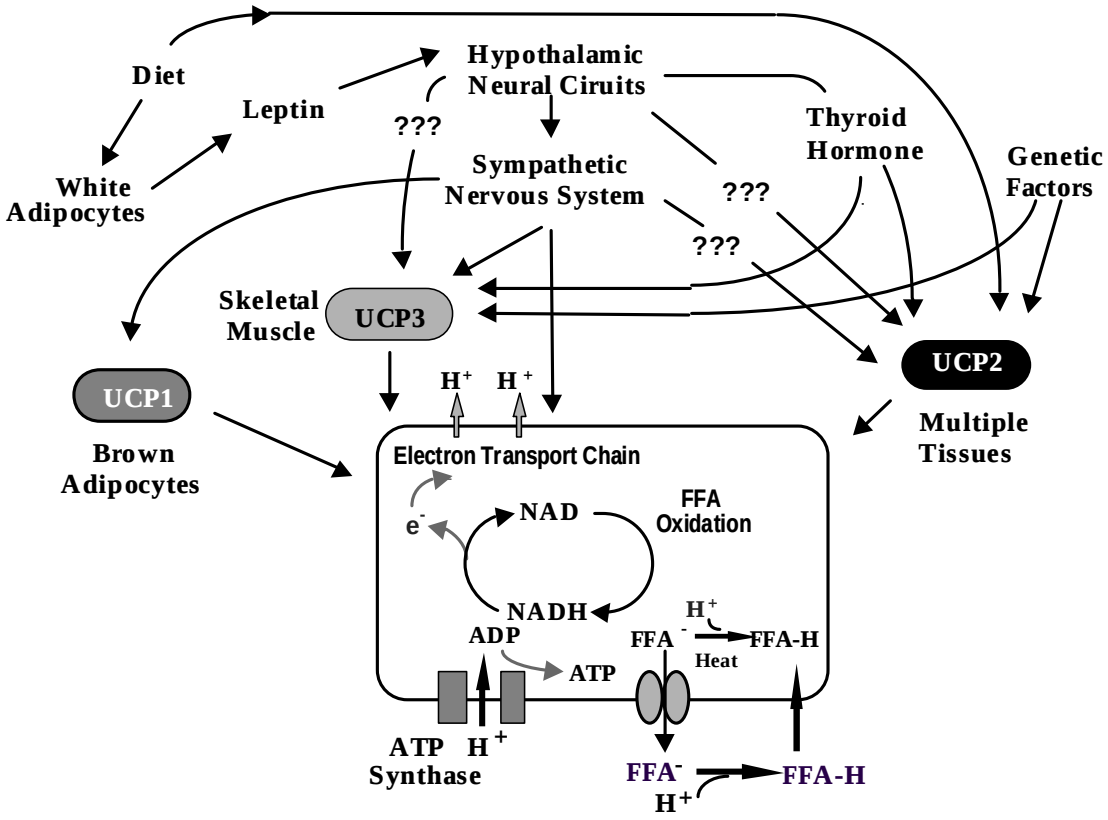


Fig. 1-6 Lines of evidence of uncoupling proteins potentially involved in the molecular pathogenesis of human obesity.

Enerback *et al.* (1997) determined the role of UCP1 in the regulation of body mass by targeted inactivation of the UCP gene in mice. They found that UCP1-deficient mice consumed less oxygen after treatment with a beta-3-adrenergic receptor agonist and that they were sensitive to cold, indicating that thermoregulation was defective. However, this

deficiency caused neither hyperphagia nor obesity in mice fed on either a standard or a high-fat diet. Enerbäck *et al.* also proposed that the loss of UCP may be compensated by UCP2 which is induced in the brown fat of UCP-deficient mice. Adrenaline and noradrenaline, the main effectors of the sympathetic nervous system and adrenal medulla, respectively, are thought to control adiposity and energy balance through several mechanisms. They promote catabolism of triglycerides and glycogen, stimulate food intake when injected into the central nervous system, activate thermogenesis in brown adipose tissue, and regulate heat loss through modulation of peripheral vasoconstriction and piloerection.

Condition	**Change in UCP1 mRNA**
Cold exposure	↑
Thermoneutrality	↓
Fasting (24-48 h)	↓
Food restriction	↓
Refeeding (24h)	↑
High hat diet	↑
Endurance	→
Obesity	↓ *(fa / fa)*
Leptin	↑ ↓ *(ob / ob)*
Hypothyriodism	↑
Thyriod hormone (T3)	↑
Glucocoticods	↓
Insulin (3 h)	↑

Tab. 1-2 Regulation of UCP1 mRNA expression in BAT.

fa / fa = obese *fa / fa* Zuker rats, *ob / ob*= obese *ob / ob* 57BL mice (Boss *et al.*, 1998)

Thermogenesis in brown adipose occurs in response to cold and overeating, and there is an inverse relationship between diet-induced thermogenesis and obesity both in humans and animal models. As a potential model for obesity, Thomas and Palmiter *et al*. (1997) generated mice that could not synthesize noradrenaline or adrenaline by inactivating the gene that encodes dopamine beta-hydroxylase. These mice were cold intolerant because they had impaired peripheral vasoconstriction and were unable to induce thermogenesis in brown adipose tissue through UCP1. The mutants had increased food intake but did not become obese because their basal metabolic rate (BMR) was also elevated. The unexpected increase in BMR was not due to hyperthyroidism, compensation by the widely expressed UCP2, or shivering.

The expression of UCP1 is regulated at the transcriptional level, and its control has been extensively studied. Norepinephrine is a strong physiological activator of UCP1 expression. A activation of ß1-, ß2-, ß3-,and α1-adrenergic receptors as well as inhibition of α2-adrenergic receptor has been shown to increase the expression of UCP1. The thyroid hormone tri-iodothyronine (T3) has been reported to act as a permissive factor for the full induction of UCP1 gene expression by norepinephrine. Expression of UCP1 is also increased by retinoic acid and by peroxisome proliferator-activated receptor (PPAR) agonists like thiazolidinediones (Boss *et al.,* 1998).

1.8.1 The expression of UCP3 varies like that of UCP1 in BAT

As shown in Tables 1 and 2, changes in environmental temperature, variation in food intake and administration of T3 or glucocorticoids affect identically UCP3 and UCP1 mRNA expression in BAT. One exception is the absence of effect of the ß3-adrenergic agonist CL 316.243 on UCP3 mRNA expression in BAT (Gong *et al.,* 1997). In BAT of hereditary obese ob/ob mice and fa/fa rats both UCP1 and UCP3 mRNA expressions are decreased compared with lean controls. The administration of leptin to *ob/ob* mice increases BAT UCP1 and UCP

3 mRNA levels (Ricquier *et al.,* 1984). These observations support the hypothesis that both UCP3 and UCP1 contribute to thermogenesis in BAT.

Condition	Change in UCP2 mRNA			
	Skeletal muscle	**BAT**	**WAT**	**Heart**
• Cold (48 h)	→ ↑	↑ →	/	↑
• Fasting (24-48 h)	↑ →	→	/	→
• Severe food restriction (90%)	↑	/	↑	/
• Food restriction	/	↓	↓	/
• Refeeding (24h)	→	→	/	/
• High heat diet	→	→	→ ↑	↓
• Endurance training	↓	→	→	/
• Obesity	↓ *(fa /fa)* → *(ob / ob)*	↑ *(fa / fa)* ↑ *(ob / ob)*	↑ *(ob / ob)* ↑ *(db / db)*	/
• Leptin	→ *(ob / ob)*	↑ ↓ *(ob / ob)*	↑ →	/
• Hypothyroidism	→	→		/
• Thyroid hormone (T3)	↑		↑	/
• Glucocorticoids	→	→	↓	/
• Insulin (3 h)	→	/	→	/

Tab. 1-3 Regulation of UCP2 mRNA expression in skeletal and BAT.

Condition	Change in UCP3 mRNA	
	Skeletal muscle	**BAT**
• Cold (48 h, 10 days)	→	↑
• Thermoneutrality	→	↓
• Fasting (24-48 h)	↑	↓
• Severe food restriction	↑	/
• Food restriction	↓	↓
• Refeeding (24h)	↓	↑
• High hat diet	↓	→
• Endurance training	→	→
	↓ *(fa/ fa)*	↓ *(fa/fa)*
• Obesity	→ *(ob/ob)*	↓ *(ob/ob)*
	↑	↑
• Leptin	↓ *(ob/ob)*	→ *(ob/ob)*
• Hypothyriodism	↓	→
• Thyriod hormone (T3)	↑	↑
• Glucocoticods	↑	↓
• Insulin (3 h)	→	/

Tab. 1-4 Regulation of UCP3 mRNA expression in skeletal and BAT (Boss *et al.*, 1998).

As shown in Table 3, UCP2 in BAT differs from UCP1 and UCP3 in the way its expression varies with metabolic changes (Muzzin *et al.*, 1989). For instance UCP2 mRNA expression in BAT is insensitive to fasting (Millet *et al.*, 1997), refeeding (Dulloo *et al.*, 1990) and administration of glucacorticoids. In BAT as well as in white adipose tissue (WAT) UCP2 mRNA expression is increased in all rodent models of hereditary obesity tested so far and a significant positive correlation has been reported between UCP2 mRNA levels in WAT

and body mass index in humans (Oberkotter *et al.,* 1992). The pattern of regulation of UCP2 expression supports the idea that UCP2 might have functions other than that of thermogenesis (Camirand *et al.,* 1998).

1.8.2 Expression of UCP3 often varies in opposite directions in skeletal muscle *vs* BAT

In contrast to what is observed in BAT. changes in environment temperature do not affect UCP 3 mRNA expression in skeletal muscle. These results are in line with the notion that muscle is not involved in nonshivering thermogenesis in rodents. It has been shown that after 10 days of food restriction (50%) and during the refeeding phase thereafter the metabolic efficiency of rats is increased and remains elevated until the animals have recovered their body fat stores (Boss *et al.,* 1998). There are two components in this enhanced metabolic efficiency. The first component is due to a decrease in BAT sympathetic activity during rood restriction. and this activity goes back to control levels within 1 day of refeeding. The second component seems to be muscular and persists for as long as the body fat stores have not been recovered. In fact, in rodents the levels of UCP3 mRNA decrease both in BAT and skeletal muscle after food restriction and during refeeding, go back to control levels within 1 day in BAT, but remain very low (or decrease even further) in skeletal muscle (Weigle *et al*., 1997).

During endurance exercise training a decrease in metabolic rate should allow for a better reconstitution of energy stores between exercise bouts. Also under this condition UCP 3 mRNA expression has been shown to be decreased in skeletal muscle .

These findings support the hypothesis that futile cycles (via UCP 3) are turned off in muscle under metabolic conditions which dictate sparing of energy. The mechanisms of the above-described decreases in UCP 3 mRNA expression in skeletal muscle are not yet understood. but some hypotheses can be proposed. Circulating levels of insulin should be decreased by food restriction, but this hormone does not seem to affect UCP3 mRNA expression in muscle. Insulin is therefore an unlikely mediator of the effect of food restriction

on UCP 3 expression in muscle, the levels should be decreased by food restriction, and it has been shown that hypothyroidism decreases and administration of increases UCP3 mRNA expression in skeletal muscle. Thus T3 might play a role in the effects of food restriction on UCP 3 expression (Millet *et al.,* 1997).

An unexpected exception in this pattern of regulation in skeletal muscle is fasting or severe food restriction. In contrast to what is observed after 4-7 days of 40%- 50% food. 24-48 h of fasting in rodents or 5 days of severe (90%) food restriction in humans increases dramatically UCP3 mRNA expression in muscle (Boss *et al*., 1997, 1998).

This increase in UCP 3 gene expression could be mediated by free fatty acids (FFAs), whose circulating levels rise during fasting but not during moderate food restriction. In obese humans the results of a recent study suggest that FFAs play a role in the regulations of muscle LTCP 3 expression, as a significant positive correlation was found between the level of plasma FFA and that of UCP 3 mRNA in vastus lateralis. As FFAs are ligands for PPARγ. The latter might be expected to mediate the effects FFA on UCP 3 expression in muscle. In fact it has been shown recently that UCP2 mRNA expression is enhanced by PPARr agonists in pancreatic islets as well as in cultured white adipocytes, brown adipocytes and myocytes. The decrease in circulating leptin levels induced by fasting might play role in the increase of muscle UCP 3 mRNA expression (Weigle *et al.* 1997). Maintenance of supraphysiological levels of circulating leptin during a 48-h fast does not alter the rise in UCP3 mRNA levels in muscle. Furthermore. this rise could not be mimicked by the administration of pharmacological doses of the glucocoriticoid cortisol, Thus, neither leptin nor glucocorticoids seem to play a role in the fasting-induced increase in UCP3 mRNA levels in muscle.

In hereditary obese rodents UCP3 mRNA expression in BAT is decreased in *both fa /fa* rats and *ob / ob* mice, whereas in muscle it is either decreased in *fa /fa* rats or unchanged in *ob /ob* mice.

In summary, the regulation of expression uncoupling proteins is mainly contributed to thyroid hormone, leptin, ß-adrenergic hormone (ß3-agonists), glucocorticoids, cold exposure and fasting and high-fat feeding.

Genetic studies in humans provide a method to test hypotheses about the biological role of specific genes. Because of a potential functional role of human UCP2 and UCP3 in energy metabolism and body weight regulation, elucidation of the structural organization, identification and characterization of genetic variants, as well as determination of functional properties of these two gene should be of major importance for obesity research. This study focuses on uncoupling protein gene family members, human uncoupling protein –2 and -3 gene: elucidation of their structural organization, identification of genetic variants and analysis of promoter function.

Chapter 2. Materials and Methods

2.1 Determination of the Genomic Organization of the Human UCP2 Gene

2.1.1 Exon / intron mapping of the hUCP2 gene

The location of introns within the human UCP2 gene was determined using PCR and sequence analysis. Based on the genomic organization of the hUCP1 gene and the published hUCP2 cDNA sequence, 8 sets of primers (see **Tab. 3-1** for the sequences) were designed to produce 8 overlapping fragments encompassing the entire coding region including the 5'-UTR and the 3'-UTR. The PCR reaction mix of 50 µl contained: 100 ng of DNA, 25 pmol of each primer, 200µM each of dNTPs, 2.0 mM $MgCl_{2,}$ 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.001% gelatin and 1 unit *Taq* DNA polymerase (MWG, Germany) (9). Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 30 sec/95°C, 30 sec/55°C, 1 min/72°C for 35 cycles, followed by a final extension step of 7 min at 72°C. For long distance PCR (determination of the size of intervening introns), we used the *Expand™ Long Template PCR System* (Boehringer Mannheim) as recommended by the manufacturer. Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 2 min denaturation at 94°C, 35 temperature cycles were carried out consisting of 94°C for 10 sec, 65°C for 30 sec, 68°C for 3 min (cycles 1 - 10) with elongation times increasing 20 sec/cycle (cycles 11 - 35), followed by a final extension step of 7 min at 68°C. The amplification products were visualized on 1.0 % agarose gels stained with ethidium bromide.

Primers No.	**Primer sequences (Forward / Reverse)**	**Location at the cDNA level,** Nucleotide position (5'-3')	**PCR product size compared to cDNA size (bp)**
# 1135	5'- AGCCGACAGACACAGCCGCACGCACTG -3'	-304 / -278	~1,200 (137)
# 1142	5'- AGCTGCCAGTGGCTATCATGGCCCGAT -3'	-170 / -196	
# 1141	5'- ATCGGGCCATGATAGCCACTGGCAGCT -3'	-196 / -170	~3,000 (193)
# 1134	5'- ACCCAACCATGATGCTGATTTCCTGCTAC-3'	10 / -19	
# 1087	5-' ATCATGGTTGGGTTCAAGGCC -3'	-3 / 18	494 (338)
# 1090	5'- TGCTCAGAGCCCTTGGTGTAG -3'	338 / 318	
# 1091	5'- ATCGGCCTGTATGATTCTGTC -3'	289 / 09	1,110 (243)
# 1099	5'- CTTTCCAGAGGCCCCGGAACC -3'	531 / 511	
# 1093	5'- GTCAATGCCTACAAGACCATT -3'	478 / 498	238 (157)
# 1101	5'- CTGTCATGAGGTTGGCTTTCA -3'	634 / 614	
# 1100	5'- GGACCTCTCCCAATGTTGCTC -3'	533 / 53	1,162 (191)
# 1094	5'- TCTCGTCTTGACCACGTCTACA -3'	723 / 702	
# 1102	5'- ATGACCTCCCTTGCCACTTCA -3'	635 / 655	665 (296)
# 1096	5'- TCAGAAGGGAGCCTCTCGGG -3'	930 / 911	
# 1144	5'- TGAGCCTCTCCTGCTGCTGACCTGATC -3'	928 / 954	336 (336)
# 1145	5'- AGGTTGAGCTTGCTTTATGGATGACAA -3'	1,263 /1,237	

Tab. 2-1 Primer sequences used for exon / intron mapping.

2.1.2 DNA cloning

Automated sequencing of PCR products was performed to determine the sequences at the exon/intron boundaries as well as the sequence of the entire introns within

the coding region and in the 5'-flanking region. PCR products derived from human genomic DNA utilizing the primer pairs described above were isolated by agarose gel electrophoresis, the fragments were polished with T4 DNA polymerase and cloned into the *EcoRV* site of the plasmid Bluescript pBSII SK^+ with the *E.coli* host strain XL1-Blue (**Fig. 3-1**), using M13 forward (-21) and reverse (-29) primers and/or SK/KS-primers for sequence analysis.

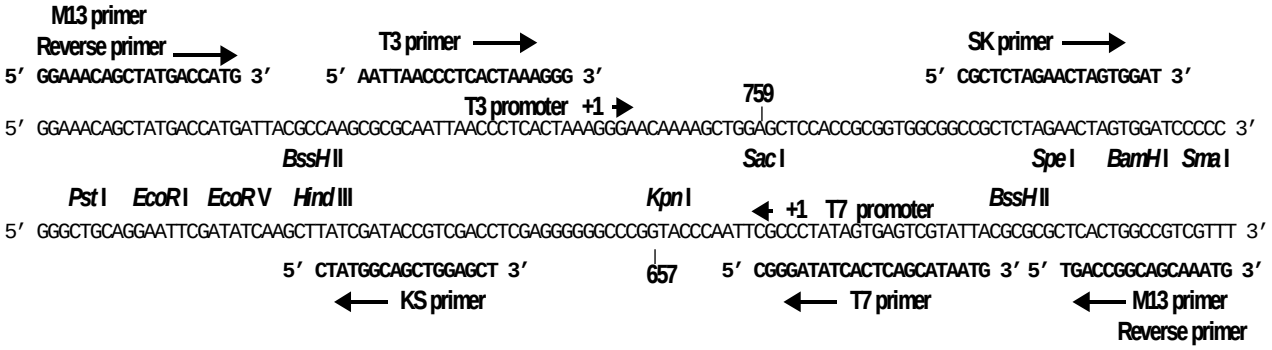


Fig. 2-1 The multiple cloning sites (MCS) of pBluescript II SK (+/-) phagemid (a 2961 bp phagemid derived from pUC19). The SK designation indicates the polylinker is oriented such that *lac Z* transcription proceeds from *Sac* I to *Kpn* I.

Alternatively, the *TOPO TA Cloning Kit* (Invitrogen Inc, USA) was used (**Fig. 3-3**), *Taq polymersae* used to generate PCR products has a nontemplate-depentent terminal transferase activity which adds a single deoxadenosin (A) to the 3' ends of PCR products, the linerized *pCR-TOPO vector* has single overhanging 3' deoxthymidine (T) residues, this allows PCR inserts to ligate efficiently with the vector. Sequence analysis was carried out with either M13 forward (-21) or reverse (-29) primers. Sequencing reactions were performed with fluorescently labelled dideoxyterminators using the *PRISM Ready Reaction Dye Terminator Kit* (PE Applied Biosystems, Weiterstadt, Germany) with minor modifications to the supplied manual. Cycle sequencing was carried out in a GeneAmp PCR System 9600 / 9700 (PE Applied Biosystems, Weiterstadt, Germany).

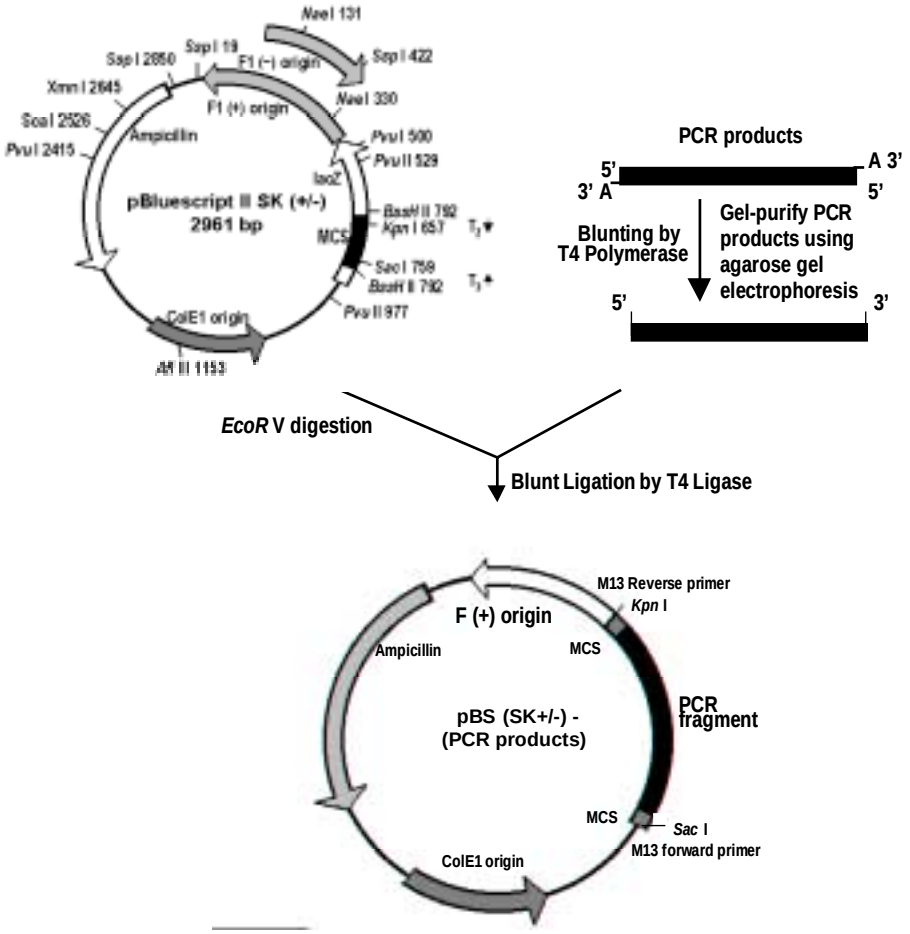


Fig. 2-2 Cloning strategy for PCR products obtained from low melting agarose gel electrophoresis, the fragments were blunted with T4 DNA polymerase and cloned into the *EcoRV* site of the plasmid Bluescript pBSII SK^+ with the *E.coli* host strain XL1-Blue.

After an initial 3 min denaturation step at 96°C, 30 temperature cycles were carried out consisting of 10 sec at 96°C, 5 sec at 55°C and 4 min at 60°C. Cycle sequencing reactions were purified by CentriSep columns and run on an ABI 310 Genetic Analyzer (PE Applied Biosystems, Weiterstadt, Germany). The *DNASTAR* computer program (DNASTAR Inc., Madison, WI, USA) was used for sequence analysis and DNA/Protein homology searches.

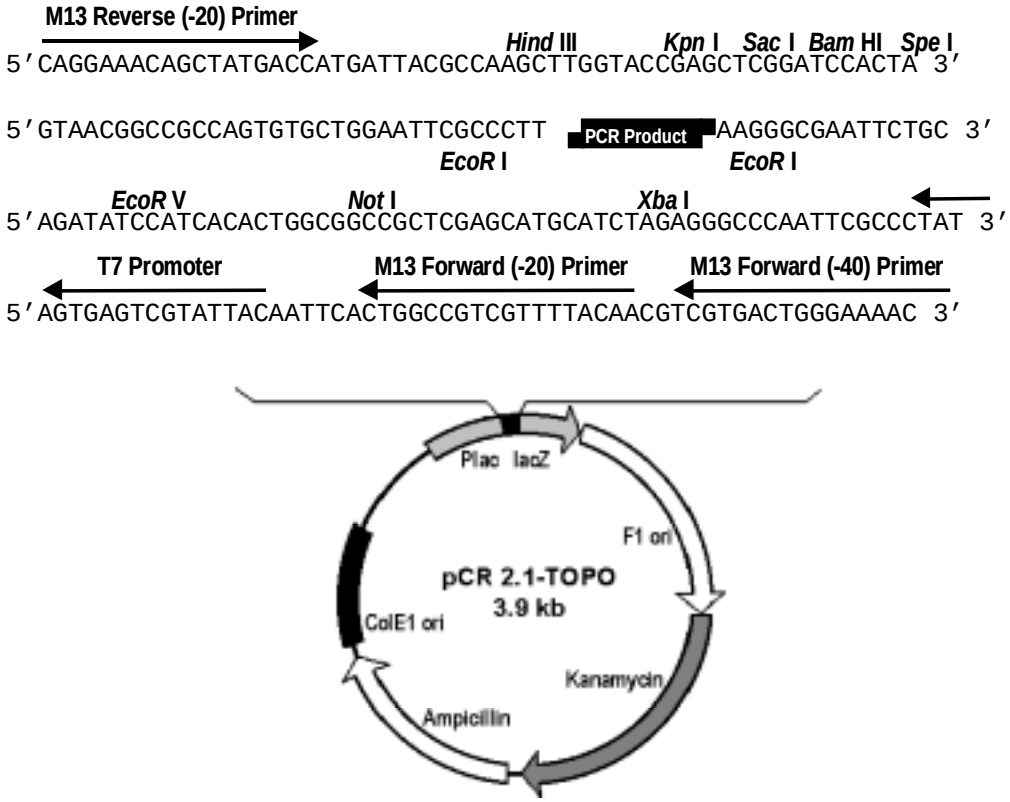


Fig. 2-3 Cloning strategy, PCR products were purified by low melting agarose gal, and then directly cloned into the polylinker region (MCS) of plasmid pCR 2.1-TOPO vector (3.9 kb) with the *E.coli* host strain XL1-Blue by using *TOPO TA Cloning Kit* (Invitrogen Inc, USA).

2.2 5'/3' - RACE (Rapid Amplification of cDNA Ends) of the human UCP –2 and -3 gene

2.2.1. 5'/3' RACE of hUCP2

To determine the 5' untranslated region of human UCP2 cDNA, 5'-RACE was carried out using human skeletal muscle Marathon ready cDNA (Clontech, USA) for mapping the transcriptional start site.

Primers No. (Forward / Reverse)	**Primer sequences for 5' / 3' RACE (Forward / Reverse)**	**Location at the Genes**	**PCR product size** (bp)
# 1133 / # Ap1	5'- AGCCCCAAGAAACTTCACAGT-3' / 5'-CCATCCTAATACGACTCACTATAGGGC -3'	Exon3 of hUCP2 The Adapter	502
# 1134 / # Ap2	5'- ACCCAACCATGATGCAGATTTCCTGCTAC-3'/ 5'- ACTCACTATAGGGCTCGAGCGGC-3'	Exon3 of hUCP2 The Adapter	438
# 1142 / # Ap1	5-'TGGTCATACTATGTGTCCGAGCCGCA -3'/	Exon2 of hUCP2	374
# 1139 / # Ap2	5'- CAGTGCGTGCGGCTGTGTCTGTCGGCT -3'/	Exon1 of hUCP2	289
# 1143 / # Ap1	5'-ATGGCTGCCTGCACTTCCCGAGAGGCT -3'/	Exon8 of hUCP2	338
# 1144 / # Ap2	5'-TGAGCCTCTCCTGCTGCTGACCTGATC -3'/	Exon8 of hUCP2	283

Tab. 2-2 Primer sequences used for 5'/ 3' RACE of human UCP2 gene.

