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Obesity is a serious human health concern in the world today. Over one third of north American adults are overweight. In animal production, to increasing the feed efficiency and to obtain less fat and more muscle in meat producing animals are the ultimate goal. So it is very important to study and control fat. Fat growth is attributed to adipose accretion, and adipose accretion can be achieved by hypertrophy and hyperplasia. Adipocyte differentiation is a very complicated development program. Several transcription factors have been identified during adipogenesis. They include CCAAT/ enhancer-binding protein α (C/EBPα), the peroxisome proliferator-activated receptor γ (PPARγ), and the retinoic X receptor (RXR)

The chicken ovalbumin upstream promoter transcription factor COUP-TF, an orphan member of the steroid / thyroid hormone receptor superfamily, has been proposed to play a key role in regulating organogenesis, neurogenesis, and cellular differentiation during embryonic development. Recently, TCDD (2,3,7,8 - tetrachlorodibenzo-p-dioxin) has been shown to inhibit adipocyte differentiation both in vitro and in vivo, and TCDD induces COUP-TF binding to a PPAR/RXR binding sequence, which is a DNA response element of the adipocyte-specific aP2 gene promoter. Transient transform assay has proved that COUP-TF is involved in the adipogenesis and plays a negative role.

But what is the molecular repression mechanism(s) of COUP-TF during adipogenesis is not known. To answer the question, four mutations have been generated by oligo-directed mutagenesis. Of these mutations, one is mutated at a position of the DNA binding domain, one is mutated at a position of the dimerization domain, one is

without the last 15 amino acids at the C-terminal, and the last one is without the last 25 amino acids at the C-terminal. By transfecting into preadipocyte cell line, these mutations will be useful to study the molecular mechanism(s) of COUP-TF-mediated repression during adipogenesis.

Site-directed Mutagenesis of Chicken Ovalbumin Upstream Promoter Transcription Factor I (COUP-TFI) in Different Functional Domain

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Zhaohong Wang, Author

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Dedication

This thesis is dedicated to my family:

To my dearest wife, Nan Hu, for her endless love and consistent patience.

To my parents, for teaching me to be a hero of indomitable spirit.

To my brothers and sisters, for their considerate care.

To my wife's family, for their understanding and support.

Site-directed Mutagenesis of Chicken Ovalbumin Upstream Promoter Transcription Factor I (COUP-TFI) in Different Functional Domain

INTRODUCTION

Obesity is a serious human health concern in the world today. Over one third of North American adults are more than 30% overweight (Scott, 1996). In animal production, to increase the feed efficiency and to obtain less fat and more muscle in meat producing animals are always the ultimate goal. So it is very important to study and control fat. Fat growth is attributed to adipose accretion, and adipose accretion can be achieved by hypertrophy, hyperplasia or combination of both (Hirsch et al., 1989). Hypertrophy is a process of increasing the fat cell size by accumulating triglycerides, and it is controlled by lipogenesis and lipolysis. Hyperplasia is a process of increasing the fat cell number, and it is regulated by fat cell replication and differentiation. Adipocyte differentiation is a very complicated development process. It has been esteemed that at least 300 hundred proteins are altered or expressed during adipogenesis (see review by Cornelius et al, 1994; MacDougald et al. 1995). Several transcription factors which may play important role during adipogenesis have been identified. They include CCAAT/ enhancer binding protein α (C/EBPα), the peroxisome proliferator-activated receptor γ (PPARγ), and the retinoic X receptor (RXR).