The following primers were employed: the adapter Ap1 and a gene specific primer (GSP1) # 1133 in the first PCR, the nested PCR was performed using 1µl of the dilution (1µl of the first PCR product to 50ul dilution) as template DNA with the nest adapter primer Ap2 and a upstream internal gene specific primer (GSP2) # 1134 (or # 1139, # 1142). The PCR reaction mix of 50 µl contained: 100 ng of DNA, 25 pmol of each primer, 200µM each of dNTPs, 2.0 mM $MgCl_{2,}$ 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.001% gelatin and 1 unit *Taq* DNA polymerase (MWG, Germany) (9). Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 30 sec/95°C, 30 sec/55°C, 1 min/72°C for 35 cycles, followed by a final extension step of 7 min at 72°C.

To detect functional polyadenylation signals in the 3' untranslated region of human UCP2 gene, the 3'-RACE was performed the condition as same as that of 5'-RACE described

above. The # 1143 was used with Ap1 in the first round PCR. The #1144 was used with Ap2 in the nested PCR.

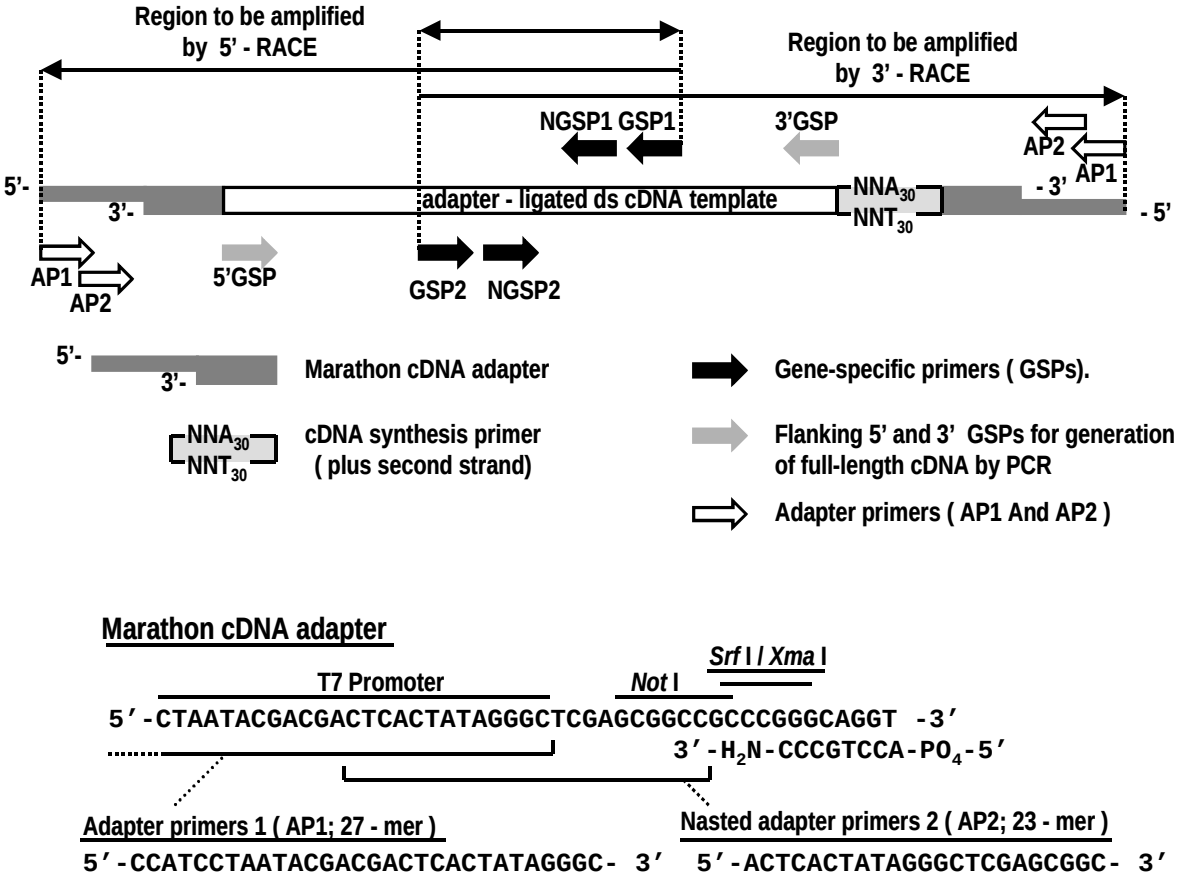


Fig. 2-4 The template and primers used in Marathon RACE reactions and sequences of the Marathon cDNA Synthesis Primer, the Marathon cDNA Adapter, the AP1 and AP2 primers.

2.2.2 5'- RACE of hUCP3 gene

To determine the 5' untranslated region of human UCP3 cDNA, 5'-rapid amplification of cDNA ends (RACE) was carried out using human skeletal muscle Marathon ready cDNA (Clonetech, USA) for mapping the boundaries of the transcripts. The following primers were employed: the adapter and a gene specific primer (GSP1) # 1136 in the first PCR, nested PCR was performed using 1ul of a 1:50 dilution of the first PCR using the nested

adapter primer and an upstream internal gene specific primer (GSP2) # 1137. The PCR reactions were performed in the condition as same as that of 5'- RACE of hUCP2 gene.

Primers No. (Forward / Reverse)	**Primer sequences for 5' / 3' RACE (Forward / Reverse)**	**Location at the Genes**	**PCR product size** (bp)
# 1136 / # Ap1	5'-TGGGAGGCACGTCTGAAGGCTTCAGT -3'/ 5'-CCATCCTAATACGACTCACTATAGGGC -3'	Exon2 of hUCP3	268
# 1137 / # Ap2	5'- AACCATAGTCCTGGAAGGCTCTGCCCAG-3'/ 5'- ACTCACTATAGGGCTCGAGCGGC-3'	Exon2 of hUCP3	227
#1148 / # Ap1	5'- GGTGAGCCTCCTCCTGCCTCCACAC- 3'	Exon6 of hUCP3S	~
#1149 / # Ap2	5'- CCCTCCCAGAGAACAGGGGCTTC- 3'	Exon6 of hUCP3S	~1.2 kb
#1151 / # Ap1	5'- CTATGAGCAGCTGAAACGGGCCCTGATGA- 3'	Exon7 of hUCP3L	~
#1152 / # Ap2	5'- AGACAAGAAGGCCACTGG- 3'/	Exon7 of hUCP3L	~
# 1158 / # Ap2	5'- GCGGTGAAGAGTACAGATGTAATGCCACA- 3'/	Exon6 of hUCP3S	~1.1 kb

Tab. 2-3 Primer Sequences used for 5'/ 3' RACE of human UCP3 and UCP3 gene.

2.3. Determination Structure Variant in the 3'-UTR of Human UCP3 Gene

2.3.1. 3'- RACE of human UCP3 gene

To detect functional polyadenylation signals in the 3' untranslated region of human UCP3 gene long and short form, the 3'-RACE was performed, respectively. The # 1151 was employed with Ap1 in the first round PCR for human UCP3 long form. The # 1152 was used with Ap2 in the nested PCR. The # 1148 was employed with Ap1 in the first round PCR for

human UCP3 short form. The # 1149 was used with Ap2 in the nested PCR. The PCR was carried out in the condition as same as that of the 5'-RACE described above.

2.3.2. Elucidation the 3'-UTR of human UCP3 short form localization in the intron 6.

Primer combination # 1153 and # 1185 were utilized to amplify the intron 6 of human UCP3 gene, with the *Expand™ Long Template PCR System* (Boehringer Mannheim). PCR parameters were: 2 min denaturation at 94°C, 35 temperature cycles were carried out consisting of 94°C for 10 sec, 65°C for 30 sec, 68°C for 3 min (cycles 1 - 10) with elongation times increasing 20 sec/cycle (cycles 11 - 35), followed by a final extension step of 7 min at 68°C.

The amplification products were visualized on 1.0 % agarose gels stained with ethidium bromide. Samples were processed in a *GeneAmp PCR System 9600* (PE Applied Biosystems, Weiterstadt, Germany).

Primers No. (Forward / Reverse)	**Primer sequences for the intron 6 of human UCP3 long form**	**Location at the Genes**	**PCR product size** (bp)
# 1153 / # 1185	5'-GTACTTCAGCCCCCTCGACTGTATGAT -3' 5'- TCAAAACGGTGTTCCCGTAACATCTGGAC- 3'	Exon6 of hUCP3 Exon7 of hUCP3	~1.8kb
Primer sequences for the RT-PCR of human UCP3			
# 1315 / # 1316	5'-GCCTACAGAACCATCGCCAGGG-3' 5'-GCTCCAAAGGCAGAGACAAAGTG-3'	Exon4 of hUCP3 Exon6 of hUCP3	~190

Tab. 2-4 Primer Sequences used for RT-PCR and amplification the inton 6 of human UCP3 long form.

2.4. RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

RT-PCR was carried out to detect the level of endogenous expression of UCP –2 and -3 in Hep-G2, NIH 3T3, CV-1, C_2C_{12}, Hela and GH_4-C1 culture cell lines. mRNA isolated from the varoius culture cells using the *µMACS mRNA Isolation Kit* (Miltenyi Biotec, Germany). The two primer combinations # 1087 / # 1090 and # 1093 / #1101 (see **Tab. 4** for the sequences) located in the coding region of UCP2 gene were utilized. in RT-PCR to test for the endogenous level of UCP3 expression in the six cell lines. The primers #1315 and #1316 that are located in exon 4 and 6 of the hUCP3 gene, respectively, were utilized Aliquots of 200 ng of mRNA were subjected to reverse transcription for 30 min at 42^{o}C using *Titan^{TM} One Tube RT-PCR System* (Boehringer Mannheim, Germany). RT-PCR amplification was performed as follows: 2 min denaturation at 94°C, 35 temperature cycles were carried out consisting of 94°C for 30 sec, 55°C for 30 sec, 68°C for 30sec (cycles 1 - 10) with elongation times increasing 5 sec/cycle (cycles 11 - 35), followed by a final extension step of 7 min at 68°C.

The *Perkin-Elmer GeneAmp RNA PCR Kit* (PE Applied Biosystems, Germany) was used in RT-PCR for UCP3. Aliquots of 200 ng of mRNA was reverse transcribed for 45 min at 60^{o}C. PCR profile times and temperature were as follows: 2 min denaturation at 94°C, 40 temperature cycles were carried out consisting of denaturation at 94°C for 15 sec, 55°C for 45 sec for annealing and elongation, followed by a final extension step of 7 min at 68°C. The amplification products were visualized on 1.5 % agarose gels stained with ethidium bromide. Samples were processed in a GeneAmp PCR System 9600 (PE Applied Biosystems, Weiterstadt, Germany). The amplification products were analyzed on 1.5 % agarose gels stained with ethidium bromide.

2.5. Molecular cloning of promoters of human UCP –2 and –3 gene

2.5.1 Elucidation the 5' flanking region of the human ucp2 gene by screening a human genomic library

A *Human Genomic Library in the Lambda FIX II Vector* (Stratagene. USA) was used in this study. The Lambda phage FIX II vector containing active *red* and *gam* genes are unable to grow on host stains that contain P2 phage lysogens such as XL1-Blue MRA (P2). The *red* and *gam* genes in the Lambda FIX II DNA are located on the stuffer fragment, therefore, the wild-type Lambda FIX phage cannot grow on XL1-Blue MRA (P2). when the stuffer gragment is replaced by an insert, the recombinant Lambda FIX II becomes *red* $^{-}$/*gam*$^{-}$ and is able to grow on the P2 lysogenic strain. The cloning region of the vector is flanked by T3 and T7 bacteriophage promoters (see **Fig. 2-5**).

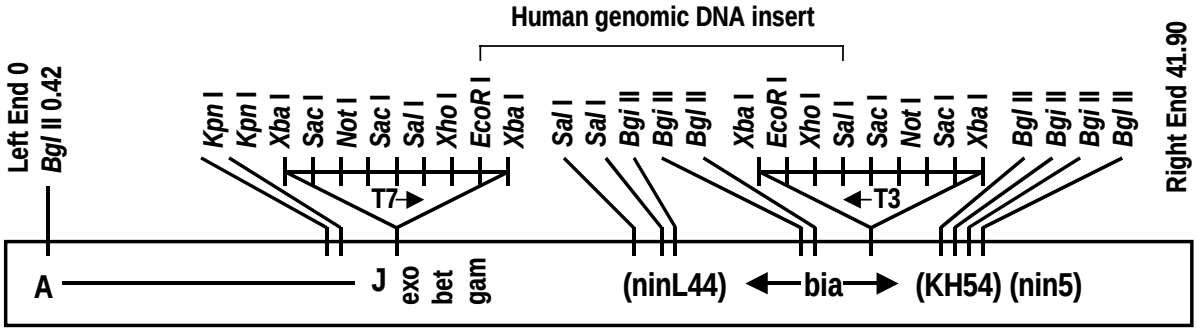


Fig. 2-5 Map of the Lambda FIX II replacement vector.

After titering the library was plated at a density of 50,000 pfu/plate with 600 µl of OD_{600} = 0.5 XL1-Blue MRA (P2) host cell/plate and 6 ml top agar on two large 150-mm NZY plates. From plate lysates approximately $1X10^6$ recombinants were used for preparation bacteriophage DNA, which was subsequently used in polymerase chain reaction (PCR) as template DNA. Based on the sequence analysis resulting from the 5'RACE of human UCP2

gene, 2 sets of gene specific primers were designed and used, the primer # 1207 and the primer T3, the nested gene specific primers #1208 (or # 1140, # 1182) and the primer T3 (see **Tab. 2-5**) were utilized in the first PCR and the nested PCR, respectively, with the *Expand™ Long Template PCR System* (Boehringer Mannheim). Samples were processed in a GeneAmp PCR System 9600 (PE Applied Biosystems, Weiterstadt, Germany). After 2 min denaturation at 92°C, 35 temperature cycles were carried out consisting of 92°C for 10 sec, 65°C for 30 sec, 68°C for 3 min (cycles 1 - 10) with elongation times increasing 20 sec/cycle (cycles 11 - 35), followed by a final extension step of 7 min at 68°C. The amplification products were visualized on 0.8 % agarose gels stained with ethidium bromide.

Primers No. (Forward / Reverse)	**Primer sequences for the library screening (Forward / Reverse)**	**Location at the Genes**	**PCR product size** (bp)
# 1207 / # T3	5'- CTGGGGCCAGGCTCACCGAGCCGA-3' 5'- AACCCTCACTAAAGGG-3'	Intron1 of hUCP2 The right arm of Lambda FIX II	~ 3.5
# 1208 / # T3	5'- CTCACCGAGCCGCAGGGAGAACAC-3'/	Exon1 of hUCP2	~ 3.5
# 1140 / # T3	5'-CAGTGCGTGGGCTGTCTGTCGGCT -3'/	Exon1 of hUCP2	~ 3.4
# 1182 / #T3	5'-ACGAGCCGGGCGAGCGTGGACAGTCAATC -3'/	Exon1 of hUCP2	~ 3.4

Tab. 2-5 Primer sequences used for PCR-screening the Human genomic Lambda FIX II Library cloning of hUCP2 gene promoter.

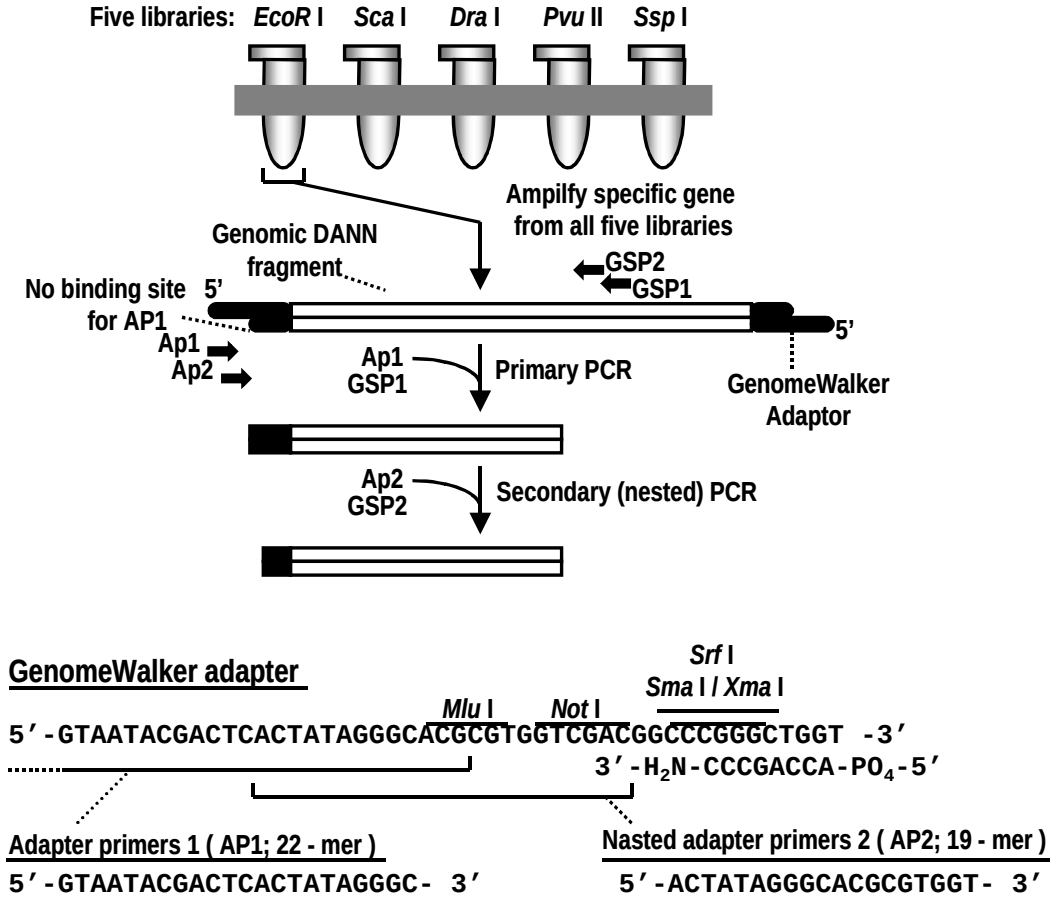


Fig. 2-6 General principle of the GenomeWalker kit and sequence of the GenomeWalker Adapter and adapter primer. The Adapter has been ligated to both ends of the genomic DNA fragment in all five GenomeWalker Libraries with kit.

2.5.2. Genome Walking of 5'-Flanking Region of Human UCP3 Gene

Cloning of the 5'-flanking region of the human UCP3 gene was performed using the*GenomeWalker Kit* for human (Clontech, USA) according to the manufacturer's recommendation. PCR amplifications were carried out with all six different libraries (*EcoR* I, *Sca* I, *Dra* I, *Pvu* II and *Ssp* I) utilizing the following primers: the adapter primer Ap1(G) (5'-GTAATACGACGACTCACTATAGGGC-3') and the gene-specific primer #1146 (5'-ACAG-GGGCTCCCTAGGGCTCCATG-3') in the first PCR; the adapter primer Ap2(G) (5'-

ACTA-TAGGGCACGCGTGGT-3') and the nested gene-specific primer #1184 (5'-AGGTGGCA-GCAGGGATTGGATGGCCCCTCC-3') for the second PCR. PCR conditions were as follows: 2 min denaturation at 92°C, followed by 35 temperature cycles consisting of 10 sec at 92°C, 30 sec at 65°, 3 min at 68°C (cycles 1-10) with elongation times increasing 20 sec/cycle (cycles 11-35), and a final extension step of 7 min at 68°C. The amplification products were visualized on 1.0 % agarose gels stained with ethidium bromide.

2.6. Sequences Determination

PCR products derived from the 5'-/3'-RACE, RT-PCR, Gemoe-walking and library PCR-screening were purified by the low melting agarose gel electrophoresis, the fragments were blunted with T4 DNA polymerase and subcloned into the *EcoR* V site of the plasmid *Bluescript pBS II SK*$^+$ (Stratagene, USA) with the *E.coli* host strain XL1-Blue, using M13 forward (-21) and reverse (-29) universe primers and/or SK/KS-primers for sequence analysis. Alternatively, the *TOPO TA Cloning Kit* (Invitrogen, USA) was used; sequence analysis was carried out with either M13 forward (-21) or reverse (-29) universe primers. Sequencing reactions were performed with fluorescently labelled dideoxyterminators using the *PRISM Ready Reaction Dye Terminator Kit* (PE Applied Biosystems, Germany) with minor modifications to the supplied manual. Cycle sequencing was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, Germany). After an initial 3 min denaturation step at 96°C, 30 temperature cycles were carried out consisting of 10 sec at 96°C, 5 sec at 55°C and 4 min at 60°C. Cycle sequencing reactions were purified by CentriSep columns and run on an ABI 310 Genetic Analyzer (PE Applied Biosystems, Weiterstadt, Germany). The *DNASTAR* computer program (DNASTAR Inc., USA) was used for sequence analysis and DNA/Protein homology searches.

2.7. Promoter function analysis of human UCP –2 and -3 gene using pCAT-3 reporter gene system

2.7.1. pCAT®-3 reporter vectors

Promoter activity in transfected eukaryotic cells is generally studied by linking the promoter sequence to a gene encoding an easily detectable "reporter"- protein. The bacterial enzyme chloramphenicol acetyltransferase type1(CAT), which has no eukaryotic equivalent, has become one of the standard markers used in transfection experiments with eukaryotic cells. The plasmids used in the *in Vitro* transient expression assays for the promoter deletion analysis in human UCP2 gene were constructed utilizing the CAT reporter gene fusion expression system. The pCAT basic and enhancer vector (Promega, USA) is a promoterless CAT expression vector. The pCAT control vector contains a SV40 virus promoter, which was used to monitor transfection efficiency in each cell line and as an internal standard between different experiments.

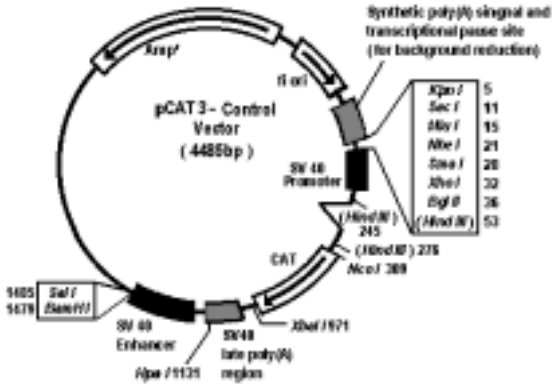


Fig. 2-7 Map of the pCAT®-3 Control Vector with the SV 40 promoter. Additional description: position of intron; CAT, cDNA encoding the chloramphenicol acetyltransferase

gene; Amp^r, gene conferring ampcillin resistance in *E. coli;* f1 oil, origin of replication derived from filamentous phage; ori, origin of plasmid replication in *E. coli*. Arrows within CAT and the Amp^r gene indication of transcription; the arrow in fi oil indicates the dorection of ssDNA strand synthesis. Restriction sites shown in parentheses are not unique sites.

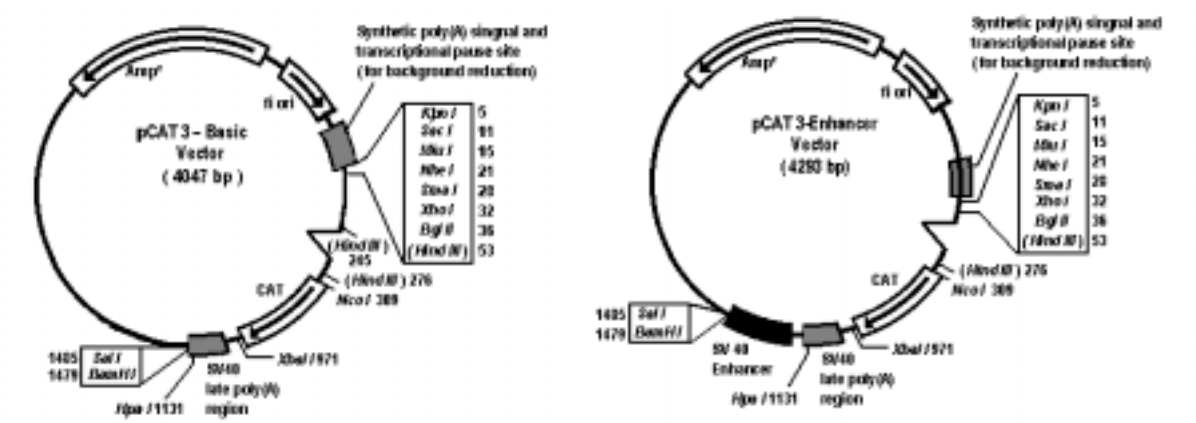


Fig. 2-8 The pCAT ®-3 Basic and Enhancer Vector maps.

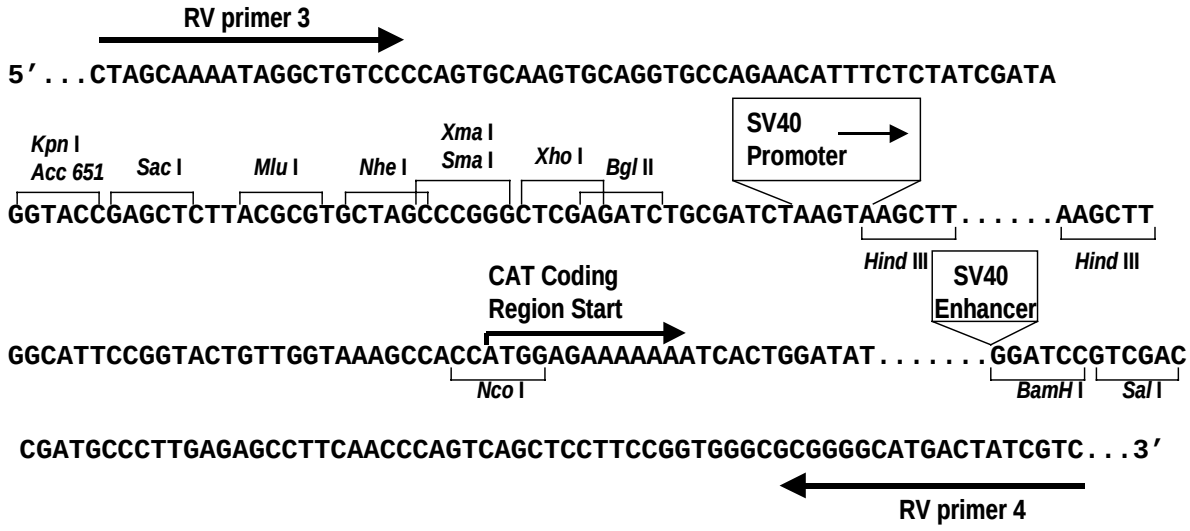


Fig. 2-9 Multiple cloning site of the PCAT ®-3 vector. Shown are the upstream and downsream cloning sites and the locations of the sequencing primers RVprimer3 and RVprimer4. The position of the promoter (pCAT ®-3 Control Vector) and the enhancer (in the pCAT ®-3 Enhancer and pCAT ®-3 Control Vectors) are shown as insertions into the sequence of pCAT ®-3Basic Vector. The sequence shown is of the DNA stand generated from the f1 ori.

2.7.2. hUCP2-pCAT promoter fusion plasmid constucts

3.3 kb of the 5' flanking region of human UCP2 gene was moved from *pCR-2.1 TOPO Vector* into pCAT ®-3 Enchancer Reporter Vector. 14 different hUCP2 promoter-CAT fusion constructs were generated for functional analysis of the human UCP2 promoter by transient expression assays.

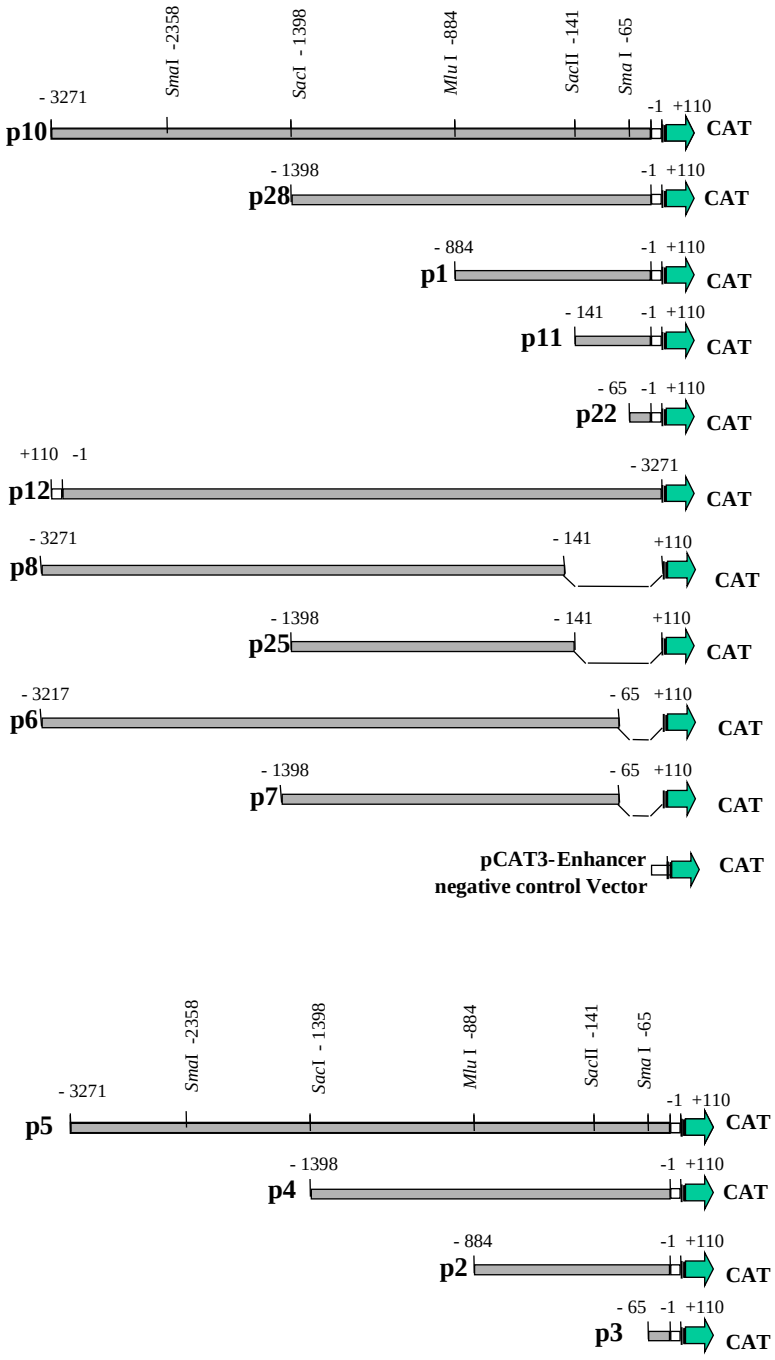


Fig. 2-10 Schematic representation of the human uncoupling protein 2 promoter-CAT fution plasmids (in the pCAT®-3 reporter enhancer and basic vectors, respectively).Numbers show the nucleotide positions relative to the tanscription-initiation site numbered as +

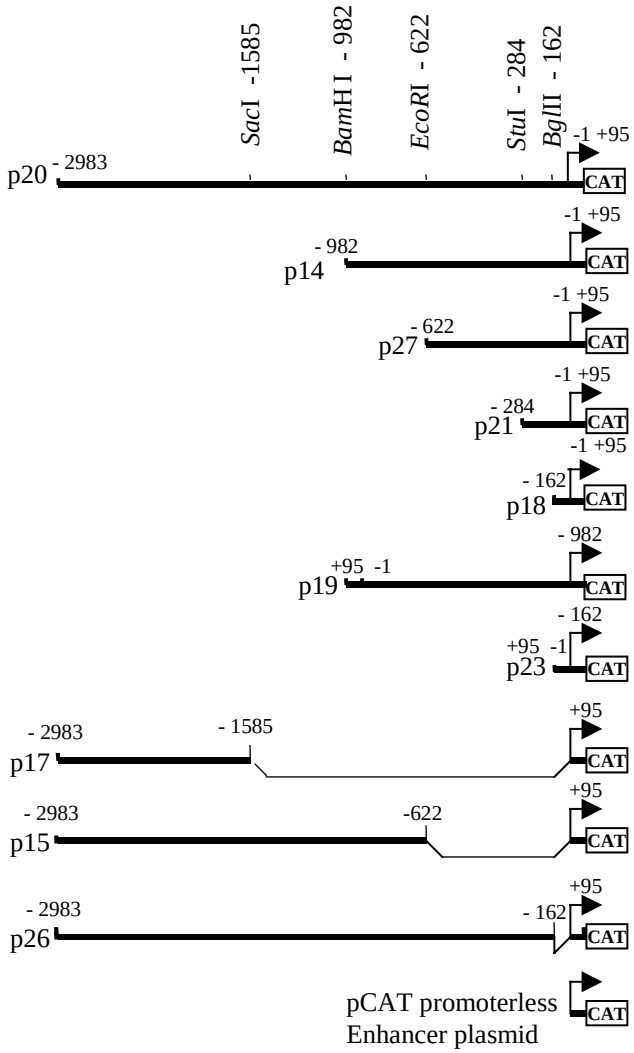


Fig. 2-11 Schematic representation of the human uncoupling protein 3 promoter-CAT fusion

enhancer plasmids Numbers show the nucleotide positions relative to the tanscription-initiation site numbered as +1.

2.7.3. hUCP3-pCAT fusion plasmids constructions

All hUCP3 promoter fragments were inserted into the *pCAT-3 reporter enhancer vector* (Promega, USA) which is a promoterless CAT expression vector. The *pCAT-3 reporter control vector* (Promega, USA) which contains a SV40 virus promoter was used to monitor transfection efficiencies in each cell line and served as an internal standard between the different experiments. The *pCAT-3 basic vector* (Promega, USA) served as a negative control.

2.7.4. Ultrapure plasmid DNA purification

All the constructed plasmids were purified using *the QIAfilter Plasmid Kit* (QIAGEN, USA) for high-quality, low-cellular toxicity DNA.

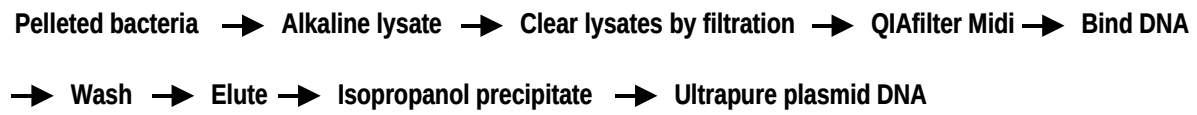


Fig. 2-12 Flow chart for the ultrapure preparing plasmid DNA purification using the *QIAfilter Plasmid Kits.* This protocol is based on a modified alkline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

2.7.5. Confirmation the sequences of each hUCP2-pCAT constructs.

The inserts of various lengths promoter in the constructs were confirmed by sequence analysis using the RVprimer3 and RVprimer4 primers (Fig. 10) . Sequence analysis as described proviously.

2.8 Cell culture and in vitro transient transfection

2.8.1. Cells culture

Transient expression assays of the human UCP2 promoter-CAT constructs were carried out in 6 different cell lines. Hela (human cervix carcinoma) cells were cultured in modified Eagle's medium (MEM), GH4-C1 (rat pituitary adenoma, Interlab Cell Line Collection, Genova, Italy) cells were cultured in Ham's F-10 medium, NIH-3T3 (Swiss mouse embryonic fibroblastes) and HepG2 (human hepatocellular carcinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM); CV-1 (Africa green monkey, kidney) cells were cultured in RPMI 1640 medium. All media were supplemented with 10% (v/v) FCS and 100units/ml penicillin and 100ug/ml streptomycin. All cell lines were incubated at 37°C, 5% CO_2 and 95 % humidity.

For differentiation into myotubes C_2C_{12} myoblasts were maintained in DMEM containing 10% FCS; when cells reached confluency, the medium was switched to a medium containing DMEM supplemented with 2% horse serum. Medium was changed every second day, after four additional days, the differentiated C_2C_{12} cells had formed myotubes. They were then subjected to transfections. Transient transfection

2.8.2. transient transfection

For transfection cells were plated at $2X10^5$ cells in 35-mm collagen-coated tissue culture dishes. 24h after seeding, the culture was washed extensively to remove non-

adherent cells and the medium was replaced. Transfections were carried out with *FuGENE 6 Transfection Reagent* (Boehringer Mannheim, Germany), according to the manufacturer's recommendation. Briefly, 2 µl (4 µl) *FuGENE 6 Reagent* were diluted with 98 µl serum free medium, after incubated for 5 min at room temperature, and then added to a tube containing 0.5 µg (2 µg) of plasmid DNA of the corresponding hUCP2-CAT (hUCP3-CAT) fusion constructs. After a 30 min at room temperature, the mixture was then added dropwise to the cells for transfection. 24 h after transfection, the medium was replaced and culturing was continued for an additional 24h. The CAT-ELISA and protein determination assays were performed 48h post-transfections.

2.9. CAT-ELISA and protein determination

2.9.1. CAT-ELISA (Enzyme-Linked Immunosorbent Assasy)

Traditionally, CAT (chloramphenicol acetyltransferase type 1) activity is measured using a radioactivity CAT assay. However, the determination of CAT levels using the CAT-ELISA has been shown to produce a sensitivity comparable to that of isotopic protocol.

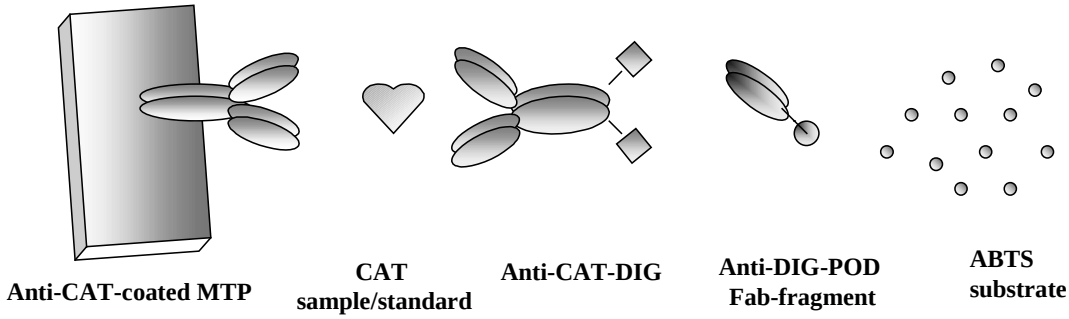


Fig. 2-13 Principle of the CAT ELISA**.** The CAT-ELISA is based on the sandwich ELISA principle. Antibodies to CAT (anti-CAT) are probound to the surface of the microtiter plate

modules (MTP modules). Following lysis of the transfected cells, the cell extracts are added to the wells of the MTP modules, all the CAT contained in the cell extracts binds specifically to the anti-CAT antibodies, next, adigoxigenin-labed antibody to CAT (anti-CAT-DIG) is added and binds to CAT. Then an antibody to digoxigenin conjugated to peroxidase (anti-DIG-POD) is added and binds to digoxigenin. In the final step, the peroxidase substrate ABTS is added. The peroxidase enzyme catalyzes the cleavage of the substrate yielding a colored reaction product. The absorbance of the sample is determined using a microtiter palt (ELISA) reader and is directly correlated to the level of CAT present in the medium supernatant. The sensitivity of the assay is enhanced by using the peroxidase substrate (ABTS) with substrate enhancer.

CAT-ELISA kit (Boehrunger Mannheim, Germany) was used in this study. 48-72 h after the transfections, the culture medium was carefully removed and cells were washed with 2 ml of pre-cooled PBS three times. Added 1 ml of lysis buffer to each dish to stand for 30 min at room temperature. Spined the cell extract in a microfuge at maximum speed for 10 min to remove the cellular debris. After the preparing the standard working dilution series, pipetted 200 µl of 200 cell extracts or 200 µl of CAT standard working dilution per well of the microtiter plate (MTP), the MTP with cover foil was incubated for 1 h at 37°C. Removed the solution and rinse wells 5 times with 250 µl of washing buffer for 30 sec each. Pipetted 200 µl of anti-CAT-DIG working dilution per well and incubate for 1 h at 37°C. then remove the solution, and rinse wells 5 times with 250 µl of washing buffer for 30 sec each. Pipetted 200 µl of anti-DIG-POD working solution per well and incubate for 1 h at 37°C. Removed the solution, and rinse wells 5 times with 250 µl of washing buffer for 30 sec each. 200 µl of POD substrate with substrate enhancer into each well incubate at room temperature for 4 – 10 min, then. Photometric detection was carried out with a *MR 7000 Microplate Reader*

(DYNATECH, Germany). Data are represented as the means ± S.D. of triplicate assays from six independent experiments.

2.9.2. Protein determination.

The results from CAT-ELISA were normalized with respect to protein concentration and cell number. Protein concentrations were determined with the *Protein Assay ESL* (Boehringer Mannheim, Germany). Spectrophotometric determination was carried out in the linear range of the calibration curve.

2.10. Polymorphism Analysis

2.10.1. Recruitment of index probands and controls

For a total of 68 obese and 104 non-obese children in the age of 7-13 years ascertained in Trier and Kaufbeuren by school physicians, pediatricians and via advertisements in local newspapers, Resting Metabolic Rates (RMR) were determined by indirect calorimetry using the *DELTATRAC MBM-100* ventilated hood system (Hoyer, Bremen, Germany). Probands were grouped according to percent deviation (up to 25% above or below) of their estimated RMR values calculated on the basis of the Harris & Benedict equation. Classification to the phenotype obese/non-obese was performed based on the criteria of Must *et al.* (1991) with BMI centiles ranging from 85-100 for the obese and from 5-85 for the normal weight children. Written informed consent was obtained from all participants (in all cases their parents). The investigation was approved by the ethics committee of the University of Trier.

2.10.2. Genomic DNA Isolation

EDTA anticoagulated venous blood samples were collected from index probands and controls. Leukocyte DNA was isolated by the salting out procedure according to Miller *et al*. (1988) or with a commercially available kit of GENTRA Systems Inc., Minneapolis, USA.

2.10.3. Polymorphism detection

Index probands (25) with significantly reduced RMR were selected for a first polymorphism analysis of the hUCP2 gene. PCR amplification of each individual exon (coding region including 3'-UTR) was performed from genomic DNA. PCR products were subsequently analyzed for possible polymorphisms by direct sequence analysis using automated DNA sequencing as described above. In case of the insertion polymorphism PCR products from exon 8 were first subcloned using the *TOPO TA Cloning Kit* (Invitrogen Inc., USA). For sequence analysis M13 forward (-21) and reverse (-29) primers were used.

2.10.4. Genotyping

2.10.4.1. Base transition in exon 4 (Ala55Val)

Genotyping for the distribution of the Ala55Val polymorphism in the hUCP2 gene was performed by allele-specific PCR (ASP) and/or direct sequence analysis. Allele-specific PCR (ASP) was carried out in a volume containing 100 ng DNA, 10 pmol of each primer (primer combination #1129 / #1130 for the wildtype allele, and #1129 / #1131 for the mutant allele, respectively; see **Tab. 3-6** for sequences). Samples were processed in a GeneAmp PCR 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 30 sec at 95°C, 30 sec at 61.5°C and 30 sec at 72°C for 35 cycles, followed by a final extension step of 7 min at 72°C. After PCR, aliquots were run on 1.5% agarose gels to determine the respective genotype.

2.10.4.2. Insertion polymorphism (3'-UTR)

PCR reaction mix of 50 µl contained: 100 ng of DNA, 25 pmol of each primer (#1203 / #1204), 200µM each of dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.001% gelatin and 1 unit *Taq DNA polymerase* (MWG, Germany). Samples were processed in a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Weiterstadt, Germany). After 7 min denaturation at 95°C, 35 temperature cycles were carried out consisting of 95°C/30 sec, 55°C /30 sec, 72°C/1 min for 35 cycles, followed by a final extension step of 7 min at 72°C. The PCR amplification products were visualized on 2.0 % agarose gels stained with ethidium bromide. Genotyping was performed according to the size of the resulting PCR product (301 bp versus 256 bp for the insertion polymorphism being present or absent).

Primer No.	**Primer sequences**	**Location at the cDNA level, necletide position (5'-3')**
# 1129	5'- TGGATACTGCTAAAGTCCGGTTA -3'	(within the intron 3)
# 1130	5'- ACCGCGGTACTGGGCGCTGG -3'	183 / 164
# 1131	5'- ACCGCGGTACTGGGCGCTGA -3'	183 / 164
#1203	5'- GAGCAGCTGAAACGAGCCCTCAT -3'	3'- UTR
#1204	5'- TGTCAACTCCACCAGCACTGAGAC - 3'	3'- UTR

Tab. 2-6 Primer sequences for allele-specific PCR and the insertion polymorphism analysis.

Chapter 3. Research Objectives

1. Determination of the genomic organization of the human UCP2 and UCP3 genes;

2. Comparison of the human UCP1, UCP2 and UCP3.

3. Mutation and polymorphism analysis in human UCP2 gene; Evaluation of the genotypes (allele frequencies, genotype/phenotype correlation).

4. Mapping the boundaries of hUCP2 mRNA and human UCP3L / UCP3S mRNA by 5'- / 3'- RACE (Rapid Amplification of cDNA Ends)

5. Comparison of uncoupling protein gene family in human and rodents.

6. Identification of the potential transcription initiation site and determination of the polyadenylation signal in the 5'- / 3'-UTR of human UCP2 and UCP3L / UCP3S cDNAs.

7. Cloning and characterization the promoter region of the human UCP –2/-3 gene by screening a human genomic DNA library and Genome walking.

8. Functional characterization of the 5'-flanking region and the promoter region of the human UCP –2/-3 gene

Chapter 4. Genomic Structure and Mutational Analysis of the Human UCP2 gene.

In order to better understand a possible implication of human UCP2 in the development of human obesity, the genomic structure of the hUCP2 gene has been deduced utilizing PCR and direct sequence analysis, and genetic variants which may be of functional relevance are identified.

4.1. Result

4.1.1. Genomic Organization of the Human UCP2 Gene

4.1.1.1 Demonstration the location of exon /intron bondaries

Sequence analysis confirmed the presence of introns and defined the exact location of the exon/intron boundaries. The hUCP2 gene (coding region plus 5'-/3'- flanking regions) spans over 8.7 kb, distributed on 8 exons and 7 introns. Intron 1 and intron 2 located in the 5'-untranslated region. The size and location of each exon and intron are shown in **Tab. 4-1**. The AG / GT splice site consensus sequence is conserved at the most of junctions.

4.1.1.2. 5'-/3'- Rapid Amplification cDNA Ends (RACE) of human UCP2 gene

To map the boundaries of human UCP2 transcribes 5'-/3'-RACE were carried out for at least 5 times to determine the potential mRNA transcription initiation site and polyadenylation signal (AATAAA) utilizing a preparation of human skeletal muscle cDNA. The longest transcript identified from 5'-RACE contained 364 bp 5'-upstream of the start codon ATG, suggesting that this represents the potential transcription initiation site.

No.	Exon		Sequences	Intron
	Size (bp)	Position	3'acceptor site - 5'donor site	Size (bp)
1	> 104	-361/ -258	.....CGCGCCTTG... .....GGCTCG**gt**gagcc..	~1,400
2	157	-257/ -100	.....cccac**ag**GACACAT..... .....GCCGG**gt**aagag....	~3,000
3	225	-99/ 126	....ttggctt**ag**ATTCCGG..... .....TACAG**gt**gaggg...	156
4	212	127/ 338	.....cttgc**ag**ATCCAAG..... .....GAGCA **gt**gagta....	868
5	194	339/ 532	....cccaca**ga**TGCCAG..... .. .... GAAAG**gt**gtgta....	81
6	102	533/ 634	.....cctacagGGACCTC..... .....GACAG**gt**gagt.....	971
7	181	635/ 815	.....ttggc**ag**ATGACCT..... .....AAAGG**gt**gagc....	369
8	> 450	816/ 1265	....tcctct**ag**GTTCATG ....CTCAACCTTG	

Tab. 4-1 Exon/intron structure of the hUCP2 gene. (Sequence of the exon-intron boundaries and size of the exons and introns sre indicated. Nucleotide position +1 is assigned to the ATG translational start codon as shown in **Fig. 4-1**)

3'-RACE indicated: the 3'-UTR for hUCP2 extends 336 bp downstream of the stop codon followed by the polyadendylation signal. Genomic organization of the human UCP 2 gene (transcripted region) is shown in **Fig. 4-1.**

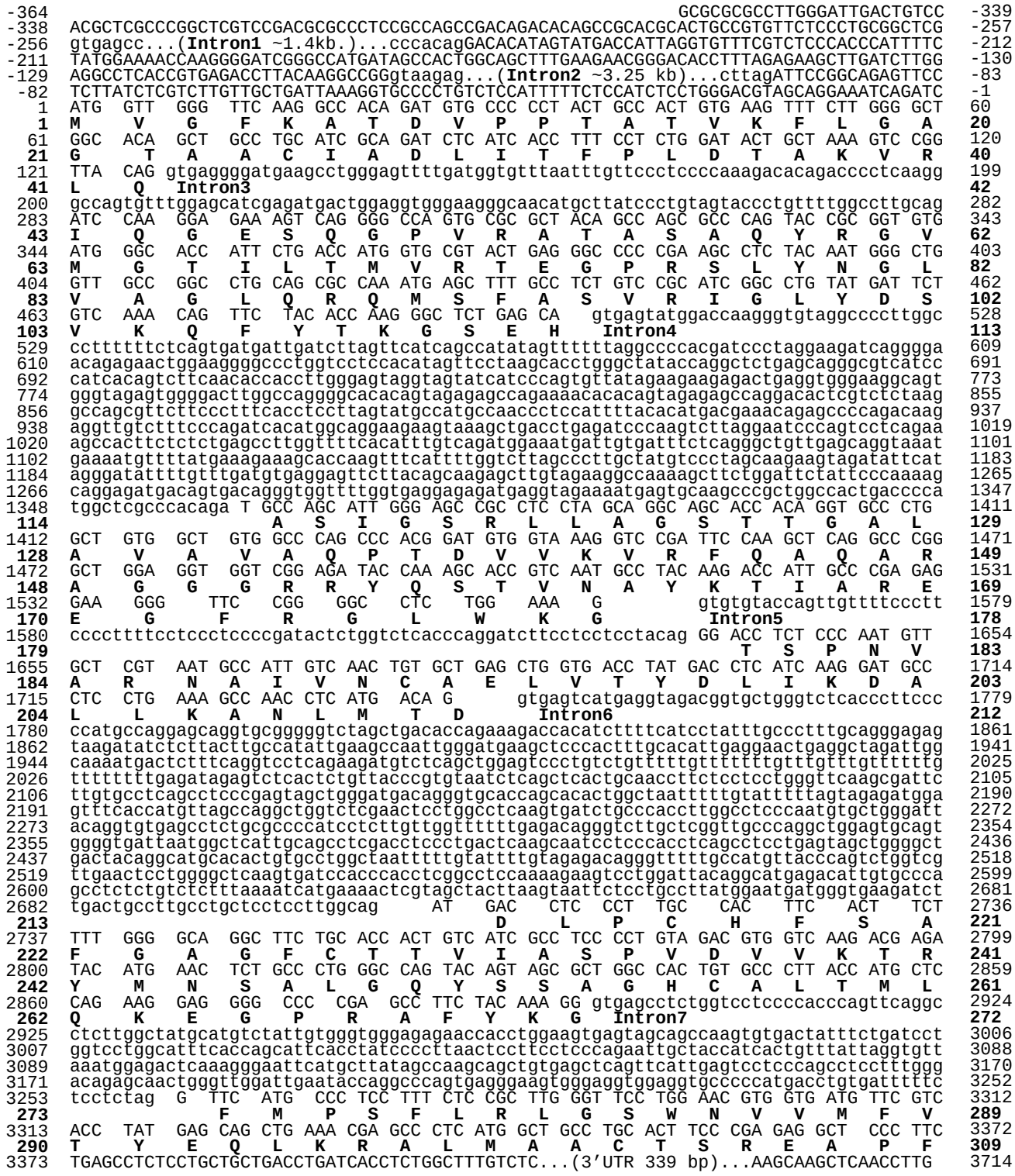


Fig. 4-1 Genomic organization of the human UCP 2 gene**.** The start codon and stop codon are marked in bold. Sequence data have been deposited in the GeneBank under the following accession number AF132536, AF132537, AF132538, AF132539, AF132540.

4.1.1.3. Comparison of UCP2 in human, mouse and rat

At the amino acid level human UCP2 has about 83.3% homology to mouse UCP2 and is 88% identical to rat UCP2, while hUCP2 is 55% identical to human UCP1. Both the human and mouse UCP2 gene are distributed on 8 exons and 7 introns, and the exon/intron structure is exactly matched within the coding regions of gene. The 5'-untranslated region of hUCP2 are interrupted by 2 introns (intron-1/-2), unlike the hUCP1 gene, which consists of 6 exons and 5 introns, no intron located in the 5'untranslated region.

4.1.2. Identification of a point mutation and an insertion polymorphism

4.1.2.1. A transition mutation (C / T) in exon 4

Because genetic variations in members of the human UCP gene family may have effects on energy metabolism, body weight regulation and thermogenesis, we have performed a mutational analysis of the hUCP2 gene. DNA sequence analysis of the hUCP2 gene in a cohort of 25 children (aged 7-13) characterized by significantly reduced RMR (based on the Harris & Benedict equation) revealed a base transition in exon 4 (coding region) consisting of a C to T exchange in codon 55 changing an alanine (GCC) to a valine (GTC).

4.1.2.2 An insertion polymorphism in exon 8

An insertion polymorphism was discovered in exon 8 (3'-UTR) by PCR and sequence analysis, which consisted of a 45 bp repeat located 150 bp downstream of the stop codon in the 3'-UTR (**Fig. 4-2)**.

Fig. 4-2 The insertion polymorphism consists of a 45 bp repeat (boxed residues) located at bp 1077 to 1122 (+1 for the translational start codon ATG), 150 bp downstream of the stop codon TGA in the 3'-UTR of the hUCP2 gene.

4.1.3. Evaluation of the genotypes (Allele frequencies, genotype/phenotype correlations)

4.1.3.1 Allele frequencies

Genotyping the Ala55Val polymorphism in 68 obese and 104 normal weight children revealed allele frequencies of 0.63 for the Alanine and 0.37 for the Valine allele. The allele frequencies for the insertion polymorphism were 0.71 for the wildtype and 0.29 for mutant allele, respectively. No significant differences in allele frequencies were found between the obese and non-obese groups.

4.1.3.2 Genotype/Phenotype correlations

Although a first set of samples indicated a higher frequency of the Valine allele in children with significantly reduced RMR this finding could not be confirmed when the allele frequencies were compared grouped according to RMR:

Group 1 (up to -25% deviation of predicted RMR): p = 0.351 (57)*

Group 2 (no deviation of predicted RMR): p = 0.388 (58)*

Group 3 (up to +25% deviation of predicted RMR): p = 0.360 (57)*

*= number of cases studied, with p-values ≤ 0.01 considered as being significant

The rate of the Ala55Val polymorphism was independent of percent deviation RMR (measured versus predicted). There was no direct effect of the polymorphism and the amount of deviation RMR, only sex had a significant effect on RMR (male >female), p = 0.001; a possible direct interaction of sex and the hUCP2 polymorphism on RMR turned out to be not significant (with p = 0.084).

Similar results were obtained for the insertion polymorphism in the 3'-UTR. So far a direct correlation of the observed genotype with reduced RMR and BMI was not evident.

4.2. Discussion

Uncoupling proteins (UCPs) are mitochondrial membrane transporters which are involved in dissipating the proton electrochemical gradient thereby releasing stored energy as heat. This implies a major role of UCPs in energy metabolism and thermogenesis which when deregulated are key risk factors for the development of obesity and other eating disorders. The recent molecular cloning of two new members of the human UCP gene family, UCP2 and UCP3, somehow has revolutionized obesity research namely because of their potential physiological role as mediators of energy metabolism, body weight regulation and thermogenesis. Flier and Lowell *et al.* (1997) themselves characterized the identification of the UCP2 homologue as a major breakthrough towards understanding the molecular basis for energy expenditure, and considered these findings likely to have important implications for the cause and treatment of human obesity. This is largely due to the fact that hUCP1 containing brown adipose tissue is unlikely to be involved in weight regulation in adult large-

size animals and in humans living in a thermoneutral environment (Fleury *et al*. 1997), primarily because of its very limited abundance. In contrast, UCP2 mRNA is ubiquitously expressed in human and rodents (Flier *et al.,* 1997), implicating a major role in body thermogenesis. UCP3 as a new member of the UCP gene family is preferentially expressed in skeletal muscle and brown adipose tissue; it therefore represents a candidate protein for the modulation of the respiratory control in skeletal muscle (Boss *et al.* 1997). The chromosomal mapping of both hUCP2 and hUCP3 to human chromosome 11q13 in close proximity to each other that have been linked to obesity and hyperinsulinaemia. Norman *et al.* (1997) showed positive linkage data in Pima Indians indicating that this region may contain genetic markers responsible for energy expenditure and body weight regulation, have made hUCP2 and hUCP3 strong candidate genes in the molecular pathogenesis of human obesity.

In order to better understand a possible implication of hUCP2 in the development of human obesity, the genomic structure of the human UCP2 gene has been deduced utilizing PCR and direct sequence analysis, and genetic variants which may be of functional relevance are identified. The hUCP2 genespans 8.4 kb distributed on 8 exons. The potential transcription initiation site located 364 bp upstream of the start codon ATG. At the amino acid level hUCP2 has about 55% identity to hUCP1 while hUCP3 is 71% identical to hUCP2. The localization of the exon/intron boundaries within the coding region matches precisely that of the hUCP1 gene and is almost conserved in the hUCP3 gene as well.

With regard of UCP2 being a potential mediator of energy metabolism and body weight regulation we have screened for possible polymorphisms within the hUCP2 gene. Two frequent genetic variants could be detected; genotyping of these variants (an Ala55Val polymorphism in exon 4 as well as a 45 bp insertion polymorphism in exon 8) in a cohort of 68 obese and 104 non-obese children did not show any significant differences in allele frequencies between the two groups. No direct interaction of these variants with RMR or BMI could be observed. So far a direct correlation of the observed genotype with RMR and BMI

was not evident. In terms of function, the Ala55Val polymorphism in the hUCP2 gene most likely has no effect on membrane potential (D. Ricquier, personal communication). This polymorphsim has recently also been described in other populations, see **Tab. 4-2.**

Study Type	Ascertainment	Alleles	Results	Reference
Linkage	French Canadian	D11S916, D11S1321	RMR P<0.000002, no linkage to BMI	Bouchard *et al.*, 1997
	American Caucasian NIDDM	7 Markers on chromosome 11	No linkage to BMI or WHR	Elbein *et al.*,1997
Association	Pima Indians	UCP2 (A55V)	Sleeping Metabolic Rate with A55V	Walder *et al.*, 1998
	Japanese NIDDM or obese	UCP2 (A55V)	No association with NIDDM or obsity	Kubota *et al.*, 1998
	American Caucasian and African American	UCP2 (A55V)	No significant association	Urhammer *et al.*, 1997
	Obese Swedish with low BMR and healthy controls	UCP2 (A55V)	No significant association with BMB	Argyropulos *et al.*, 1998
	French NIDDM, morbidly obese or healthy control	6UCP2 alleles, including A55V, and exon 8 insertion / deletion	No significant association	Klannemark *et al.*, 1998
	Caucasian and African American	UCP3 splice site	Respiratory quotient	Otabe *et al.*, 1998
	Random Danish young adult	UCP3 (G84S)	No significant association with BMI	Urhammer *et al.*, 1998

Tab. 4-2 Summary of linkage and association studies of human UCP-2 and -3 gene polymorphism.

The insertion polymorphism in the 3'-UTR probably has also no obvious functional consequences however it may have an effect on UCP2 mRNA stability. This does not completely exclude a potential role of hUCP2 in energy metabolism and body weight regulation. Study of a further 790 full-blooded Pima Indians revealed that individuals >45 years of age who were UCP2 exon 8 insertion/deletion heterozygotes had lowest body mass indices (BMIs) (p=0.004) (Walder *et al.*, 1998); In contrast, Examination of 966 French people including many morbidly obese for the exon 8 insertion/deletion variant did not reveal any association with weight gain, BMI, RMR, or body composition characteristics (Otabe *et al.*, 1998).

The close proximity of hUCP3 to hUCP2 on chromosome 11q (Pecqueur *et al*, 1999) implies that instead of hUCP2 a malfunctionning of hUCP3 or a deregulation of its gene expression may well be a causative factor for the development of human obesity. Therefore the identification of genetic variants in the hUCP3 gene and its flanking regions will be of great importance to determine whether and to what extent UCP3 may play a role in energy metabolism and thermogenesis.

In a Gullah-speaking African-American woman with severe obesity and type 2 diabetes, Argyropoulos *et al.* (1998) found heterozygosity for a Val102Ile (V102I) mutation of the UCP3 gene, located in the first cytosol-oriented extramembranous loop. Three overweight children in this family were found to be homozygous for the V102I polymorphism. The fourth child, a 9-year-old male with a body mass index (BMI) of 18.5, was heterozygous for the V102I polymorphism. No paternal sample was available but the father was presumed to be at least heterozygous for the V102I polymorphism. The polymorphism was not found in genomic DNA from 128 Caucasian Americans. However, examination of 280 African Americans revealed that 4% of individuals were homozygous and 28% heterozygous for the polymorphism.

Fragment	Position	Alteration	Method
Exon 3	Codon 93	C/T (Ser93Ser)	SSCP/Sequencing
Exon 3	Codon 102	G/A (Val102Ile)	Direct Sequencing
Exon 4	Codon 143	C/T (Arg143X)	Direct Sequencing
Exon 6	Splice donor junction	Ggt - Gat	Direct Sequencing
Intron 3	-46	a / t	SSCP/Sequencing
Intron 3	- 47	c / t	SSCP/Sequencing
Intron 3	-143	t / c	SSCP/Sequencing
Intron 3	-96	g / a	SSCP/Sequencing
Promoter	near TATA box	c / t	SSCP/Sequencing

Tab. 4-3 Genetic alterations in the human UCP3 gene (Argyropoulos et al., *J. Clin. Invest.* 102(7), 1345-1351, 1998)

In a 16-year-old with morbid obesity (BMI = 51.8) and type 2 diabetes, Argyropoulos *et al.* (1998) found compound heterozygosity for a 427C-T transition in exon 4, resulting in the introduction of a premature stop codon at residue 143, Arg143 to Ter (R143X), in the third, matrix-oriented loop. In addition, the patient was heterozygous (G/A transition) for a guanine to adenine polymorphism at the splice donor site of exon 6 (Ggt-Gat), resulting in loss of the splice junction and premature termination of the protein product in the sixth, matrix-oriented loop and truncation of the protein product at the first TGA stop codon of the adjacent intron. In their study, a putative protein resulting from this mutation would be

identical to that encoded by the short transcript of UCP3 mRNA. Pedigree analysis and DNA sequence determination of family members showed that the R143X mutation was transmitted to the compound heterozygous proband from the grandmother, through the mother, in typical mendelian fashion. They examined an additional 168 individuals comprising both African Americans and Caucasians for the 2 nucleotide changes showed that the 2 genotypes were independent. The frequency of the G/A heterozygous genotype was nearly twice as high (P=0.04) in obese (30%) compared with lean (16%) individuals. The heterozygotes for the exon 6 splice donor polymorphism had a 50% reduction in fat oxidation adjusted for lean body mass and a marked elevation in the nonprotein respiratory quotient, compared with wildtype subjects. V102I and the exon 6 splice donor Ggt-Gat. Among unrelated individuals, no significant differences were found between heterozygotes and wildtype individuals for BMI, percentage of body fat, and resting energy expenditure adjusted for lean body mass. The same analyses performed for the V102I polymorphism showed no significant differences between heterozygotes and wildtype individuals for any of the aforementioned quantitative traits.

In conclusion, the genomic structure of the human UCP2 (hUCP2) gene shows great homology to the other known members of this family of mitochondrial carrier proteins, hUCP1 and hUCP3. Using direct sequence analysis we have identified a missense mutation in exon 4 of the hUCP2 gene (Ala55Val) which is quite abundant and does not exhibit association to obesity in any studied population. Because of the similarity in allele frequencies a direct correlation of the Ala55Val mutation with regard to the phenotype *obese* is not evident. The results suggest that the hUCP2 exon 8 insertion/deletion polymorphism shows linkage with BMI only in populations ascertained for obesity. The set-up of suitable expression systems (e.g. yeast) should be of extreme value to determine functional correlates

of UCP2 as well as for the characterization of newly identified genetic variants effect on protein levels.

Chapter 5. Human UCP 3 and Uncoupling Protein Family

Comparison of UCPs in human and rodents, despite of differences among UCP counterparts by the number of exons in the 5'-UTR, shows the high conservation of the exon boundaries within the coding region and the high degree of similarity at the amino acid level.

5.1. Result

5.1.1. The transcription initiation site of the human UCP3 gene

To map the cap site of the human UCP3 mRNA 5'- RACE was carried out for at least 6 times utilizing a preparation of human skeletal muscle cDNA. The longest transcript identified contained 186 bp 5'-upstream of the start codon ATG, suggesting that this represents the potential transcription initiation site. At the genomic level, the 5'-flanking region is interrupted by a single intron (intron 1, 1.9 kb). This is in contrast to the genomic organization of the human UCP2 gene which contains two introns within this region.

5.1.2. Identification differing in the 3'-UTR of two human UCP3 gene transcripts

The 3'-UTR of hUCP3L/S mRNAs were mapped by 3'-RACE using cDNA isolated from human skeletal muscle. Comparing the 3'-UTR region of the human UCP3 short form (hUCP3-S) determined by both 3'-RACE and RT-PCR with the sequence of intron 6 revealed that the 3'-UTR of hUCP3-S is part of the 5'-end of intron 6, indicating that hUCP3-S is generated by incomplete transcription caused by the presence of a cleavage and a polyadenylation signal (AATAAA) in intron 6 terminating message elongation. Alternatively, elongation continues through exon 7 up to a AATAAA signal located 1.1 kb downstream of the stop codon of hUCP3-L. The genomic structure of human UCP3 gene short form thus differs from its long form by the absence of exon 7 at the C-terminus of the protein (**Fig. 5-1**).

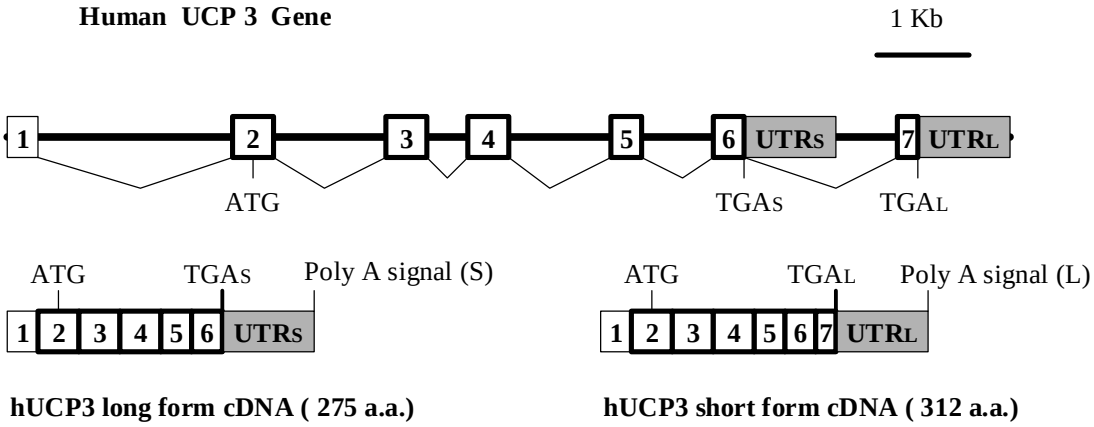


Fig. 5-1 Schematic representation the variation of 3'-UTR of hUCP3L and hUCP3S. The 3'-UTR of hUCP3S localizes within the intron 6 of hUCP3L. Human UCP3 gene with start codon (ATG), stop codons and cleavage poly (A) adenylation signal. The coding region between the start codon and stop codon for hUCP3 long / short form are boxed in blot line, respectively.

5.1.3. Comparison of Human UCP1 , UCP2, UCP3

At the amino acid level human UCP1 has about 55% identity to human UCP2 and about 54.7 similarity with human UCP3, while hUCP2 is 71% identical to hUCP3 (**Fig. 5-2**). In spite of that, the localization of the exon/intron boundaries within the coding region of human UCPs gene matches precisely each other (excepts a singly base pair shifts on the boundary of exon 4 / intron 4). The high degree of homology at the nucleotide level and the conservation of exon/intron boundaries among the three UCP gene suggests that they may have evolved from a common ancestor or are the result from gene duplication events. However, the size of each of the introns differs among hUCP1, hUCP2 and hUCP3 counterparts. Furthermore, within the 5'-flanking region there is no additional intron in the hUCP1 gene, which distinguishes it from hUCP2 and hUCP3 for which two additional

introns (hUCP2) or only one (hUCP3) can be detected. The three mitochondrial carrier protein motifs present in hUCP1 are conserved in hUCP2 and in hUCP3. At the carboxyterminus of the hUCP2 protein a Purin-Necleotide Binding Domain (PNBD) is found, similar to hUCP1 and hUCP3L (not present in hUCP3S) (**Fig. 5-3**). The comparison of genomic structure of these tree genes is showed on **Tab. 5-1**.

	hUCP1	**hUCP2**	**hUCP3**
Gene Map Locus	4q31	11q13	11q13
Coding sequence	924 bp 6 Exons / 5 Introns	930 bp 8 Exons / 7 Introns	936 bp hUCP3L: 7 Exons / 6 Introns hUCP3S: 6 Exons / 5 Introns
Protein	307 aa	309 aa	hUCP3L: 312 aa hUCP3S: 275 aa
Expression	exclusively in BAT	widely in BAT, WAT, heart, etc.	preferentially in skeletal muscle

Tab. 5-1 Human uncoupling protein family

Analyzing the overall genomic structure of hUCP3 and its counterparts hUCP1 and hUCP2 revealed a high degree of homology, particularly within the coding region. Major differences of the exon/intron structure are only found at the boundaries of these genes. Cassard *et al.*, (1990) note that the human UCP1 gene spans 13 kb and contains a transcribed

region that covers 9 kb of the human genome (similar to hUCP2), which is split into 6 exons only. hUCP3 contains 7 exons spread over 8.5 kb.

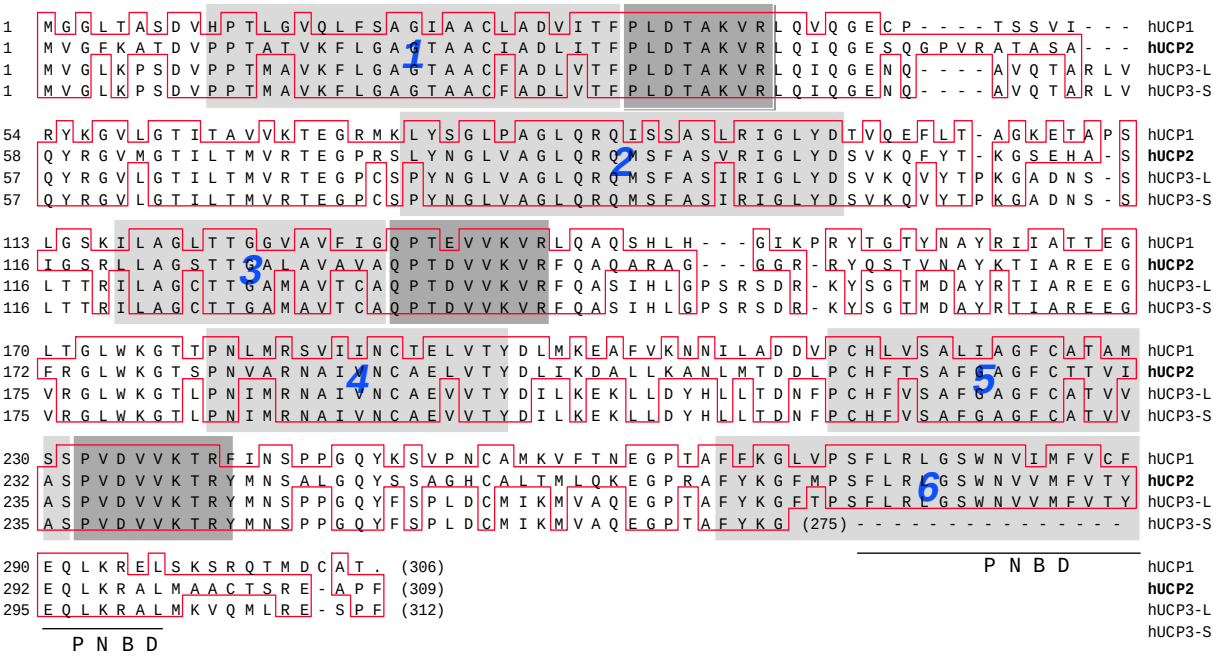


Fig. 5-2 Comparison of human UCP1, UCP2 and UCP3 protein sequences. Residues that match the hUCP2 sequence exactly are boxed. The three mitochondrial carrier protein motifs present in UCP1 and conserved in UCP2 and UCP3 are highlighted in dark gray. Potential transmembrane helices (1-6) are marked in light gray. The potential purine-nucleotide binding domain (PNBD) is underlined.

In contrast to hUCP2, hUCP3 generates two transcripts, UCP3L and UCP3S which are predicted to encode long and short forms of the UCP3 protein differing by the presence or absence of 37 amino acid residues at the C-terminus. These 37 residues are encoded by exon 7 which is missing in UCP3S. Very recent data from Pecqueur *et al*. (1999) reported that the hUCP3 gene maps 5' to the hUCP2 gene and that the extreme 3'-end of exon 7 of hUCP3 and

the transcriptional start site of hUCP2 are less than 7 kb apart from each other. This strongly implies that the organization of the UCP3/UCP2 gene locus is a result of a gene duplication event. The hUCP1 gene on the other hand was assigned to human chromosome 4 (4q31) (Cassard *et al.*, 1990). However, despite their sequence similarity, all UCPs are distinguished by their different pattern of expression: hUCP1 with a 1.9 kb mRNA expressed exclusively in human perirenal brown adipose tissue, plays an important role in generating heat and burning calories, as well as in the regulation of body temperature, body composition, and glucose metabolism.

Gene	**hUCP1** (Size, bp)		**hUCP2** (Size, bp)		**hUCP3 (**Size, bp)			
					Long Form		**Short Form**	
No.	Exon	Intron	Exon	Intron	Exon	Intron	Exon	Intron
1	342	612	> 104	~ 1,400	> 88	~ 1,800	> 88	~1,800
2	199	> 833	157	~ 3,250	221	~ 750	221	~ 750
3	200	105	225	156	211	~ 240	211	~ 240
4	102	?	212	867	204	~ 1,200	204	~1,200
5	181	?	194	81	102	~ 470	102	~ 470
6	> 115		102	971	181	~ 1,800	331	
7			181	369	~ 1,100		~ 1,200	
8			450					
Total	> 1,139		> 1,625	~ 7,094	> 2,107	~ 6,260	>2,357	4,460
	~ 9,000		> 8,719		> 8,367		> 6,817	

Tab. 5-2 Comparison of the Genomic Organization of human UCP1, UCP2 and UCP3

Compared to hUCP1, a 1.6 kb hUCP2 mRNA is widely expressed in adult human tissues (Fleury *et al.,* 1997) including tissue rich in macrophage; expression levels are upregulated in white fat in response to fat feeding. hUCP3, the third analogue discovered by Vidal-Puig *et al.* (1997) and Boss *et al.* (1997), is distinguished from hUCP1 and hUCP2 by its abundant and preferential expression in skeletal muscle, little in heart of humans, and brown adipose tissue and skeletal muscle in rodents. Since skeletal muscle and brown adipose tissue are believed to be important sites for regulated energy expenditure in humans and rodents, respectively, hUCP3 may be an important mediator of adaptive thermogenesis (Vidal-Puig *et al.,* 1997).

5.2. Discussion

Human UCP1 is 76.2% identical to rat UCP1 (306 aa) at the anino acid level. The mouse UCP1(302 aa) is 77.2 % homologous to human UCP1 and 98.6% identical to the rat UCP1. The mouse UCP1 gene was mapped in murine chromosome 8 (Jacobson *et al.*, 1985).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9		
1		66.5	68.1	77.2	66.0	69.4	76.2	66.5	69.1	**1**	Human UCP1.SEQ
2	44.7		79.8	66.0	98.2	84.4	65.5	97.3	84.0	**2**	Human UCP2.SEQ
3	41.8	23.5		64.2	79.8	93.4	63.2	79.8	93.0	**3**	Human UCP3.SEQ
4	27.3	45.8	48.9		65.5	67.5	98.6	66.0	67.2	**4**	Mus UCP1.SEQ
5	45.5	1.8	23.5	46.7		84.4	65.0	99.1	84.0	**5**	Mus UCP2.SEQ
6	39.6	17.4	6.9	42.9	17.4		66.5	84.4	99.5	**6**	Mus UCP3.SEQ
7	28.6	46.7	50.9	1.5	47.5	44.7		65.5	66.2	**7**	Rat UCP1.SEQ
8	44.7	2.8	23.5	45.8	0.9	17.4	46.7		84.0	**8**	Rat UCP2.SEQ
9	40.1	17.9	7.4	43.5	18.0	0.5	45.3	17.9		**9**	Rat UCP3.SEQ
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		

Fig. 5-3 Percent similarity and percent divergence among the members of the uncoupling protein family in human, mouse and rat . For alignment the *Clustal Multiple* Sequence

Alignment (*DNA STAR*, USA) was used. The Gene Bank accession numbers are as follows: human UCP1, U28480; human UCP2, U76367; human UCP3, A001787; mouse UCP1, U63481; mouse UCP2, U69135; mouse UCP3, AB010724; rat UCP1, M11814; rat UCP2, AB010734; rat UCP3, RNU92096.

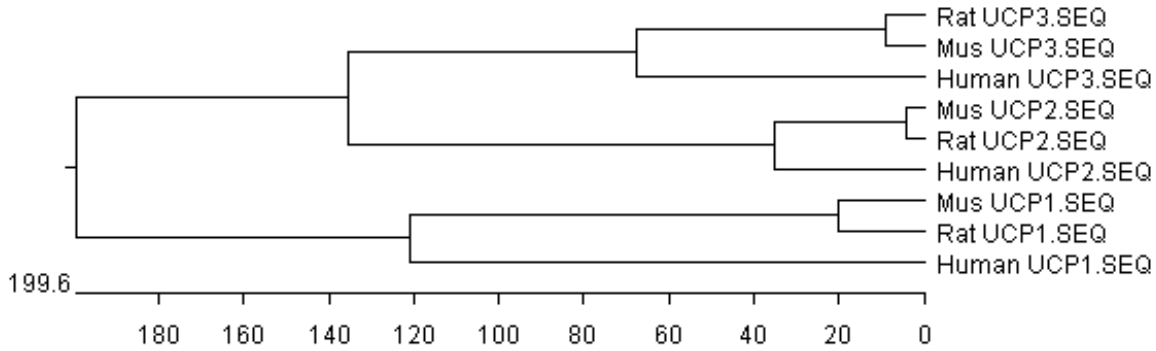


Fig. 5-4 Phylogenetic tree of human, mouse and rat uncoupling proteins. The amino acid sequences alignment were generated with the *Clustal multiple sequence alignment* (*DNASTAR*, USA).

The mouse homologue UCP2 (308 aa), which is 98.2% identical to human UCP2, and 99.1 homologous to the rat UCP2, maps to murine chromosome 7, tightly linked to the 'tubby' mutation, in an area of homology of synteny to 11q13. Human UCP2 is 97.3 % homologous to rat UCP2 (308 aa) at the amino acid level. Human UCP3 is 93.4% identical to mouse UCP3 (307 aa) and 93.0% homologous to rat UCP3 (307 aa) at the amino acid level, whereas the mouse UCP3 is 99.5% identical to rat UCP3.

Human UCP3 gene was initially reported located within 100 kb of the hUCP2 on chromosome 11q13 (Gong *et al.,* 1997 and Solanes *et al.,* 1997), Pecqueur *et al.* (1999) very recently claimed that the exact sequence between the TGA codon of the human UCP3 gene and transcriptional start site of the UCP2 gene is 6987 bp long, whereas the corresponding intergenic region for mouse UCP-2/3 is 8249 bp long on murin chromosome 7.

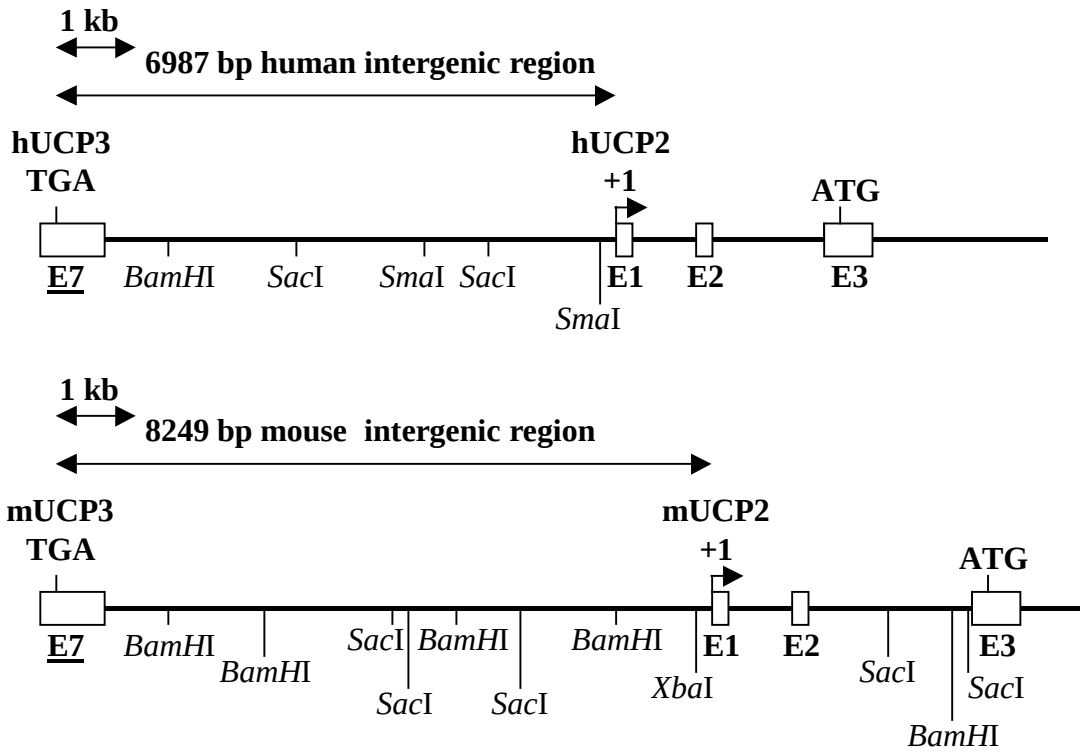


Fig. 5-5 The intergenic region of human and mouse UCP3/UCP2 juxtaposed gene loci is shown from the stop codon (TGA) in exon 7 of UCP3 gene to the transcriptional start site (+1) of UCP2. Exonic structure are indicated as E1, exon 1; E2, exon 2; E3, exon 3; E7, exon 7. Human and mouse intergenic region is rich in repetitive DNA sequences (Alu repeats).

In conclusion, comparison of UCPs in human, mouse and rat, although differences among UCP counterparts by the number of exons in the 5'-UTR, the conservation of the exon boundaries within the coding region and the high degree of similarity at the amino acid level among the UCP genes in human, mouse and rat, particularly of UCP2 and UCP3, suggests that they may have evolved from a common ancestor or are the result from gene duplication events. This has been further substantiated recently by the mapping of human (or mouse) UCP –2 and -3 gene in the juxtaposed loci to human chromosome 11q13 (or murin chromosome 7).

Chapter 6. Molecular Cloning and Functional Characterization of the Promoter Region of the Human UCP2 Gene

As a member of the uncoupling protein family, UCP2 is ubiquitously expressed in rodents and humans, implicating a major role in thermogenesis. To analyze promoter function and regulatory motifs involved in the transcriptional regulation of UCP2 gene expression, 3.3 kb of 5'flanking region of the human UCP2 (hUCP2) gene have been cloned and analyzed.

6.1 Results

6.1.1. Cloning and characterization of the 5' flanking region of the hUCP2 gene

The 3.3 kb promoter region of the hUCP2 gene obtained by PCR-screening was cloned into *pCR2.1-TOPO Vector* (**Fig. 6-1**). About 3.4 kb of the human UCP2 gene promoter region were sequenced using M13 forward and reverse primers. Analysis of the 5'-UTR of hUCP2 by long-range PCR revealed the existence of two introns upstream of the translation initiation codon.

Computer-assisted sequence analysis of the 3.3 kb 5' promoter region of the UCP2 gene revealed that the promoter region of UCP2 were not homologous to hUCP1 and hUCP3. Neither a typical TATA box nor a CAAT box can be found, however a GC-rich region consisting of several Sp-1 binding sites, and AP-1 and AP-2 motifs are located near the transcription initiation site.

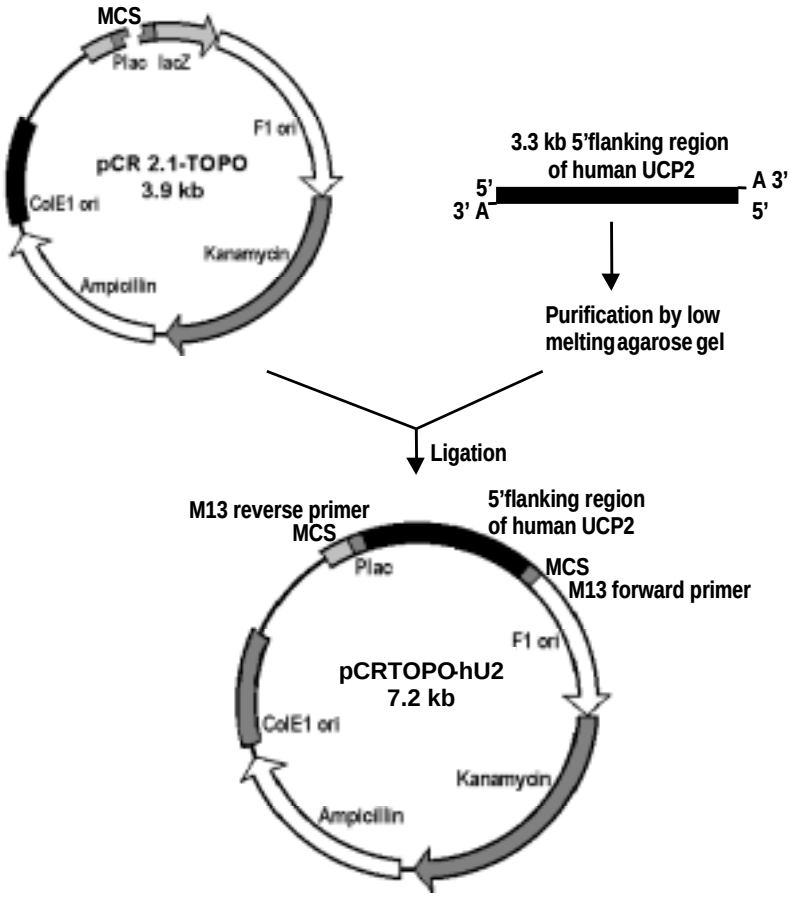


Fig. 6-1 Cloning strategy of the human UCP2 promoter region. The 3.3 kb 5'flanking region of human UCP2 gene were obtained by PCR-screening and cloned into *pCR2.1-TOPO Vector*.

The presence of potential consensus binding sites for transcription regulatory elements was considered to be important for UCP2 expression. A consensus CCAAT/ enhancer-binding protein beta (C/EBP-beta) binding site can be found at position -174 to –188, (numbers are based on +1 for the transcriptional start site), CCAAT/ enhancer binding protein beta was described as a nuclear factor for IL-6 expression with a consensus binding sequence RNRTKNNGMAAKNN (Akira *et al.*, 1990), which was compiled from 21 binding sites of 14 cellular genes and 3 viral genomes.

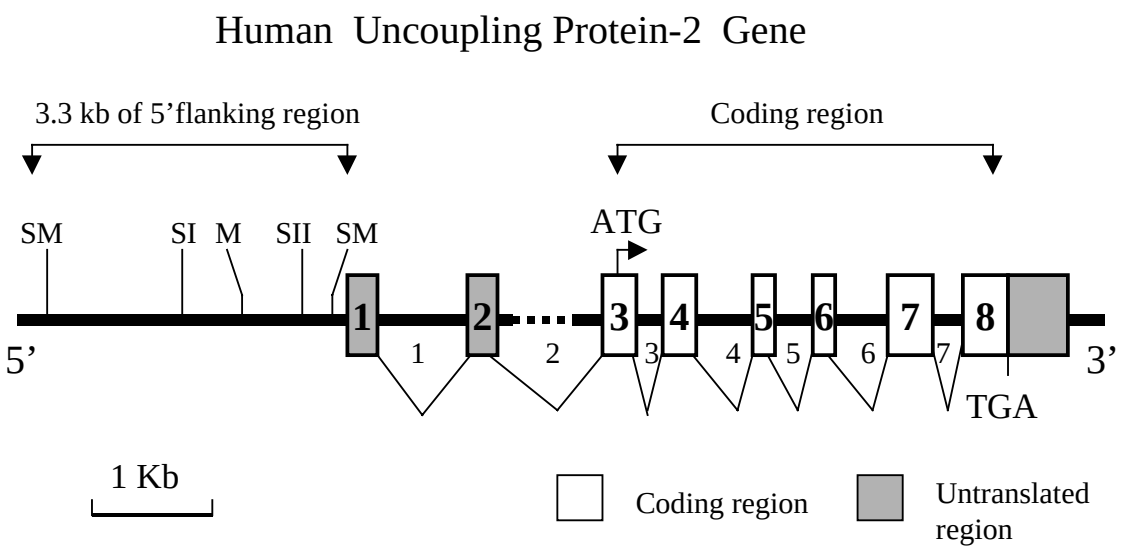


Fig. 6-2 Human UCP2 gene structural organization (5'flanking region and transcribed region). The transcribed region between the start codon (ATG) and stop codon (TGA) is shown in open boxes, the untranslated regions are shown in grey boxes. *Sma*I (SM), *Sac*I (SI), *Mlu*I (M) and *Sac*II (SII) restriction sites are indicated.

Three motifs for cyclic AMP (cAMP) response element binding protein 1 (CREB-1) were found, one with a core consensus sequences CGTCA (Kozak *et al.*, 1994) located at –1241 to –1247, and two with a consensus sequence TGASSTCA (Barton *et al.*, 1992) were present at –1079 to –1089 and –2580 to -2590. Multiple CREBs (4 CREB-1 sites) have been reported in the mouse UCP1 promoter with a core sequence CGTAC. Two peroxisome proliferator activated receptor gamma (PPARγ) response elements (PPRE) were present at -720 to –726 and 779 to –785, containing a DR1 half site AGGTCA. Two putative thyroid hormone response elements (TRE) with a core sequence of YRRGGTCA (Zavacki *et al.*, 1996) are located at position –718 to –725 and -3200 to –3208. A consensus binding motif for the muscle regulatory protein MyoD or E box is present at -594 to –604. Two GATA elements can be found at position –2828 to –2833 and –1546 to –1551 (**Fig. 6-3**).

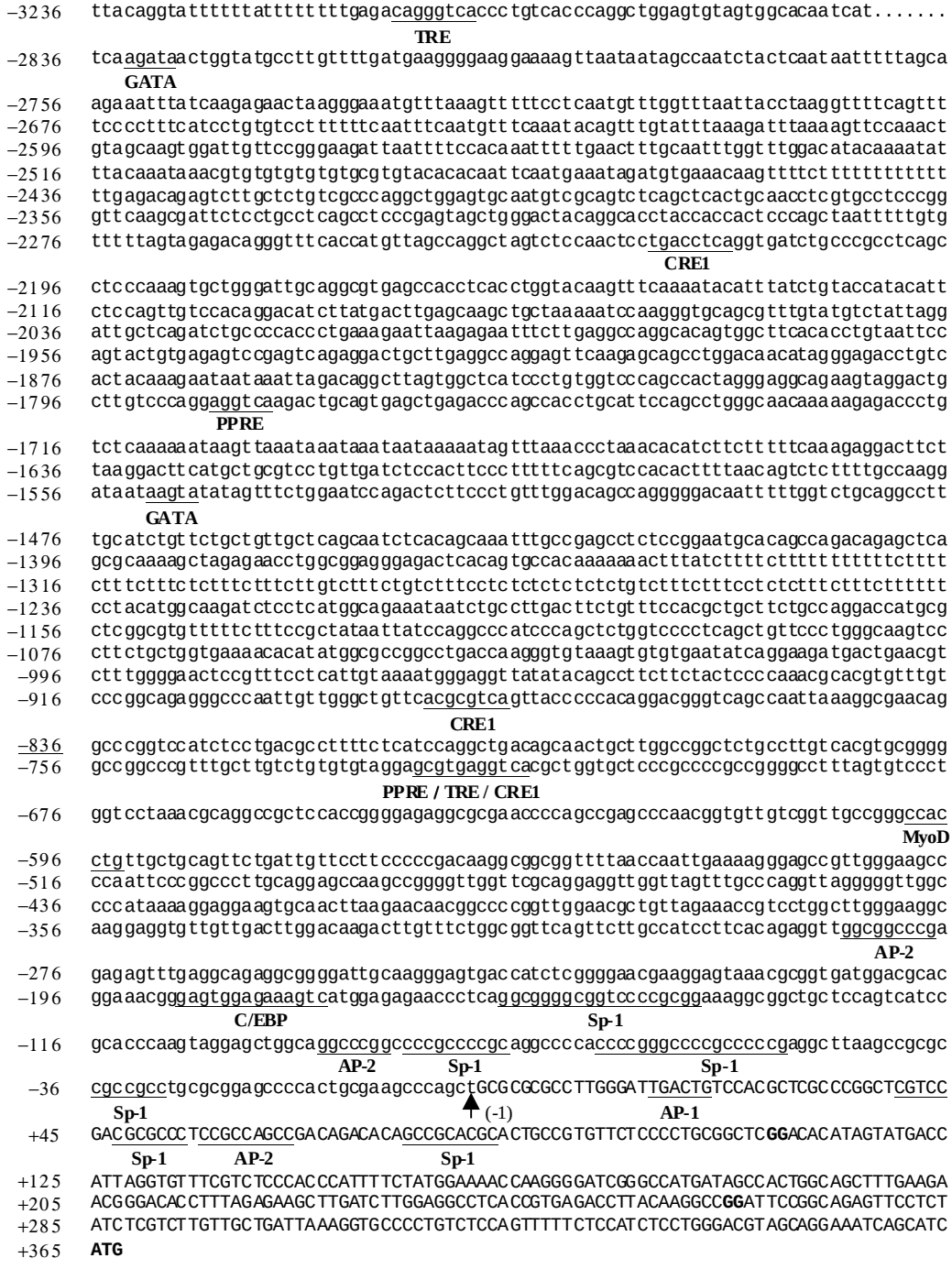


Fig. 6-3 Proximal promoter of human UCP2 gene and regulatory sequences. Numbering +1 is the transcription initiation site marked by an arrow. Intron 1 and 2 splicing site are bold in uppercase.

6.1.2. Analysis of Human UCP2 Promoter Function

6.1.2.1 hUCP2 promoter-CAT fusion constructs

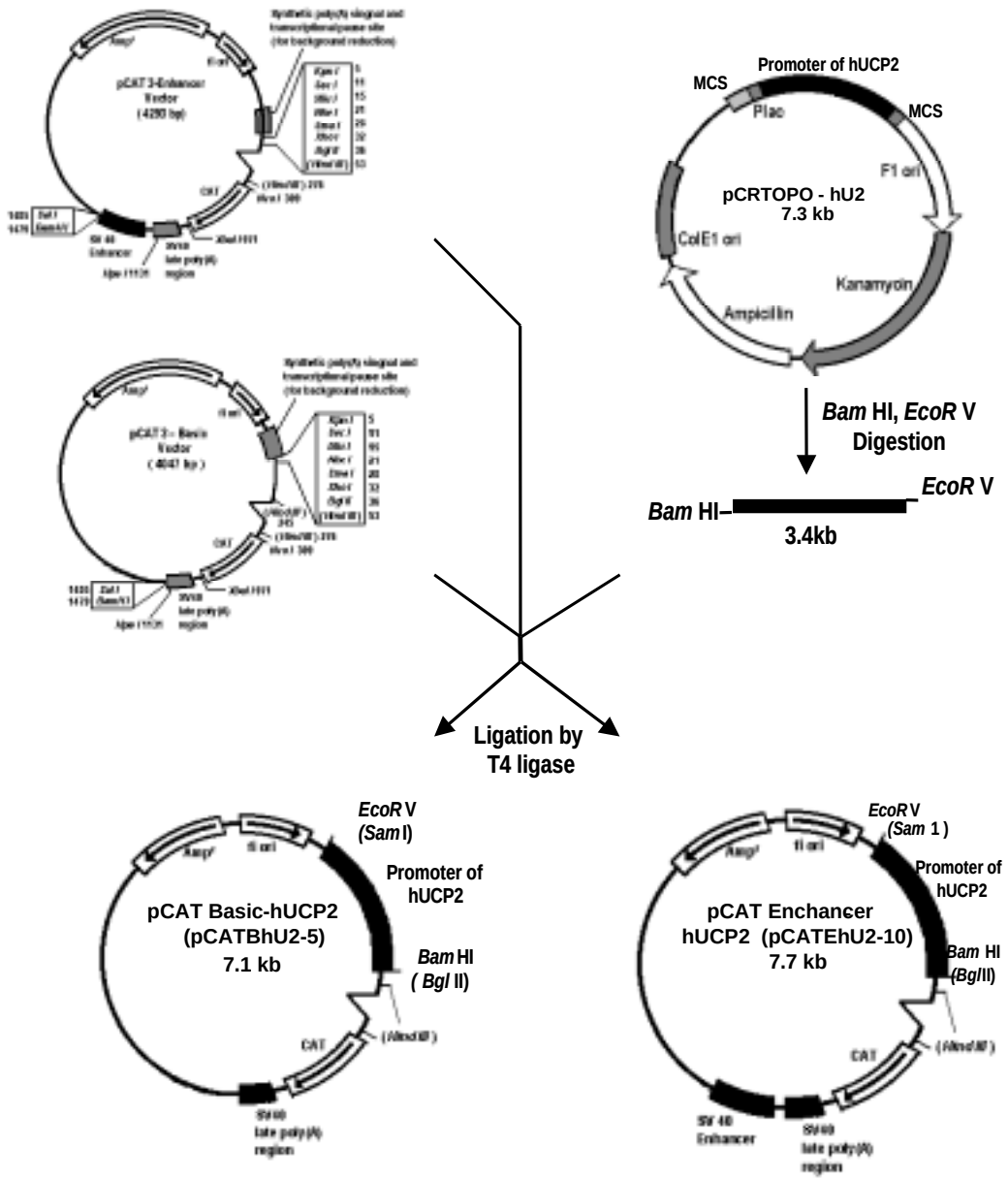


Fig. 6-4 Cloning strategy for pCATBhU2-5, pCATEhU2-10.

For a functional analysis of the above mentioned regulatory elements possibly controlling human UCP2 expression, transient expression assays were carried out utilizing 14 various hUCP2 promoter-CAT fusion constructs **(Fig. 6-4)**.

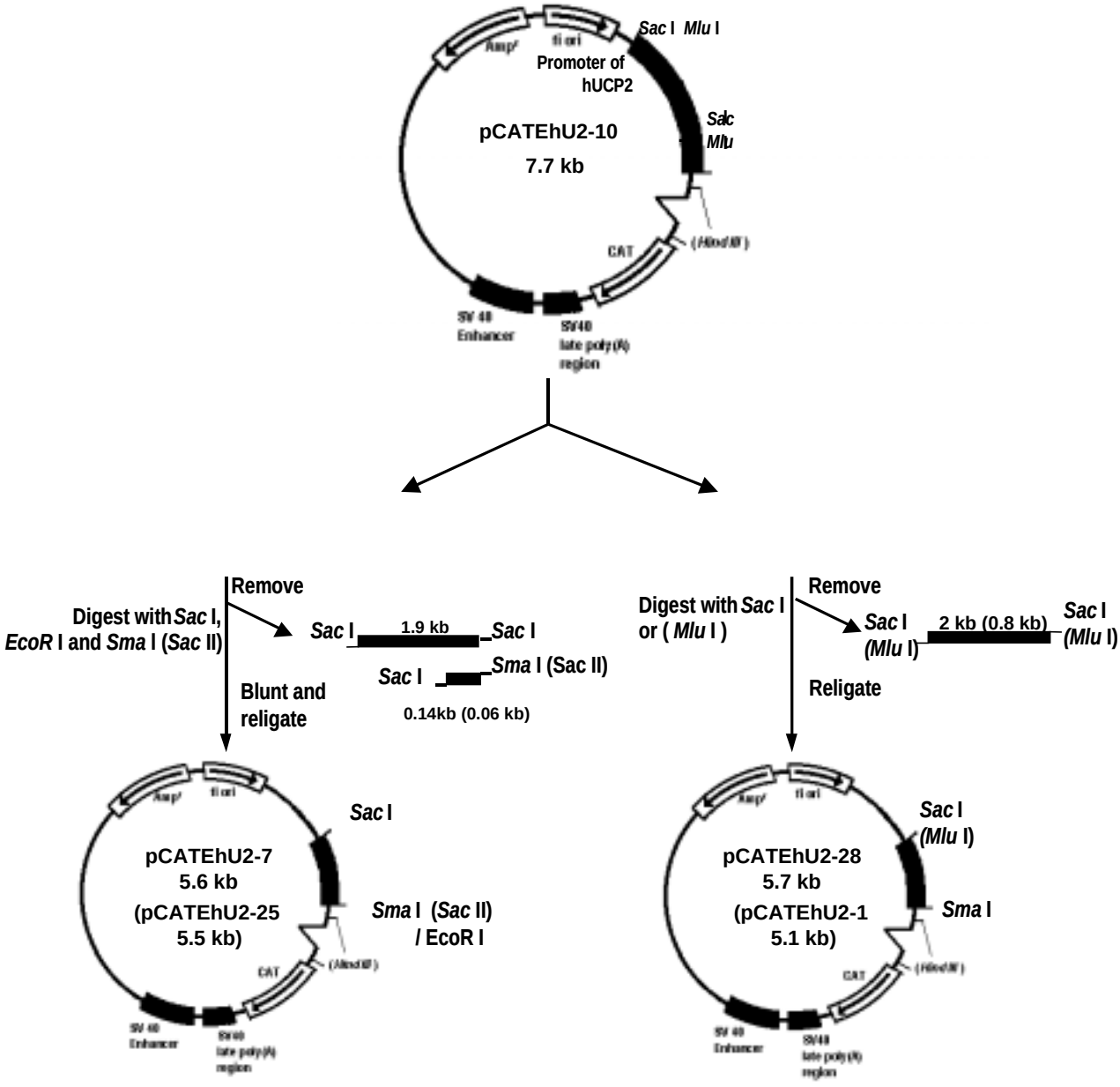


Fig. 6-5 Cloning strategy for constructs of pCATEhU2-7, pCATEhU2-25, pCATEhU2-28, pCATEhU2-1 from pCATEhU2-10.

A 3.4 kb fragment of the 5' flanking region of hUCP2 gene was inserted into the *Sma*I/*Bgl*II sites of the polylinker region of the pCAT-3 Enhancer and Basic vector,

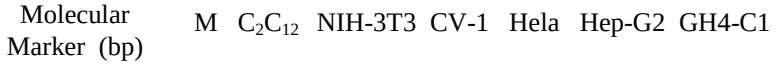
designated as pCATEhU2-10 (p10) and pCATBhU2-5 (p5) (**Fig. 6-5**). The following numbers are based on +1 for the transcriptional start site. pCATEhU2E-1 (p1) was created by a *Mlu*I digest removing sequences between –3271 and –884 of p10 and the plasmid was religated. pCATEhU2-28 (p28) was generated in a similar way using *Sac*I (-1398).

CATEhU2-12 (p12) was created from p10 by *EcoR*I digest and the resulting fragment cloned into the pCAT-3 Enhancer Vector in opposite orientation. pCATEhU2-7 (p7) was constructed by using *Sma*I (-68) and *Spe*I (remaining polylinker site from the *TOPO Vector*) which removes the sequences between –65 and + 110 of p28. pCATEhU2-25 (p25) was created in a similar way using *Sac*II (-146) and *Spe*I. pCATEhU2-22 (p22) was generated from p10 using a *Sac*I (in the polylinker of pCAT-3 vector) and *Sma*I (-65) digest to release the sequences between –3271 and –65. pCATEhU2-11 (p11) was generated using *Sac*I (-1398) and *Sac*II (-141) **(Fig. 6-6)**.

pCATBhU2-2 (p2) was created by a *Mlu*I digest removing sequences between –3271 and –884 of p5 and religation of the remaining vector. pCATBhU2-4 (p4) was generated in a similar way using *Sac*I (-1398). pCATBhU2-3 (p3) was generated from p5 using a *Sac*I(located in polylinker of pCAT-3 vector) and *Sma*I (-65) digest to release the sequences between –3271 and –65.

6.1.2.2. Endogenous expression of UCP2 in the different cell lines

Reverse transcription-polymerase chain reaction(RT-PCR) was carried out in $C_2C_{12,}$ Hep-G2, Hela, CV-1, NIH-3T3 and GH_4-C1 cell lines to check for the endogenous level of UCP2 mRNA expression. It was found that there is significant transcription of UCP2 mRNA in each cell line, with a slightly stronger signal observed in Hela cells (**Fig. 6-6**).



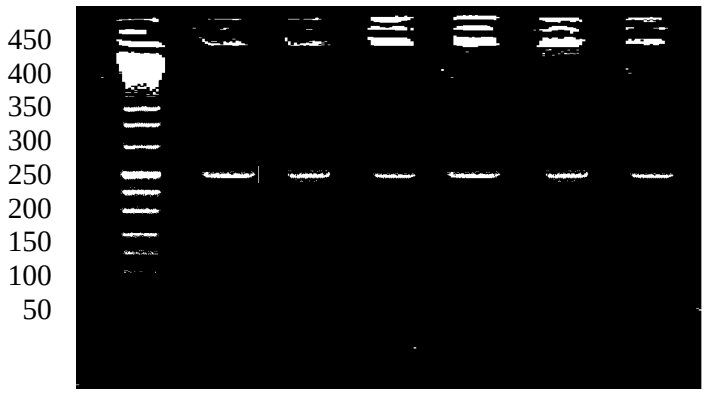


Fig. 6-6 Identification of endogenous expression of UCP2 in the different cell lines utilizing Reverse transcription-polymerase chain reaction (RT-PCR).

6.1.2.3. Transient expression assays

To analyzehUCP2 promoter fuction, transient expression assays were carried out utilizing above mentioned the hUCP2 promoter-CAT fusion constructs. Promoter activity was monitored in 6 cell lines derived from various tissues. The results of transient expression assays in Hep-G2 and CV-1 are shown in **Fig. 6-7.**

The longest construct p10, from –3271 to +110 of the human UCP2 gene promoter, showed CAT expression at the same level (100%) relative to that of the positive control (SV40 promoter). Deletion of the sequences between –3271 and –1398 (p28) exhibited greatly decreased promoter activity; further deletion to –884 (p1) resulted in the lowest CAT expression. Maximal CAT expression occurred(140% relative CAT concentration) with construct p11, which contains the hUCP2 promoter sequence from –141 to +110. The shortest construct p22 (from –65 to +110) showed between 20 to 40% expression of the positive control implying that this region could be regarded as a minimal promoter. The fact that the region from –141 and +110 (p11) exhibited maximal promoter activity suggests the presence of a strong *cis*-acting positive regulatory element (or enhancer).

At a similar location (-233 to -34) an enhancer controlling UCP2 expression has recently been described in mouse (9). A few *cis*–acting negative regulatory elements (or silencers) were located in the region from –1398 to –884 and from –884 to -141, which greatly repressed promoter activity by interacting with the above mentioned *cis*-acting regulatory element. However this negative effect could be partially overcome by interactions with regulatory motifs existing in the region from –3271 to –1398.

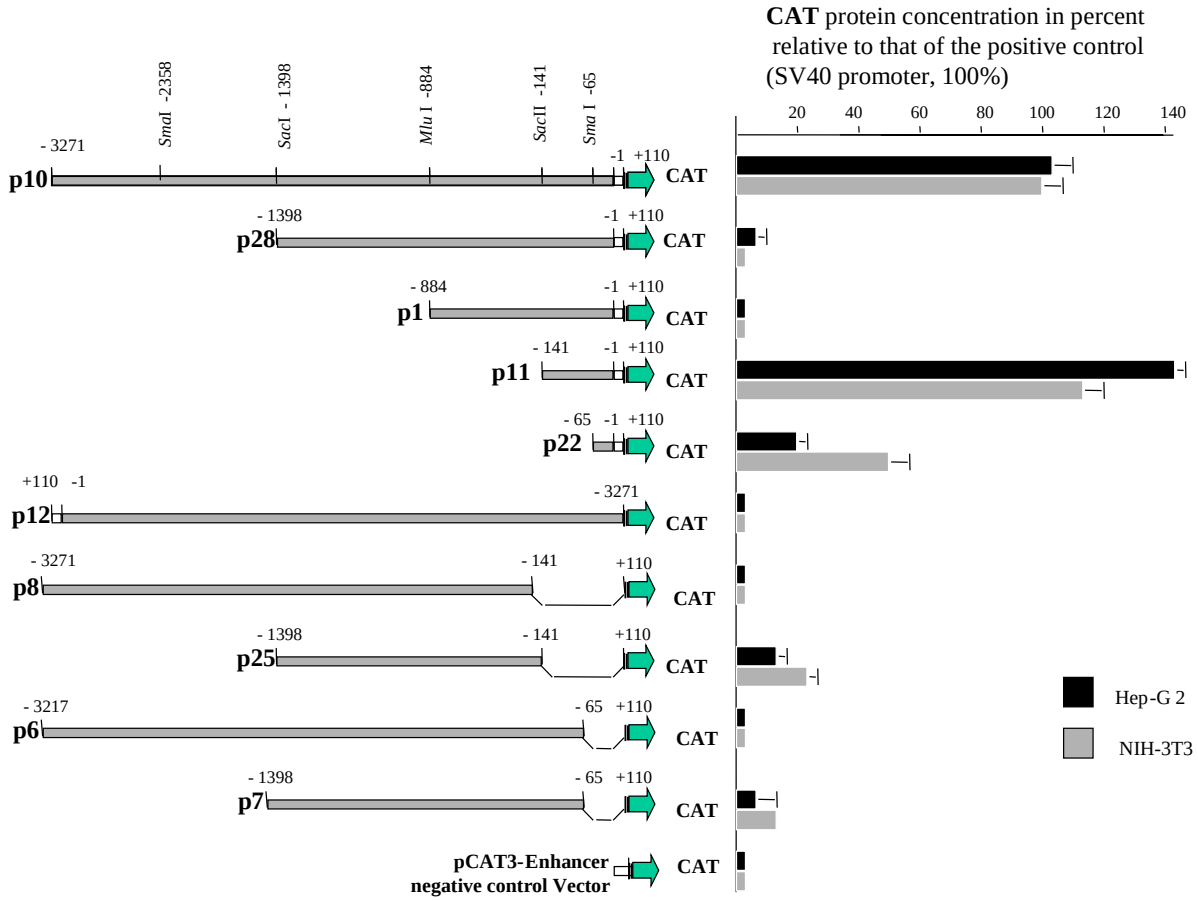


Fig. 6-7 Results of transient expression assays of pCAT®-3 derived enhancer plasmids containing various length of the 5' flanking region of the human UCP2 promoter. CAT protein concentrations are expressed in % relative to that of the control vector which was set at 100%. Data are represented as the means ± S.D. based on triplicate assays of three separate experiments.

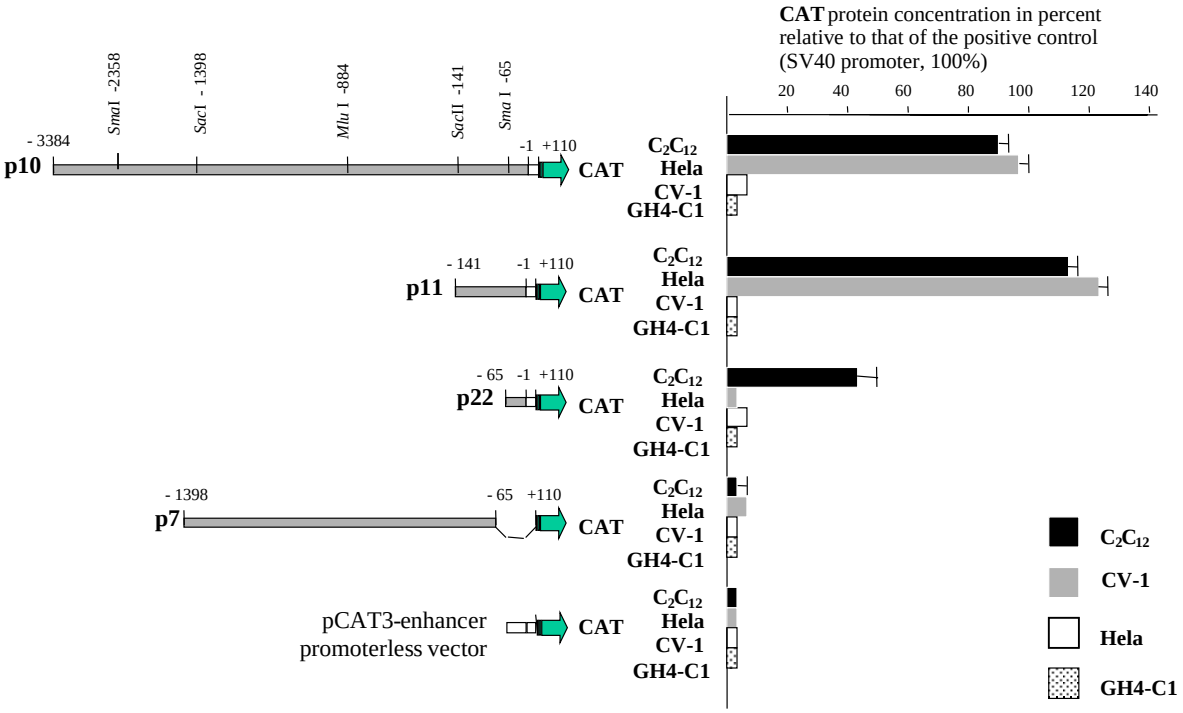


Fig. 6-8 Comparison of the promoter activity of four different human UCP2 promoter deletion constructs relative to that of the positive control vector in C_2C_{12} ,CV-1, Hela and GH4-C1 cell lines. Data are represented as the means ± S.D. based on triplicate assays of three independent experiments.)

The next series of experiments focused on elucidating precisely possible interactions among regulatory elements by comparison of the promoter activity of constructs p8, p25, p6 and p7. The constructs p25 and p7 showed 25% and 20% CAT expression relative to that of the positive control plasmid (see Fig. 3). This finding suggested the additional presence of negative elements in the region from –3271 to –1398 and positive elements in the region from –1398 to –884. If both elements are present (p8 or p6), this results in counteracting effects leading to a drastically reduced promoter acticvity. In contrast, the

existence of positive regulatory elements alone in p25 (or p7) the promoter activity will be partial recovered.

Furthermore, effects on transcription from negative elements located in the region from –1398 to -884 becomes only evident with the existence of positive *cis*-acting regulatory elements (or enhancers) located at the sequence between –141 and +110. Constructs p6 and p8 were essentially inactive indicating once again that the first 141 bp upstream from the transcription initiation site are significantly important for human UCP2 gene promoter.

6.1.2.4. Comparison of transient expression assays in various cell lines

To determine the above-mentioned regulatory elements contributing to the ubiquitous hUCP2 gene expression, the constructs of p10, p11, p22, and p7 were transiently transfected into C_2C_{12}, Hela, CV-1, GH4-C1, Hep-G2 and NIH-3T3 cell lines (see **Fig. 6-8**). The results showed that there is no obvious variation in CAT expression among Hep-G2, NIH-3T3, C_2C_{12} and Hela cell lines derived for most of the constructs, except from a relatively lower CAT expression found in p22 in Hela cells, which suggests that the possible presence of regulatory motifs contributing to the ubiquitous human UCP2 gene expression localized in the sequence between nt –3271 and –65. However, CAT expression from each construct was significantly lower in CV-1 and GH4-C1 cells. Interestingly, extremely high levels of reporter gene expression were observed for the mouse UCP2 promoter in GH4-C1 cells (Yoshitomi *et al.*, 1999).

6.1.2.5. CAT expression with pCAT®-3 reporter basic vector

Additional 4 constructs were constructed in pCAT®-3 reporter basic vector. No detectable CAT expressions variation among constructs was observed in various cell types

suggesting that SV40 enhancer present pCAT®-3 enhancer vector is necessary for transient expression CAT protein (**Fig. 6-9**).

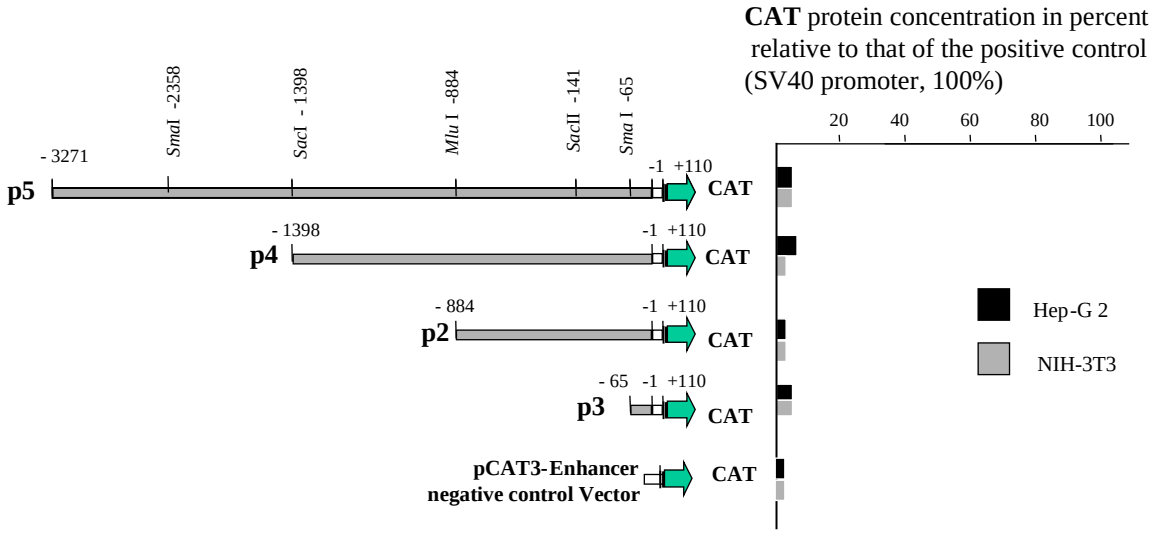


Fig. 6-9 Results of transient expression assays of pCAT®-3 derived basic plasmids containing various length of the 5' flanking region of the human UCP2 promoter. CAT protein concentrations are expressed in % relative to that of the control vector which was set at 100%. Data are represented as the means ± S.D. based on triplicate assays of three separate experiments.

6.2. Discussion

UCP1 is expressed exclusively in human brown adipose tissue, which is very scarce in adult humans. UCP1 therefore is unlikely to be involved in weight regulation in human and adult large-size animals living in a thermoneutral environment because there is little brown

adipose tissue present in adults (Huttunen *et al.*, 1981). UCP2 is markedly different from UCP1 in the way that UCP2 mRNA is widely expressed in skeletal muscle, lung, heart and kidney as well as in tissue of the immune system. Three microsatellite markers encompassing the UCP2 locus and spanning a 5 cM region on 11q13 were found to be linked to resting energy expenditure in adult humans (Fleury *et al.*, 1997). In UCP1 knock-out mice the level of UCP2 expression complementarily increased five-fold in BAT (Enerbäck *et al.*, 1997). In A/J mice given a high fat diet UCP2 mRNA was overexpressed in WAT (Suiwit *et al.*, 1998). These results strongly suggested a role of UCP2 in thermogenesis and energy balance.

Uncoupling proteins are well known to be under strict transcriptional regulation. The UCP1 gene is mainly regulated at the trancriptional level and is positively regulated by the sympathetic nervous system in rodents (Riquier *et al.*, 1985; Arch *et al.*, 1988; Bianco *et al.*, 1997). More recent studies suggest that UCP3 may indeed be regulated in a similar way as UCP1 (Riquier *et al.*, 1986; Muzzin *et al.*, 1989; Gong *et al.*, 1998; Boss *et al.*, 1998). Thyroid hormone (T3) and insulin activate UCP1 gene transcription. The ability of a high fat diet to induce obesity and diabetes in mouse is related to the expression of UCP1 in BAT and UCP2 in WAT, and is unrelated to differential expression of UCP2 and UCP3 in skeletal muscle or BAT. In rat, expression of UCP3 mRNA is similar to that of UCP1, markedly increased in BAT of obese Zucker rats after chronic treatment with ß3-adrenoceptor agonists, but no change in WAT and skeletal muscle could be found. It seems that insulin may play a role in the control of UCP2 and UCP3 mRNA expression in WAT, which actually increase UCP2 and UCP3 expression in rat adipose tissue and muscle (Gimeo *et al.*, 1997; Millet *et al.*, 1997).

Transgenic animals, primary cultures and cell lines have been used to determine tissue-specificity, hormone regulation and *cis-/trans*-acting elements in the upstream region of mouse and rat UCPs. However so far the transcription regulation of human UCP-2 and -3 expression have yet not been described. In the present study, we have cloned 3.3 kb of the 5'-

flanking region of the human UCP2 gene and characterized its promoter function using deletion analysis of hUCP2-CAT fusion constructs transiently expressed in various cell lines. Analysis of regulatory motifs in the 5-flanking region of hUCP2 gene reveals that the ubiquitous expression of UCP2 involves complex interactions of positive and negative regulatory elements. Deletion analysis identified five regions (A,B,C,D,E) for hUCP2 mRNA regulation. 1. The region showing minimal promoter activity extends to –65 bp (region A) in the UCP2 gene; it lacks a classical promoter factor TATA or CAAT box; however is GC-rich resulting in the presence of Sp-1 and AP-1/AP-2 motifs, which is comparable to the mouse UCP2 promoter but different from the UCP1 and UCP3 promoter (Kozak *et al.,* 1994; Yoshitomi *et al.,* 1998; Yoshitomi *et al.,* 1999). 2. A strong *cis*-acting regulatory element (or enhancer) with significant improvement of the basal promoter activity is located between –141 and –65 (region B). A similar location for positive elements controlling UCP2 expression in mouse has recently been described by Yoshitomi *et al.* They found that the region located between nt –233 to –34 in the mouse UCP2 promoter revealed especially strong enhancer activity and that at least 601 bp of the 5' flanking region contributed to the ubiquitous expression of mouse UCP2 (9). An enhancer within 220 bp located in the promoter region of the murine UCP1 gene was reported to be critical for tissue-specificity and regulation via ß-receptors (Kozak *et al.,* 1994). 3. The presence of a few *cis*-acting negative regulatory elements in the region from –1398 to –884 (region D) and the region from –884 to –141 (region C), which greatly repressed the promoter activity by interacting with the *cis*-acting regulatory element located in region B, as well as positive regulatory motifs existing in the region between –3271 and –1398 (region E) and the region from –1398 to –884 (region D). 4. A comparable working model with murine UCP1 gene for the regulatory regions of human UCP2 gene expression, involves a complex series of interactions between the five regions.

Potential consensus binding sites for transcription factors within the 5'flanking region considered to be of functional importance in the regulation of human UCP2 expression are shown in **Fig. 6-10**.

Evidence from in vivo and in vitro studies suggests that norepinephrine binds to ß1- and ß3-adrenergic receptors to initiate a cAMP signal transduction pathway which activates UCP1 gene transcription (Rehmark *et al.,* 1990; Akira *et al.,* 1990). Multiple CREBs have been identified associated with many genes, but UCP genes have more than most other genes that may imply their putative regulatory function. Four CREB1 sites have been reported in the mouse UCP1 promoter with a core sequence CGTAC resulting in essential functions: CREB1-2 was critical for enhancer function, and mutation of CREB1-4 completely knocked out expression (Kozak *et al.,* 1994). While seven are present in the rat UCP1 gene which all were active (Cassard-Doulcier *et al.,* 1993), only two were found in the mouse UCP2 gene (Yoshitomi *et al.,* 1999). Three CREBP1 motifs were found in the regions C, D and E, (nt –3271 to –141) of the hUCP2 gene, similar to the corresponding mouse gene, where they seem to play a role in the positive regulation of UCP2 mRNA after induction by ß-3 agonists. The ß3 agonist Ro16-8714 could increase UCP2 mRNA expression levels in rat brown adipocytes and skeletal muscle, a similar phenomenon was also observed for rat UCP1 and UCP3 (Gong *et al.,* 1998; Boss *et al.,* 1997), which supports the role of cAMP as a positive regulator of UCP2 expression.

A consensus CCAAT/enhancer-binding protein beta (C/EBP-beta) binding site is located in region B (from –141 to -65). The observed strongly upregulated basal promoter activity provided from this region may mainly be contributed to the presence of this C/EBP-beta element, which was involved in C/EBP dependent transcription regulation, especially the action of thyroid hormone and cAMP stimulus in primary brown adipocytes (Giralt *et al.,* 1991; Park *et al.,* 1993).

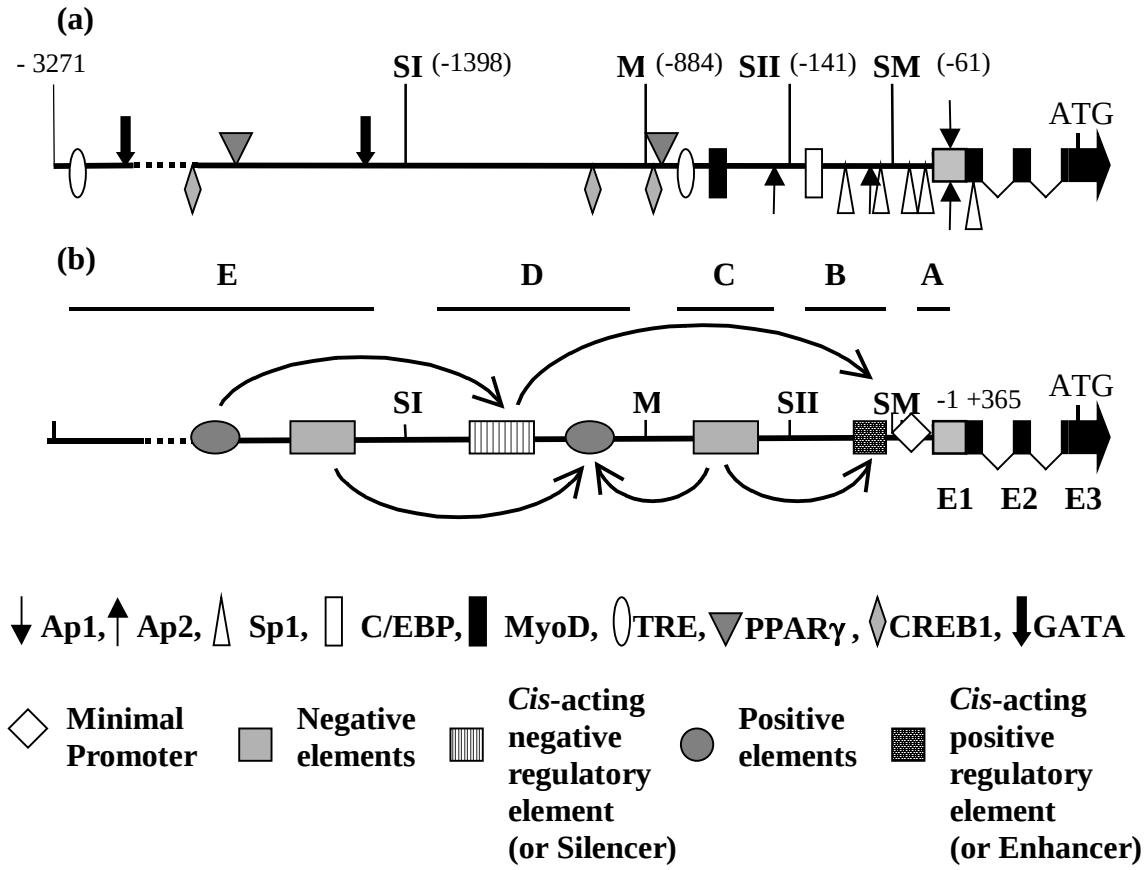


Fig. 6-10 (a) Classical promoter elements and potential transcription factor motifs in the promoter region of human UCP2 are mapped as follows: PPAR, black triangle; AP-1, downward arrow; C/EBP, grey rectangle; TRE, white oval; CRE1, grey diamond; AP-2, upward arrow; Sp-1, white upward arrow; MyoD site (E box), black rectangle. The transcription -initiation site is indicated as +1. Restriction endonucleases: EV, *EcoR* I; SI, *Sac* I; SII, *Sac* II; SM, *Sma* I; exonic structure are abbreviated as: E1, exon1; E2, exon2; E3, exon3. (b) Possible model of interactions involved in human UCP2 mRNA regulation.

DNAse-I footprint analysis showed the presence of two C/EBP binding sites in the 5' proximal region of rat UCP1 (Kozak *et al.*, 1994), and which was also present in the promoter of the mouse UCP2 gene (Yoshitomi *et al.*, 1999). We found that a putative thyroid hormone response element (TRE) consensus site located in region E (from –3271 to –1398) might be a positive element to override the repression from negative elements located in the downstream

region. Triiodothyronine (T3) potentiates the effect of norepinephrine and is essential for the adaptive response of UCP1 to cold. T3 can stimulate the transcription of UCP1 gene and amplifies the effect of cAMP acting directly on the gene (Bianco *et al.,* 1988). Masaki *et al*.(1997) described a significant increase of rat UCP2 expression by daily infusions of T3 in skeletal muscle and brown adipocytes. Much more evidences provide a clear role for thyroid hormone on UCP2 expression in rodents (Gong *et al.,* 1997).

Moreover two peroxisome proliferator activated receptor gamma response elements with half DR1 sites (PPRE) are present in region E (-3271 to -1398) and C (-884 to -141). PPRE containing half DR1 site was reported to be involved in transcription regulation (Forman *et al.,* 1990), which could provide a mechanism of the positive regulation of hUCP2 expression via free fatty acids (FFAs), which are ligands of PPARγ. In fact it has been shown recently that UCP2 mRNA expression is enhanced by PPARγ agonists in pancreatic islets as well as in cultured white adipocytes (Shimabukurio *et al.,* 1997; Aubert *et al.,* 1997; Camirand *et al.,* 1997). Other regulatory motifs such as GATA box and MyoD could also be involved in the specific regulation of human UCP2 expression.

In conclusion, maximal promoter activity was localized within 251 bp (from –141 to +110) of the 5’ flanking region of the hUCP2 gene, which may be mainly contributed to a *cis*-acting positive regulatory element (or enhancer) localized in the region from –141 to –68. A *cis*-acting negative regulatory element (or silencer) located between –1398 and –141 greatly repressed the downstream positive regulatory element activity. The UCP2 expression is subjected to complex interactions among positive and negative regulatory elements distributed over a minimum of 3.3 kb. Possible additional regulatory factor binding motifs may be localized in the region further upstream. Future studies will be directed to the identification of individual regulatory motifs localized in the entire 7 kb intergenic region

between the UCP2 and UCP3 locus on human chromosome 11q13 (Pecqueur *et al.*, 1999), in order to identify the underlying mechanisms orchestrating human UCP2 gene transcription.

Chapter 7. Functional Characterization of the 5'-Flanking Region and the Promoter Region of the Human UCP3 Gene

The newly discovered member of the uncoupling protein family, UCP3, is considered as important regulators of energy expenditure and thermogenesis in humans. However, up to now little is known about the mechanisms regulating gene expression of this homologue. In this study, about 5 kb of the promoter region of the human UCP3 (hUCP3) gene have been cloned by genome walking, and the transcription initiation site has been mapped. Sequence analysis of the promoter region revealed typical classical promoter regulatory elements as well as consensus motifs for various transcription factors and muscle-specific genes which may influence hUCP3 gene transcription in vitro and in vivo. Deletion analysis of a 3 kb hUCP3 promoter fragment subcloned into a pCAT reporter plasmid was performed and the resulting basal promoter activity monitored after transient transfection of the deletion constructs into different cell lines using a commercially available CAT-ELISA.

7.1 Result

7.1.1. Cloning and characterization of the 5’ flanking region of the hUCP3 gene

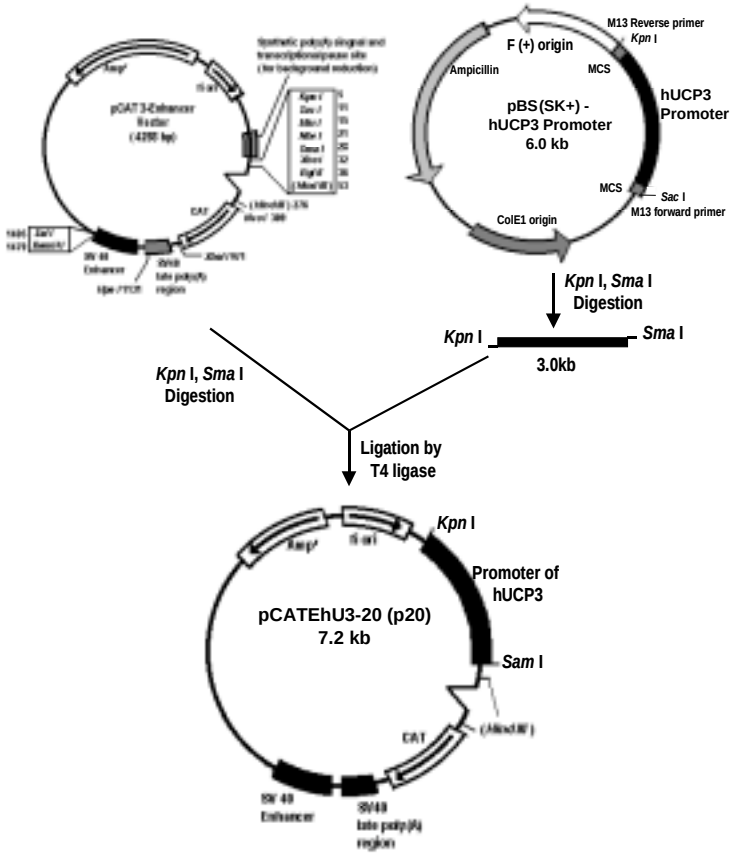


Fig7-1 Schematic representation for the construction of pCATBhU3-20.

To identify sequences of potential functional significance, putative regulatory elements in the promoter region of human UCP3 gene were searched. Computer-assisted analysis of the promoter region revealed a consensus sequence for a TATA box at position -231, numbering based on +1 for the transcriptional start site. No GC-rich Sp1-like sequences were found (**Fig. 7-1**).

-4373 accctccgtcagctttgtccagcttgcaggaggcaccaggtctggctccttcagggctgtcacagtcctgaaaccacca

CRE

-4293 ttgcctaggccacggatgcctcaagagacccggtcagcccagactggaggagtgccccaaaccagtccagtgtccactt

-4213 gccagaatttcagctccttttttctgtggtgaggcaaaggctagaaataaagtggaanccgcgcccaagtgggacccaa

-4133 caggcatctttcggtggagaaacgccccccagtgtccttgacacagctgactgctggcatgagcccaccctctgcctga

-4053 gatgcaggaccatttttgccgtaaaagttggggaggagggacatgtaattgtgtgcatngtgcatagtcaaggaaatcc

-3973 aggggccacctccaagttcatttgttgtgggaacaaggatattttctagatacaaattatttttatgctgtgttgaatt

-3893 atcaattaggagaggaaggggagatcacttccttcaaacttttatctgattgtctaaaattctaaccatgcttttaac

-3813 tattatttttacccagctctgaaggtcattgttcttgcctgtgtttgaataaaatcatattggtgtttgtaatctgtgc

-3733 caagtgtttcgctgggagtggggatgctaggaattcgtgtgtgccctaactgccagagagaaagattaacaaccataca

-3653 cccacgctagacgaaagatcaatcaccaatgagacttctgggctcctccagaaatcagggttgggtccaagatgacaag

-3573 gaacacccaagttatttcctttgctgatgcttgaaatcccatcagacaaagcccccgtggggaggacaggaaaagggcc

-3493 tggatgggtgcagtggctcacacctgtaatccaaacactttgggaagccgaggcaggcagatcatgaggtcaggagtt

CRE2 / TRE

-3413 tgagactaccctggccaacctggcaaaaccccgtctctactaaaaaaaaaaaaaaaatacaaaaattagctgggcgtggt

-3333 cacgcctgtagtcctagctactcaggaggctgaggcaggagaatcacttgaacccaagaggtggaggttgcagtgaacc

-3253 agaccacgccactgcactccagcctggacaacagagcaagactccgtatcacaaaaaaaaaaaaaaaaatggggtcgcg

-3173 gagccaaatgtgcagagtgagcagtgtggctgggcaacaggctttcacccccaagtacatgccacttcactcagtcctc

-3093 aaacagctctctaactctagatattatgatatctagagtttctgtaagttcagtgttttgcctctgtttgtgtagttag

-3013 aagtgattaaccacacaagaaatcccagaggtattgcaaggctactgctttcagtaatcaaaacaaggtacagccaata

-2933 gtgctctgaatttagggacttcatggagaaagaccatgtgggctggggcagtcagggaaggcttcctagaagagacagg

-2853 cttggggtgagcacagagaattggcccaggctgtggacagctatagagagagagccaggaaagcctgtgagcagtgtgc

-2773 gctggagatagagaccagtctcacgtcgtctgagcctctgtggcctcgtctgtaaaacagagataatcaccccagcttca

-2693 tggggttgtgaggaagatcaaatgagatgacatttgtgaaagtgcttcgcctacttttggcacatggtggaagcttagg

-2613 aatgtcagccccacccactctcctgactttcgggagcaaaccagtagagaggcaagcatgcagccactggggcaggcag

-2533 gcactcctgggcctcagagggtgcaaaatgatgcgggaacctcaaggaaggagtgggtgctgccttcttggtgacaagc

-2453 ctcccgcttctatttatctctgctccgacgcctgccagcccacccctccccagcaccacagaggccagcctccctctca

-2373 gtcaccagcacagagcattcaaggcccagggccccacttcccttggggcccaggggacctaaagaggggcagtcagggc

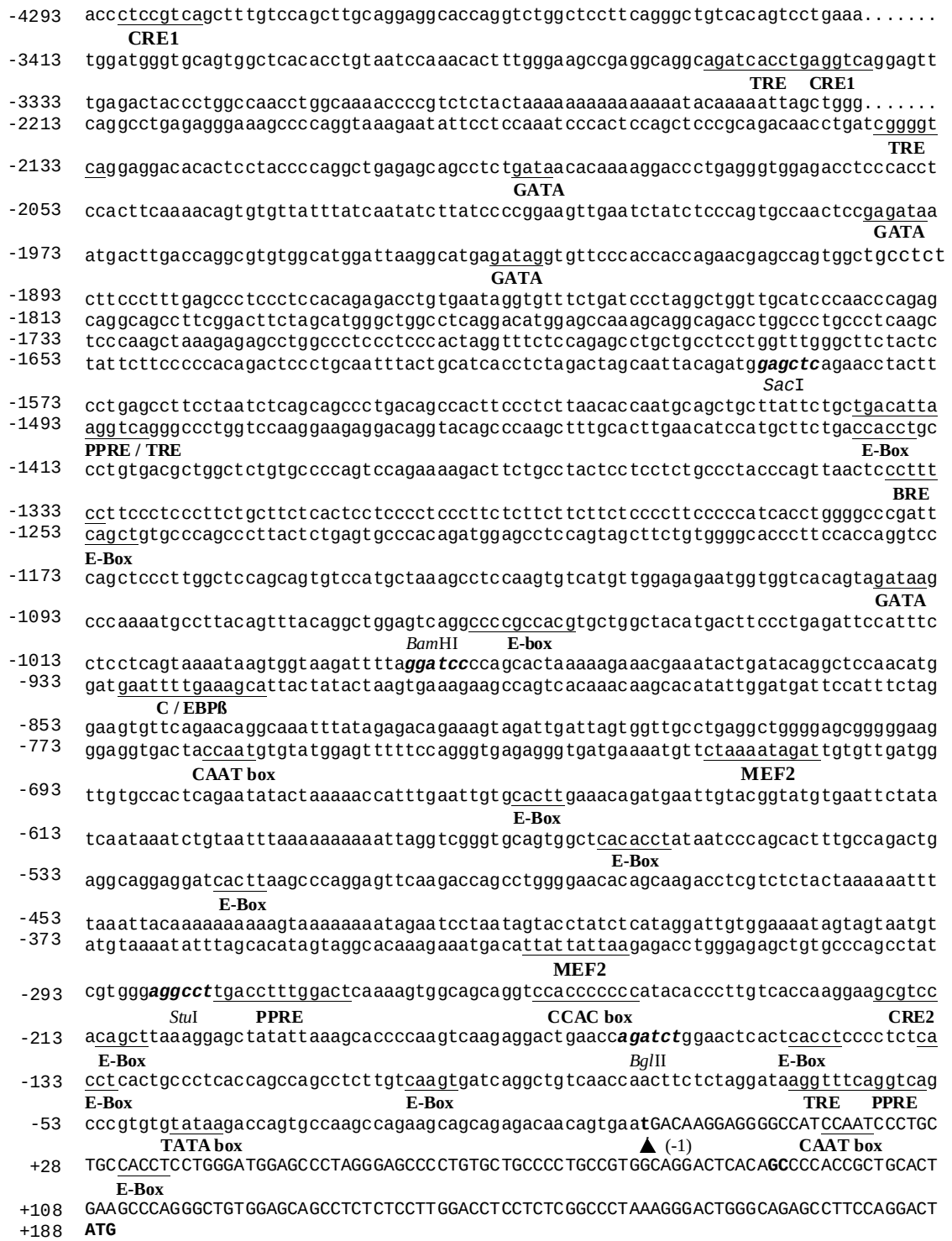


Fig. 7-2 Sequence of the human UCP3 5-flanking region. Consensus sequences for classical promoter elements and putative transcription factors present in the 5'flanking region are

underlined. Numbering is based on +1 for the putative transcriptional start site, which is marked by an arrow. The splice site for intron 1 is indicated in bold capital letters. Restriction sites within the sequence used for generating the promoter deletion constructs are indicated in bold letters.

Computer-assisted analysis of the promoter region **(Fig. 7-2**) revealed a consensus sequence for a TATA box at position -45 (numbering based on +1 for the transcriptional start site). No GC-rich Sp1-like sequences were found. Two myocyte-specific enhancer factor-2 protein (MEF2)-like consensus binding sites (Andres *et al.,* 1995) at position -332 to -323 and –715 to –705 can be detected which activate transcription via binding to a consensus A/T-rich *cis*-element widely found in the control region of muscle-specific and growth factor-induced genes (Liu *et al*., 1994). Moreover, an abundant number of recognition sites for muscle-specific factors such as E-Box (or MyoD elements, Nakayama *et al.*, 1996) are located in the proximal part of the promoter region. A CCAC box motif at position -251 to -242, which previously was described in the myoglobin promoter is necessary but not sufficient for muscle-specific gene transcription (Bassel-Duby *et al.*, 1992; 1993). The CCAC box could play a crucial role in UCP3 gene transcription. Two CCAAT boxes can be found at positions +17 to +21 and -762 to –758. Three putative response elements for PPARγ (are located at positions -67 to -56, -281 to –269 and -1501 to -1488. Since PPARγ is known to be expressed in skeletal muscle, their response elements provide a mechanism by which free fatty acids which are ligands for PPARγ enhance UCP3 expression during fasting. This is in perfect agreement with the selective expression pattern of UCP3 in skeletal muscle. In addition, several putative thyroid hormone response element consensus sites can be found. The TRE element at position -3355 (the sequence is 5'-AGATCACCTGAGGTCA-3') differs by a

single nucleotide from the canonical DR4 motif (AGGTCA(N_4)AGGTCA) described by Umesono et al. (Umesono et al., 1991).

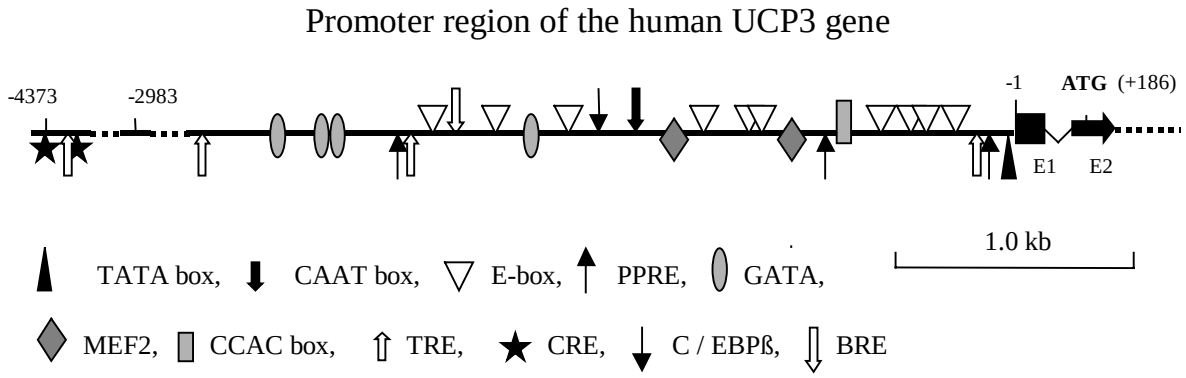


Fig. 7-3 Schematic drawing of the human UCP3 promoter region. Promoter regulatory elements and potential transcription factor binding motifs are marked as follows: TATA box, black triangle; CAAT box, downward arrow; E-box, white triangle; PPRE site, upward arrow; GATA element, grey oval; MEF2 site, grey diamond; CCAC box, grey rectangle; TRE, open upward arrow; CRE, black star; C/EBPß, black downward arrow; BRE site, open downward arrow. The transcription-initiation site at position -187 is indicated . Exons 1 and 2 are marked as black boxes.

Thyroid hormone has long been recognized as a thermogenic hormone; its effect is believed to be caused by a generalized stimulation of the metabolic activity of most tissues (Rabelo *et al.*, 1996). Within the upstream TRE element a consensus sequence for a cAMP response-like element (CRE-like) can be identified (5'-TGAGGTCA-3', Kozak *et al.*, 1994). A CRE1 site (CTCCGTCA) is localized at position -4290 to -4283, a CRE2 site at -219 to -214. This implies a regulation of UCP3 expression via intracellular changes of cAMP levels due to the activation of beta-adrenergic receptors, in particular of beta-3-adrenergic receptors. In fact, regulation of UCP3 expression has been demonstrated to be increased in rodents after

administration of selective beta-adrenergic agonists (Gong et ai., 1997; Yoshitomi et al., 1998). Furthermore a C/EBPß binding site can be found at position -930 to-917. Finally, there are four potential GATA sites and a single BRE element (-1338 to -1332). GATA-4 is an essential transcription factor in heart development (Qin *et al*., 1997, Kozak *et al.,* 1994). An overview of all regulatory elements localized in the hUCP3 promoter region is shown in **Fig. 7-3.**

7.1.2. Fuctional analysis of the human UCP3 promoter

7.1.2.1 Human UC3 gene promoter-CAT fusion constructs

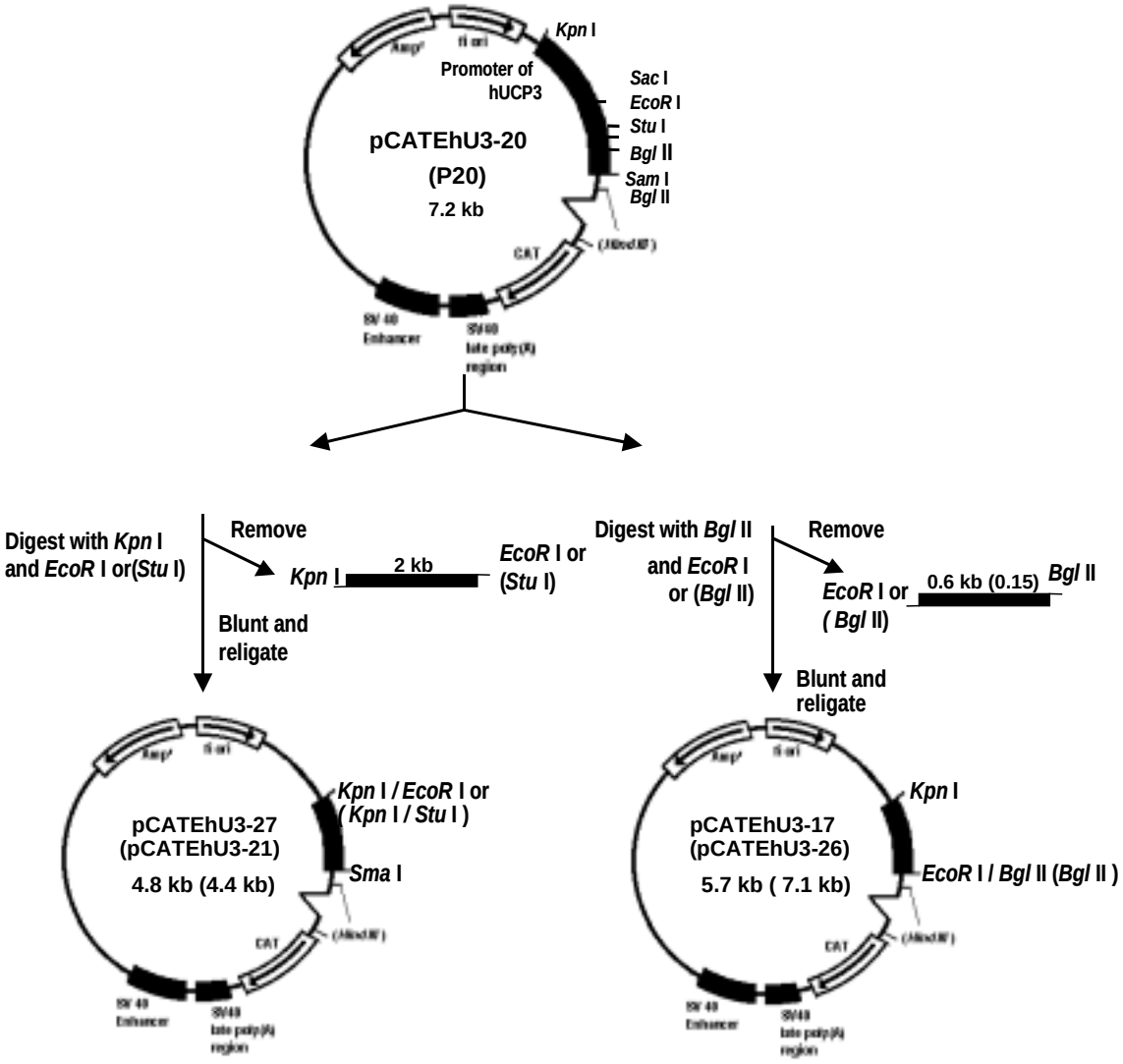


Fig. 7-4 Schematic representation of the construction of pCATEhU3-27 (p27), pCATEhU3-21 (p21), pCATEhU3-17 (p17) and pCATEhU3-26 (p26).

In order to examine the basal transcription activity of the human UCP3 promoter a 3 kb fragment of the 5' flankingregion have been subcloned into a promoterless CAT-vector (pCAT-3 Enhancer Vector) giving rise to p20.

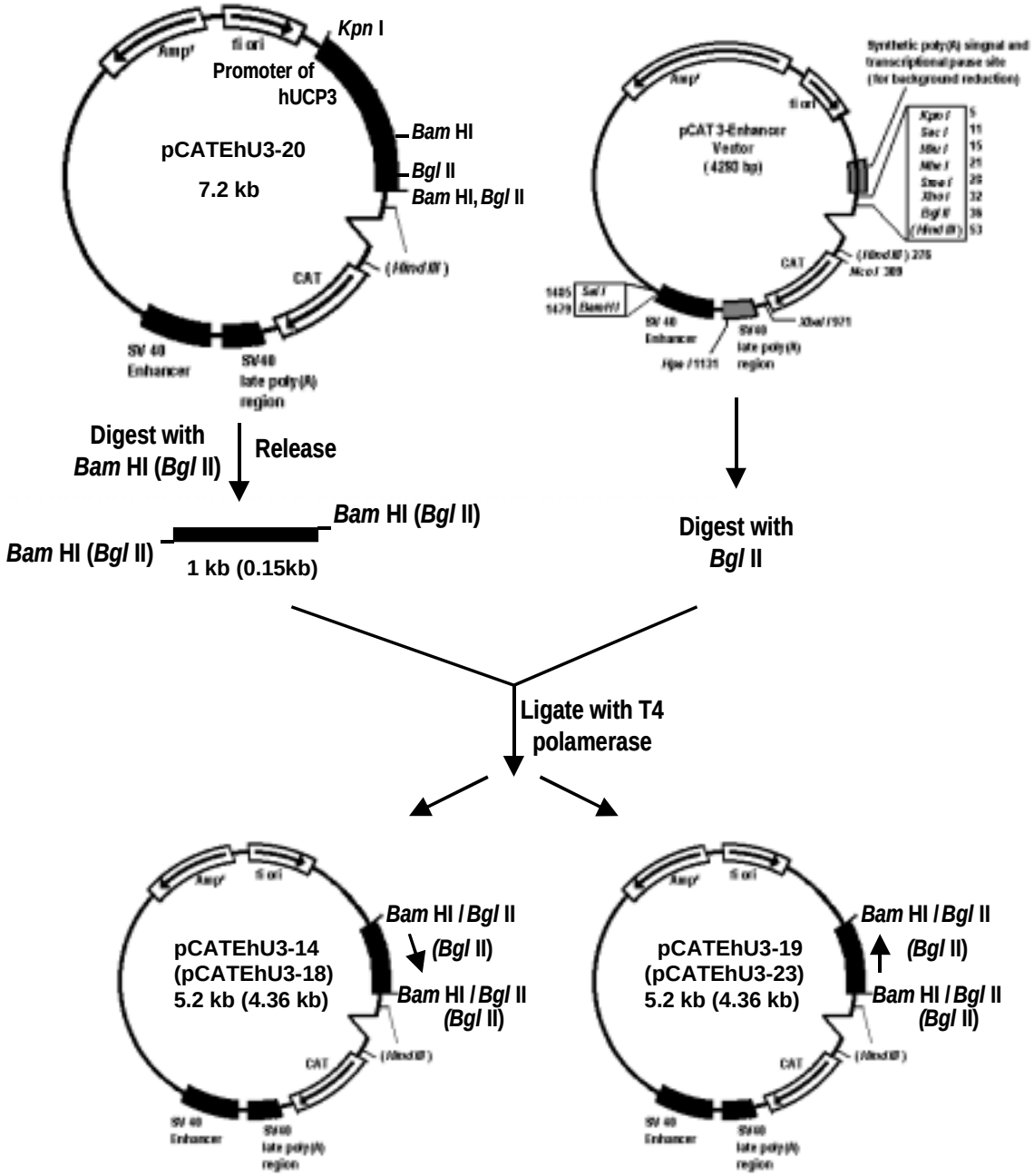


Fig. 7-5 Construction of pCATEhU3-14 (p14), pCATEhU3-18 (p18), pCATEhU3-19 (p19) and pCATEhU3-23 (p23).

Various deletion constructs including nested deletions were generated from p20 utilizing unique restriction sites located within that promoter fragment. To test for the presence of possible enhancer activities some of the constructs were fused in opposite orientation to the CAT gene.

7.1.2.2. Endogenous expression of UCP3 in the cell lines

To check for the endogenous level of UCP3 mRNA expression reverse transcription-polymerase chain reaction (RT-PCR) was carried out in C_2C_{12}, HepG2, Hela, CV-1, NIH-3T3 and GH_4-C1 cells. A strong signal of UCP3 transcription was detected in C_2C_{12} cells, weak signals were observed in CV-1 and NIH-3T3 cell lines. However, no message was detectable in HepG2, GH4-C1 and Hela cell lines.

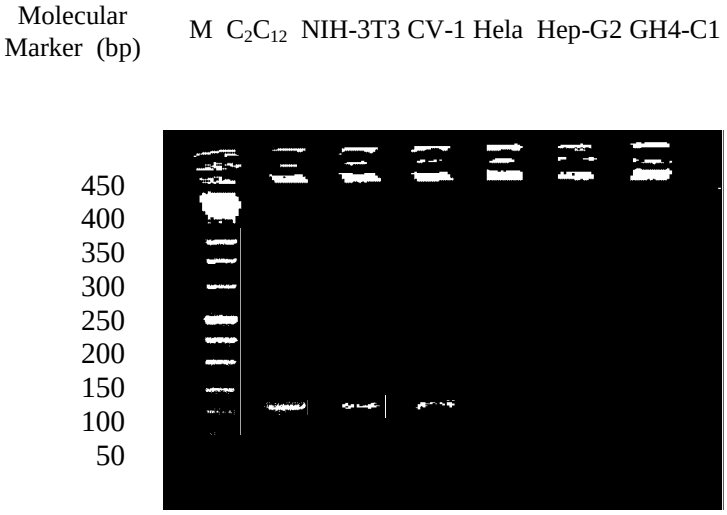


Fig. 7-6 Endogenous expression of UCP2 in C_2C_{12}, Hep-G2, Hela, CV-1, NIH-3T3 and GH_4-C1 cell lines.

7.1.2.3 CAT expression assays

After transient transfection of all generated CAT fusion constructs in various cell lines the resulting CAT expression was monitored. To compare the level of CAT expression, a positive control plasmid where CAT expression was placed under the control of the SV40

promoter as well as a negative, promoterless basic vector were used. Levels of CAT expression were calculated as fold induction of the negative control (promoterless vector) after normalization to protein concentration and/or cell number. The results of the CAT assay are shown in **Fig. 7-7** and **Fig. 7-8**.

From the six different cell lines tested (C_2C_{12}, CV-1, Hep-G2, Hela, NIH-3T3, GH_4C_1) CAT expression level were highest in C_2C_{12} and CV-1. The longest promoter fragment p20 exhibited only a moderate activity. Deletion of about 2000 bp (construct p14) resulted in a strong increase of basal promoter activity comparable to that of the positive control plasmid containing the SV40 promoter. Further deletion of sequences between -982 and -622 (p27) and -622 to -284 (p21) led to a 50% reduction of promoter activity as compared to p14. However, the levels of CAT expression were quite similar between p27 and p21. These findings indicated not only that a strong enhancer element of basal promoter activity is located between nt -982 and -622 in the human UCP3 promoter, but also that a *cis*-acting negative regulatory element is localized within the region from nt -2983 to -982. Deletion of sequences from nt -284 to -162 (p18) reduced the promoter activity further to a level at least 5 fold higher than that of the promoterless construct implying that this region can be regarded as a minimal promoter. The presence of a strong enhancer element between nt - 982 and -622 is well documented by the fact that in construct p19, in which the orientation of the insert of p14 is reversed, considerable promoter activity could be detected which was comparable to that of p21. This effect completely disappeared in construct p23 where the minimal promoter fragment of p18 was fused in reverse orientation to the CAT gene.

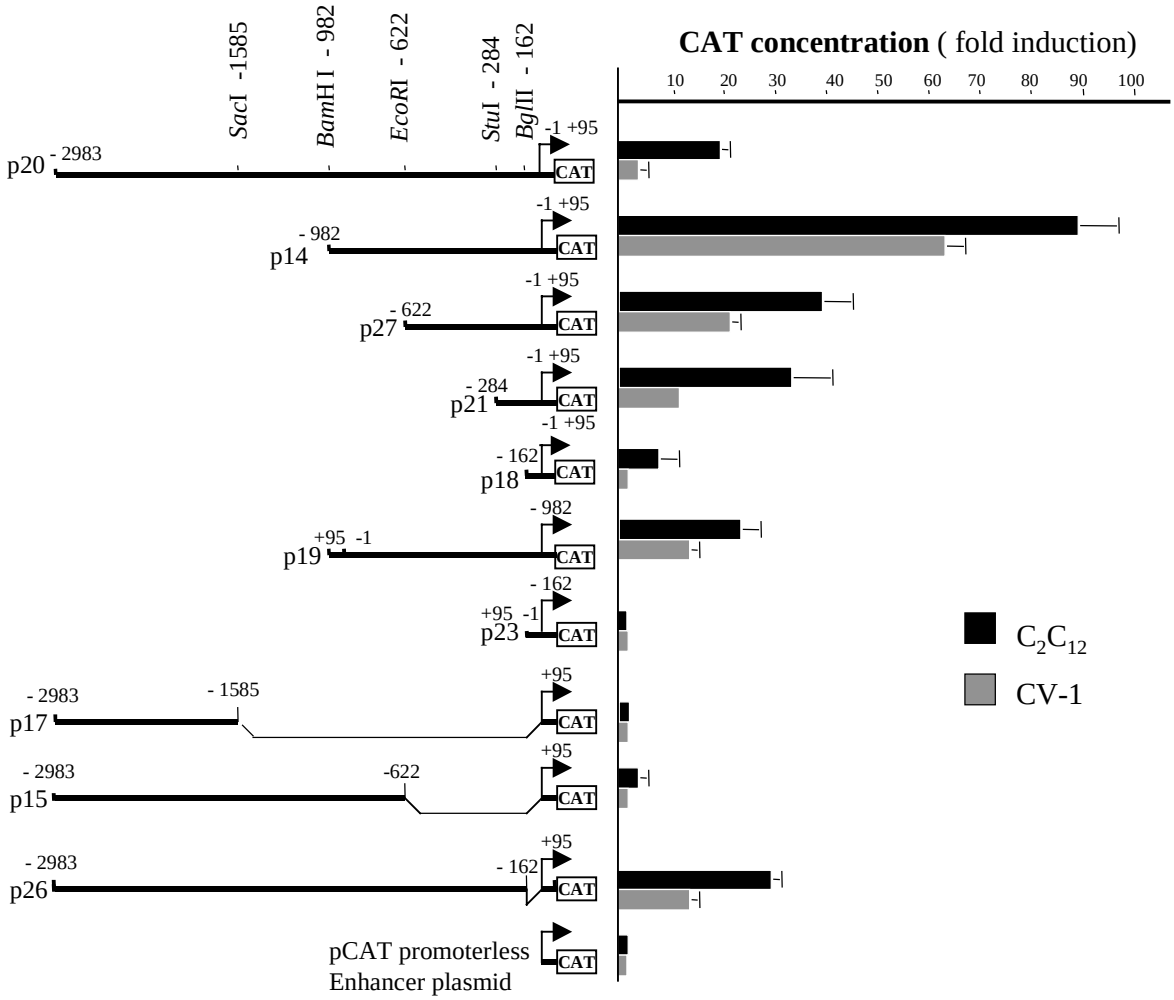


Fig. 7-7 Result of the CAT expression assays of pCAT®-3 derived enhancer plasmids containing various lengths of the 5' flanking region of the human UCP3 promoter in C_2C_{12} and CV-1 cells. CAT protein concentrations are expressed as fold induction of that of the basic vector. Data are represented as the means +/- S.D. based on triplicate assays of three separate experiments.

The presence of a *cis*-acting negative regulatory element in the upper part of the hUCP3 promoter region can clearly be demonstrated by successive nested deletions of p20 (see Fig. 3). In p17 which only contains promoter sequences of nt -2983 to -1585 the inhibitory effect is most prominent; gain of additional sequences downstream of a *Sac*I restriction site at

position -1585 (see constructs p15, p16 and p26), especially those of the region between -982 and -162, showed a progressively increasing promoter activity.

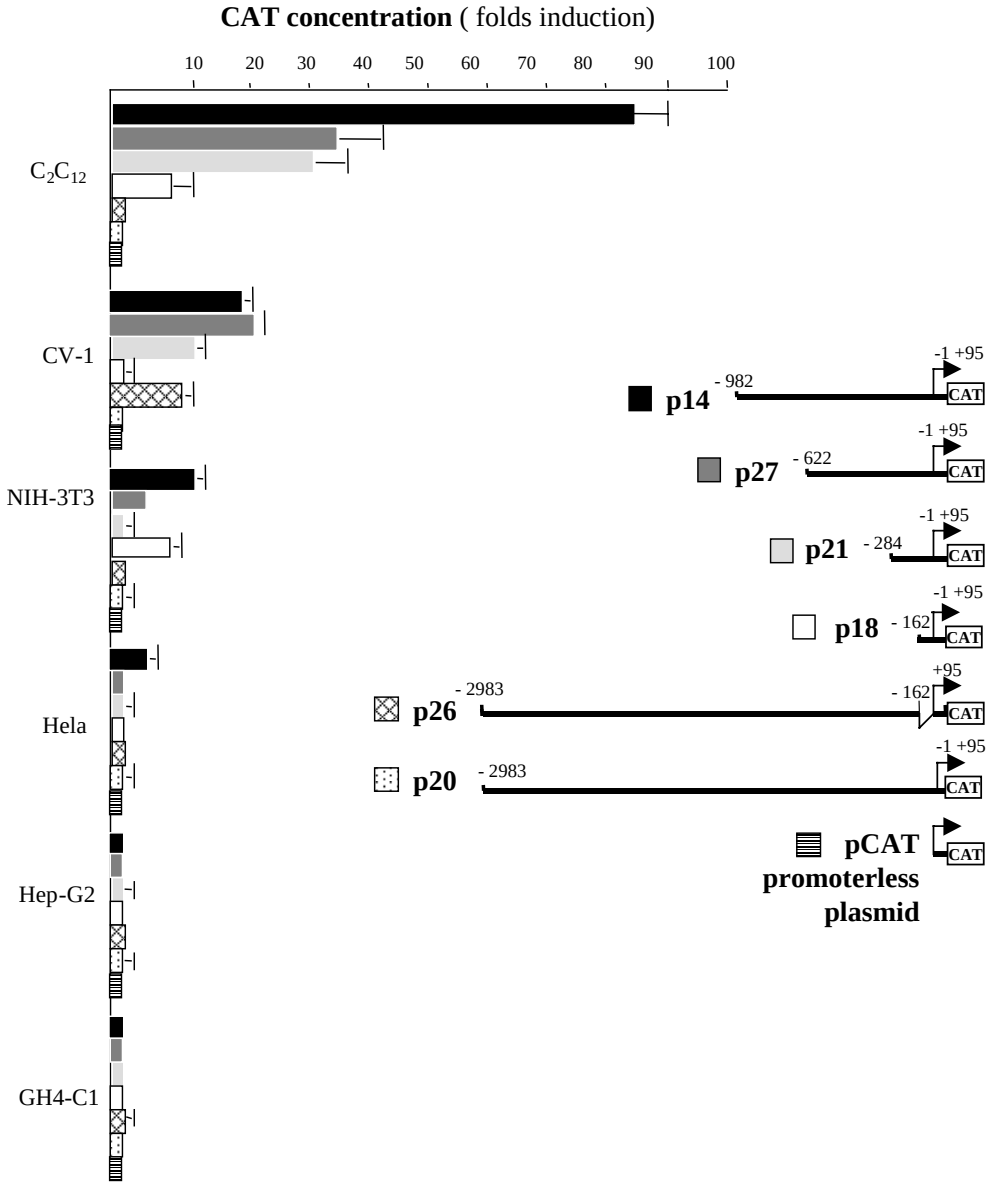


Fig. 7-8 Comparison of the promoter activity of six different deletion constructs in C_2C_{12}, CV-1, HepG2 and Hela cell lines. Data are represented as the means +/- S.D. based on triplicate assays of four independent experiments.

7.1.2.4. CAT expression in the cell lines

When comparing the CAT expression levels of six selected UCP3 promoter constructs, basal promoter activity was highest in C_2C_{12}, less pronounced in CV-1 and NIH-3T3 cells while HepG2, Hela and GH4-C1 cells exhibited the lowest activities. This is in perfect agreement with the fact that UCP3 expression is almost exclusively restricted to skeletal muscle. From the various constructs tested, p14 showed the strongest promoter activity in all cell lines with decreasing efficiencies in constructs p27, p21, p18, p26 and p20 (see Fig. 4). The overall general tendency of the six constructs with regard to their promoter activity tested in the six different cell lines was quite similar to that observed in the murine skeletal muscle cell line C_2C_{12}. An exception may be the result of construct p26 in CV-1 cells which demonstrated considerably higher promoter activity than in the other cell lines. Nevertheless, the region between -982 and -622 could contain a muscle-specific enhancer which contributes to the selective expression pattern of UCP3 in skeletal muscle and which is not active in other cells.

7.2. Discussion

The recent discovery of two new members of the uncoupling protein family, UCP2 and UCP3, somehow has revolutionized obesity research as these homologues, in contrast to its first member UCP1, may have a pivotal role in energy expenditure and thermogenesis in humans (Fleury et al., 1997; Gimeno et al., 1997; Boss et al., 1997). This is supported by linkage studies with markers flanking the human UCP2/UCP3 gene locus as well as through the discovery of genetic variants found to be associated with sleeping metabolic rate in Pima

Indians (Bouchard et al., 1997; Walder et al., 1998). In particular, the almost exclusive expression pattern of hUCP3 in skeletal muscle (little in heart) points to a direct involvement of this gene in thermogenesis. The genomic structure of the hUCP3 gene was recently elucidated (Solanes et al., 1997; Boss et al., 1998; Lentes et al., 1999). A striking feature of UCP3 when compared to its other homologues is the existence of two different transcripts, a long and a short form which are derived from the differential usage of a splice donor site at the end of exon 6 and the presence of a polyadenylation signal (AATAAA) located in intron 6 (Solanes et al., 1997; Lentes et al., 1999). Both transcripts seem to be equally expressed in humans (Solanes et al., 1997), with high levels in skeletal muscle and with low abundancy in heart (Boss et al., 1997; Vidal-Puig et al., 1997).

The finding that UCP3 diminishes mitochondrial membrane potential when expresssed in yeast or C_2C_{12} myoblasts suggests that it may contribute to thermogenesis *in vivo*. Besides being a thermogenic protein, recent studies have shown an upregulation of UCP3 mRNA in response to fasting or circulating free fatty acids indicating a possible role of UCP3 in lipid metabolism (Weigle et al., 1998, Millet et al., 1997).

Up to now, little is known about the mechanisms regulating human UCP3 gene expression *in vitro* and *in vivo*. For the corresponding mouse genes first attempts to the characterization of the 5'-flanking region and analysis of the promoter function have been made (Yamada et al., 1998; Yoshitomi et al., 1998; Yoshitomi et al., 1999). In the present study, we have determined the transcriptional start site of the UCP3 gene and sequenced about 5 kb of its promoter region obtained by genome walking. A 3 kb fragment of the promoter region was inserted immediately 5'-upstream of the chloramphenicol acetyltransferase (CAT) gene contained on a pCAT reporter plasmid and the promoter activity determined by creating deletion constructs which were transiently transfected into 6 different cell lines to assess their general potency on CAT gene transcription. In addition, the possibility of tissue-selective promoter activity was evaluated in multiple cell lines.

To study the mechanisms regulating hUCP3 transcription and to identify *cis*-/*trans*-acting DNA elements important for UCP3 mRNA expression we have mapped the transcriptional start site and analyzed its promoter region. Using a cDNA preparation from human skeletal muscle the 5'-end of hUCP3 mRNA was mapped at position -187 upstream of the first start codon ATG. Sequence inspection of a 3 kb promoter fragment identified a putative TATA-Box at position -45, but no Sp-1, AP-1 or AP-2 motifs near the transcriptional start site could be found. This is different from the corresponding regions in the human and/or mouse UCP2 promoter (Tu *et al*. 1999; Yamada *et al*., 1998; Yoshitomi *et al*., 1998)

Several potential consensus sequences for transcription factors which from *in vitro* experiments were thought to be important for human UCP3 mRNA expression are present. For example, consensus binding motifs for muscle-regulatory proteins (MyoD, MEF2) , as well as CRE1, CRE2, PPARγ, GATA and TRE motifs can be found. The MyoD-binding site (E-box) is proposed to act as a muscle-specific enhancer of murine neurofilament light chain (Molkentin *et al*., 1996; Yaworsky *et al*., 1997), the Myocyte Enhancer Factor-2 (MEF2) site as well as a CCAC-box motif are known to be important for transcriptional regulation of muscle-specific genes (Olson *et al.,* 1995; Cassard-Dpolcier *et al.,* 1994). This is of importance with regard to the selective expression pattern of UCP3 in skeletal muscle.

In order to define *cis*-acting DNA elements necessary for hUCP3 mRNA expression, we have analyzed about 3 kb of the proximal promoter region using a CAT reporter gene assay. Deletion analysis of the hUCP3 promoter in six cell lines of different origin revealed a strong negatively *cis*-acting region between nt -2983 and -982. The region between -982 to -622 on the other hand was found to contain a strong enhancer whose effect gradually disappeared after consecutive deletions of the region between -622 to -162 (constructs p27 to p18). In p18 promoter activity was reduced to the level of a minimal promoter. The presence of an effective muscle-specific enhancer located most likely within the region of -982 to -622 was clearly demonstrated in p19 which contains the region of nt -982 to +95 fused in opposite

orientation to the CAT reporter gene. This region for instance contains a MEF2 site and a MyoD element. In contrast, the localization of a negative *cis*-acting element in the hUCP3 promoter was narrowed down by creating successive nested deletions of the 3'-part. The fragment with the largest deletion of the 3'-region (p17) exhibited almost no activity when compared to the promoterless basic plasmid. Adding more and more sequences from the 3'-part led to a significant increase in CAT expression based upon the presence of the enhancer region located further downstream. Since p17 exhibited the lowest promoter activity it is very likely that the negatively *cis*-acting element resides in the region between nt -2983 and -1585.

To test for tissue-selective promoter activity we have examined promoter function of hUCP3 in different cell lines. Our results convincingly demonstrate that UCP3 as an almost exclusively expressed muscle specific gene showed highest promoter activity in the murine myoblast cell line C_2C_{12}, to a lesser extent in CV-1 and NIH-3T3 cells, and with lowest expression levels in HepG2, Hela and GH4-C1 cells. This is in contrast to the findings of Yoshitomi *et al.* (1999) who showed that mouse UCP2 promoter constructs were most active in HepG2 cells while C_2C_{12} and NIH-3T3 cells exhibited a weaker response. These results may be explained by the ubiquitous nature of UCP2 expression. The fact that considerable promoter activities could be detected in CV-1 cells is somewhat surprising as UCP3 expression in kidney so far has not been reported. Interestingly, from our own RT-PCR experiments where we checked for endogenous expression of UCP3 in the six cell lines tested we were able to detect message not only in C_2C_{12} (strong signal), but also in CV-1 and NIH-3T3 cells (weak signal).

In conclusion, the promoter region of human UCP3 gene contains both TATA and CAAT boxes as well as consensus motifs for PPRE, TRE, CRE and muscle-specific factors like MyoD and MEF2 sites. Functional characterization of a 3 kb hUCP3 promoter fragment in multiple cell lines using a CAT-ELISA identified a *cis*-acting negative regulatory element

between - 2983 and -982 while the region between -982 and -284 showed greatly increased basal promoter activity suggesting the presence of a strong enhancer element. Promoter activity was particularly enhanced in the murine skeletal muscle cell line C_2C_{12} reflecting the tissue-selective expression pattern of UCP3. The identification of consensus sequences for various transcription factors, among them PPARgammer, CRE1/2, and TRE in the 3 kb promoter fragment of hUCP3 confirms earlier findings of *in vitro* studies where administration of beta-adrenergic agonists (generation of cAMP via activation of beta-3/-4 receptors expressed in BAT and skeletal muscle), thyroid hormones, glucocorticoids, and in particular PPARγ agonists were able to increase UCP3 mRNA expression. To clarify the potential significance these elements may have on regulation of hUCP3 expression *in vitro* and *in vivo*, electromobility shift assays and DNA-footprinting analysis should be employed.

Chapter 8. Final Discussion

Throughout most of human history, weight gain and fat storage have been viewed as signs of heath and prosperity. In times of labor and frequent food shortages, securing an adequate energy intake to meet requirements has been the major nutritional concern. Today, however, as standards of living continue to rise, weight gain and obesity are posing a growing threat to the health of inhabitants from countries all over world. Indeed, it is now so common that it is one of the most significant contributors to ill health. Certainly, obesity is not recent phenomenon, Ist historical root can be traced to the Palaeolithic era more than 25,000 years ago, since stone age artifacts of corpulent women have been found in several sites acroos Europe. However, the prevalence of obesity gas never before reached such epidemic proportions as today.

Clinical evidence of obesity can be dated as far back as Greco-Roman times, but little scientific progress was made towards understanding the condition until the 20th century. In the 19th century , the work of Lavoisier and others indicated the metabolism was similar to slow combustion, and that obese and lean human obeyed the law of thermodynamics. In addition, the discovery that fat is stored in cell, the basic unit of biology, led to the idea that obesity could be caused by too many fat cells.

With the turn of the century, analysis of life insurance data indicated that obesity was associated with an increased death rate. In the 1920s, a familial basis for obesity was suggested along with descriptions of Cushing’s Disease and hypothalamic obesity. Later, the introduction of thyroid hormone, dinitrophenol and amphetamine as pharmacological treatments of obesity opened the door to the use of drugs, and the field of genetics improved understanding of several specific forms of obesity resulting from genetic defects.

In terms of pathogenesis of human obesity, today most of people believe that obesity is a consequence of an energy imbalance where energy intake has exceeded energy expenditure over a considerable period. Numerous different complex and diverse factors can give rise to a positive energy balance, but it is the interaction between a number of these influences, rather than any single factors acting alone. Genetic, environmental, psychological, and other factors all may play a part. (1) Genetic Factors. Obesity tends to run in families, suggesting that it may have a genetic cause. However, family members share not only genes but also diet and lifestyle habits that may contribute to obesity. Separating these lifestyle factors from genetic ones is often difficult. Still, growing evidence points to heredity as a strong determining factor of obesity. In one study of adults who were adopted as children, researchers found that the subjects' adult weights were closer to their biological parents' weights than their adoptive parents'. The environment provided by the adoptive family apparently had less influence on the development of obesity than the person's genetic makeup. Nevertheless, people who feel that their genes have doomed them to a lifetime of obesity should take heart. As discussed in the next section, many people genetically predisposed to obesity do not become obese or manage to lose weight and keep it off. (2)Environmental Factors. Although genes are an important factor in many cases of obesity, a person's environment also plays a significant part. Environment includes lifestyle behaviors such as what a person eats and how active he or she is. Some people tend to have high-fat diets, often putting taste and convenience ahead of nutritional content when choosing meals. Some people also don't get enough exercise. People can't change their genetic makeup, of course, but they can change what they eat and how active they are. Some people have been able to lose weight and keep it off by: Learning how to choose more nutritious meals that are lower in fat. Learning to recognize environmental cues (such as enticing smells) that may make them want to eat when they are not hungry. Becoming more physically active. (3)Psychological Factors. Psychological factors also may influence eating habits. Many people eat in response to

negative emotions such as boredom, sadness, or anger. While most overweight people have no more psychological disturbance than normal weight people, about 30 percent of the people who seek treatment for serious weight problems have difficulties with binge eating. During a binge eating episode, people eat large amounts of food while feeling they can't control how much they are eating. Those with the most severe binge eating problems are considered to have binge eating disorder. These people may have more difficulty losing weight and keeping the weight off than people without binge eating problems. Some will need special help, such as counseling or medication, to control their binge eating before they can successfully manage their weight. (4)Other Causes of Obesity. Some rare illnesses can cause obesity. These include hypothyroidism, Cushing's syndrome, depression, and certain neurological problems that can lead to overeating. Certain drugs, such as steroids and some antidepressants, may cause excessive weight gain. A doctor can determine if a patient has any of these conditions, which are believed to be responsible for only about 1 percent of all cases of obesity.

There are a number of important themes, which include:

- Obesity can result from a minor energy imbalance which leads to a gradual but persistent weight gain over a considerable period. Once the obese state is established, physiological processes tend to defend a new weight

- Body weight is primarily regulated by a series of physiological processes but is also influenced by external societal and cognitive factors.

- Recent epidemiological trends in obesity indicate that the primary cause of the obesity problem lies in environmental and behaviour changes. The rapid increases in obesity rates

have occurred in too short a time for there to have been significant genetic change within populations.

- The increased proportion of fat and the increased energy density of the diet, together with reductions in level of physical activity and rise in the level of sedentary behaviour, are thought to be major contributing factors to the rise in the average body weight of populations.

- The obesity problem can be viewed as a consequence of the massive social, economic and cultural problem.

- Epidemiological, genetic and molecular studies in many populations of the world suggest that there are people who are more susceptible to weight gain and the development of obesity than others. Genetic, biological and other personal factors such as smoking, sex and age interact to determine an individual's susceptibility to weight gain.

As regards the treatment of human obesity, considerable advances have been made in diet, exercise and behavioural approaches to treatment for obesity in first half of the 20th century, and gastric surgery has had the most effective long-term success in treating the severely obese. However, new drugs with ever better profiles of pharmacological activity continue to be introduced on a regular basis.

The discovery of the uncoupling proteins could be a breakthrough in understanding the complex mechanisms regulating energy expenditure and has given new stimuli for research in this field. The results so far strongly suggest a role for the UCPs in energy balance and obesity. Unfortunately, it is not possible yet to come up with a unifying, inclusive hypothesis on the physiological role of UCPs, a lot of questions have yet to be answered to understand

fully the importance of these proteins, which include generating antibodies for the proteins and developing assays to measure protein levels directly in vivo; what are the molecular and physiological similarities and dissimilarities among UCPs? what are mechanisms of action? are the other functions for UCP2 and UCP3?

To get more definitive proof that UCPs are involved in regulating basal metabolic rates, and thus weight gain or loss, engineering mice should be generated, in which the genes encoding the proteins are either knocked out or overactive, those race will be on to find drugs that can combat obesity by tuning up the activity of the proteins. If the level of uncoupling proteins could be slightly increased 1%-2%, then fat oxidation and thermogenesis would increased, and that could boost the resting metabolic rates of millions of people and whittle away their days of perpetual dieting.

Because obesity prevalence continues to increase sharply as people approach the next century, today the challenge to public health worker and scientists in this area has never been greater.

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Abbreviations

A

aa	Amino Acid
ABTS	Peroxidase Substrate
ADP	Adenosine Diphosphate
A/J	Obesity-Resistant Mice
AMP	Adenosine Monophosphate
AP-1 / -2 Motif	Activator protein 1 / -2 binding site
ASP	Allele-Specific PCR
ATP	Adenosine Triphosphate
ß-AR	ß-Adrenergic Receptor

B

BAT	**Brown Adipose tissue**
BMR	Basal Metabolic Rate

C

cAMP	Cyclic Adenosine Monophosphate
CAT	**Chloramphenicol Acetyltransferase Type 1 (Bacterial Enzyme)**
C_2C_{12}	Mouse Myoblasts Cell Line
C/EBPß	CCAAT/ enchancer-binding protein beta
CHD	Coronary Heart Disease

CHO	Carbohydrate
CREB2	cAMP Response Element Binding Protein 2
CVD	Cardiovascular Diseases
CV-1	**Green Monkey Kidney Cell Line**

D

Da	Dalton
db / db	Obese *db / db* C57bl Mice
DMEM	Dulbecco's modified Eagle's medium
dNTPs	Deoxyribonucleoside Triphosphates

E

ELISA	Enzyme-Linked Immunosorbent Assay

F

fa / fa	Obese *fa / fa* Zuker Rats
FFAs	Free Fatty Acids
FBS	Fetal Bovine Serum

G

GDP	Guanosine Diphosphate
GH4-C1	Rat Pituitary Adenoma

GSP	Gene Specific Primer
NGSP	Nested Gene Specific Primer

H

Hela	Human Hepatocellular Carcinoma cell line
Hep G2	Human Cervix Carcinoma Cell line

M

MC4R	Melanocortin-4 Receptor
MCS	multiple cloning site
MEF 2	Myocyte-Specific Enhancer Factor-2 Protein
MEM	Modified Eagle’s Medium
mRNA	Message Ribonucleic Acid
MTP	Microtiter Plate
MyoD	Muscle specific regulatory protein

N

NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NCDs	Noncommuniable Diseases
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NIH-3T3	Swiss Mouse Embryonic Fibroblastes Cell Line
NST	Nonshivering Thermogenesis

O

OB	Obesity
ob / ob	Obese *Ob / Ob* 57bl Mice

P

PCR	Polymerase Chain Reaction
PNBD	Puring-Nucleotide Binding Domain
Poly (A)	Poly-adenylation Signal
PPAR	Peroxisome Proliferator-Activated Receptor

Q

QTLs	Quantitative Trait Loci

R

RACE	Rapid Amplification of cDNA Ends
RMR	Resting Metabolic Rate
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

S

Sp1 Motif	Specificity Protein 1 binding site
SV 40	Simian Virus 40
SSCP	Single-Strand Conformational Polymorphism

T

T3	Tri-iodothyronine
TEF	Thermic Effect of Food
TRE	Thyroid Hormone Response Element

U

UCP	Uncoupling Protein
hUCP	Human Uncoupling Protein
UCP3L	Uncoupling Protein 3 Long Form
UCP3S	Uncoupling Protein 3 Short Form
UTR	Untranslated Region

W

WAT	White Adipose Tissue
WHO	World Health Organization

Appendixes

HONOR

- Prize awarded: der INNOVATIONSPREIS 1999, Universität Trier.

AWARDS

- Fellowship awarded: Nikolas-Koch-Stiftung, from 3/1997-1999.

- Scholarship awarded: BMBF, German Federal Ministry of Education, Science, Research and Technology, from 5/1996-2/1997.

MEMBERSHIPS

- Membership of North American Association for the Study of Obesity (NAASO)

- Membership of Chinese Environmental Mutagen Society and Chinese Toxicological Society

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EDUCATION

- April of 2000 – present: Post Doctor Training, Institute of Molecular Medicine and Genetic, Medical College of Georgia, the United States. Research area: Chromatin remodeling and transcription regulation.
- April of 2001, Dr.rer.nat. University of Trier, Germany. Dissertation Title: Obesit-Environmental Factors and / or Genetic Influence, -Genomic Structure, Mutation Analysis and Promoter Function of the Human Uncoupling Protein -2/-3 (hUCP2/hUCP3) gene
- To March of 2000, Doctoral Student (Molecular Genetics), University of Trier, Germany. Thesis Title: Genomic Structure, Mutation Analysis and Promoter Function of hUCP2/hUCP3 gene.
- July of 1993, M.Sc. (Biology), Yunnan Normal University, China. Dissertation Title: Structure-Function Relationship in B1 esterase.
- July of 1990, B.Sc. (Biochemistry), East China Normal University, China.

ACADEMIC RESEARCH EXPERIENCE

- April of 2000 – present: Post Doctor Fellow, Gene Regulation, Institute of Molecular Medicine and Genetic, Medical College of Georgia, Augusta, the United States. Research areas: Chromatin remodeling and transcription regulation, etc.
- March of 1997 – March of 2000: Ph.D Student, Laboratory of Molecular Neurogenetics, University of Trier, Trier, Germany. Study projects: Structural organization, polymorphism and promoter function analysis of the human uncoupling protein - 2 / -3 (hUCP2 / hUCP3) genes.
- May of 1996 – March of 1997: Visiting Scholar, Federal Research Center for Health and Environment (GSF), Munich, Germany. Research focus: Tumor promoters, inhibition of transforming growth factor-beta1 and UV light-induced apoptosis by prostanoids in primary cultures of rat hepatocytes.
- Cooperative research (November of 1996 to January of 1997), WHO Collaborating Center for Occupation Health, Dortmund, Germany. Research topic: Associations of polymorphic glutathione S-transferase gene with lung cancer.

- September of 1995 – May of 1996: Assistant Researcher, Project Leader, Shanghai Institute of Entomology, Chinese Academy of Sciences, Shanghai, China. Research focus: Polymorphic genotype of the glutathione S-transferase gene in Chinese population.

- July of 1993 – August of 1995: Practical Researcher, Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China. Research focus on expression of proteins from cloned the core antigen gene of hepatitis virus C and the Shiva-1 gene by a baculuvirus expression system.

- September of 1990 – July of 1993: Graduate Student. Shanghai Research Center of Biotechnology, Chinese Academy of Sciences, Shanghai, China. Study project: Structure-Function Relationship in B1 esterase; construction cDNA library of insecticide-resistant mosquito (*Culex pepienes quinquefasciatus, TemR*) and library analysis.

HONORS AND AWARDS

- Prize awarded: der INNOVATIONSPREISE (Innovation Prize) 1999, Universatät Trier.

- Fellowship awarded: Nikolas-Koch-Stiftung,

- Scholarship awarded: BMBF (German Federal Ministry of Education, Science, Research and Technology)

- Membership of North American Association for the Study of Obesity (NAASO). Membership of Chinese Environmental Mutagen Society and Chinese Toxicological Society

PUBLICATIONS

- Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. -U. Structure Organization and Mutational Analysis of Human Uncoupling Protein-2 (hUCP2) Gene. *Life Sci.*, Vol. 64, No. 3, PL 41-50, 1999.

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ABSTRACTS

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- Lentes, K. -U., Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G. and Pirke, K. M. Identification of a Mutation (Ala55Val) in the Human UCP2 Gene: Possible Effects on Energy Metabolism and Body Weight Regulation. *Obesity Research*, Vol.5, Suppl. 1, 23s, 1997

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List of Publications and Abstracts During the Doctoral Study

- **PUBLICATIONS**

1. Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K.-U. Functional Characterization of the 5'-Flanking Region and the Promoter Region of the Human UCP3 (hUCP3) Gene. *Life Sci.*, Vol. 22; 67 (18), 2267-2279, 2000.

2. Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. -U. Molecular Cloning, Functional Characterization of Promoter Region of the Human Uncoupling Protein 2 Gene. *Biochem. Biophys. Res. Commun*. ,Vol. 265, 326-334, 1999.

3. Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. -U. Structure Organization and Mutational Analysis of Human Uncoupling Protein-2 (hUCP2) Gene. *Life Sci.*, Vol. 64, No. 3, PL 41-50, 1999.

4. Lentes, K.-U., Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G. and Pirke, K.M. Genomic Organization and Mutational Analysis of the Human UCP2 Gene, a prime Candidate Gene for Human Obesity. *J. Receptor Research*, Vol. 19, No. 1-4, 229-244, 1998.

5. Kroll, B., Kunz, S., Tu, N. and Schwarz, L. R. Inhibition of Transforming Growth Factor-beta1 and UV light-induced Apoptosis by Prostanoids in Primary Cultures of Rat Hepatocytes. *Toxicol. Appl. Pharmacol*, Vol. 152, No. 1, 240-250, 1998.

- **ABSTRACTS**

1. Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. -U. Characterization of the 5'-Flanking Region and the Promoter Region of the Human UCP3 (hUCP3) Gene: Identification of a Novel Polymorphism in Close Proximity to the Putative TATA Signal. Annual Meeting of North American Association for the Study of Obesity, Nov., 14-18, 1999.

2. Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. -U. Mapping of the Promoter Region and the Boundaries of the Human UCP3 Gene by Genome Walking and 5'-/3'-RACE. *International Journal of Obesity*, Vol. 22, Suppl. 3, S6, 1998 (Oral presentation at the 8th International Congress on Obesity, Paris, Aug., 1998).

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4. Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G., Pirke, K. M. and Lentes, K. -U. Analysis of the Genomic Organization of the Human UCP2 Gene, *Obesity Research*, Vol.5, Suppl. 1, 84s, 1997

5. Lentes, K. -U., Tu, N., Chen, H., Winnikes, U., Reinert, I., Marmann, G. and Pirke, K. M. Identification of a Mutation (Ala55Val) in the Human UCP2 Gene: Possible Effects on Energy Metabolism and Body Weight Regulation. *Obesity Research*, Vol.5, Suppl. 1, 23s, 1997