The chicken ovalbumin upstream promoter transcription factor (COUP-TF), an

orphan member of the steroid / thyroid hormone receptor superfamily, has been proposed to play a key role in the regulating organogenesis, neurogenesis, and cellular differentiation during embryonic development. Recently, TCDD (2, 3,7,8 - tetrachlorodibenzo-p-dioxin) has been shown to inhibit adipocyte differentiation both in vitro and in vivo (Chen et al., 1996; and Brodie et al., 1996a), and TCDD induces COUP-TF binding to a PPAR/RXR binding sequence, which is a DNA response element of the adipocyte-specific aP2 gene promoter (Brodie et al., 1996b). COUP-TF mRNA is presented in early stage in adipogenesis and is down regulated, but not completely inhibited, after induction of 3T3-L1 cells to differentiate (Brodie et al., 1996c). Transient transfection assay has shown that COUP-TF is involved in the adipogenesis and plays a negative role (Brodie et al., unpublished data).

But the molecular repression mechanism (s) of COUP-TF during adipogenesis is not known. To answer the question, four mutations have been generated by oligo-directed mutagenesis. Of these mutations, one is mutated at a position in the DNA binding domain, one is mutated at a position of in the dimerization domain, one is without the last 15 amino acids at the C-terminal, and the last one is without the last 25 amino acids at the C-terminal. By transfecting into preadipocyte cell line, these mutations will be useful to study the molecular mechanism(s) of COUP-TF-mediated repression during adipogenesis.

In the following literature review chapter, the general background regarding recent research on adipose tissue specially on obesity gene (leptin) and its cognate receptor, adipocyte differentiation, COUP-TF and its effect on adipogenesis will be addressed.

LITERATURE REVIEW

The ob gene and db gene

For a long time, our knowledge about fat has been surprisingly little and ambiguous. Facing the adipose tissue , the most dynamic and exciting organ , scientists had been groping in the dark and showing some kind of no alternative, even after the discovery of ob/ob mice in 1950's. But with the identification of the mouse ob gene in 1994 (Zhang et al., 1994), a new era of fat research has arrived. "Nowhere has research been moving faster in the past year than in the study of obesity" as a cell biologist said (Chua et al., 1996).

The hottest topic, the biggest news in fat research over the past two years has been "the fat gene". The fat cell is now officially a hormone secreting tissue. Obese ob/ob mouse is a genetic model for the early onset obesity as a recessive trait. By means of crossbreeding and positional cloning strategies, the physical and genetic maps of the mouse ob gene and its homolog in human are established (Zhang et al., 1994), the allele is located on chromosome 6. The ob gene encodes a 4.5 kb mRNA in adipose tissue. The predicted amino acid sequence is 84% identical between human and mouse. A nonsense mutation in codon 105 is found in the ob mice gene. A recessive mutation of the obese gene in mouse (ob/ob mice) results in a syndrome that includes obesity, increased body fat deposition, hyperglycemia, hyperinsulinemia, and hypothermia. Parabiotic study suggests that ob/ob mice are deficient for a blood-borne factor that could regulate adiposity by modulation of appetite and metabolism (Tartaglia et al., 1995). The product of this obese

gene, dubbed leptin, Greek for thin, when not perturbed by mutation, is secreted from the fat cells and acts on the hypothalamus of the brain to regulate body weight and fat deposition through effects on metabolism and appetite (Pelleymounter et al., 1996; Yu et al., 1997). The leptin is a 16 KD, monomer, 146 amino acid protein in mouse and human. Its existence is not detected in the plasma from ob/ob mouse. The cloning of this ob gene has provided a basis to investigate the potential role of the ob gene product in body weight regulation. A biologically active form of recombinant mouse ob protein was overexpressed and purified to near homogeneity from a bacterial (an E.Coli) expression system. Using this recombinant leptin, a series of experiments have been conducted (Pelleymounter et al., 1996; Halass et al., 1996; and Campfield et al., 1996). Daily intraperitoneal injections of the ob/ob mouse with the recombinant leptin lowered their body weight (by 30%), percent body fat, food intake, and serum concentrations of glucose and insulin. In addition, metabolic rate, body temperature , and activity levels were increased by this treatment. The data suggest that the leptin regulates body weight and fat deposition through effects on metabolism and appetite. In other words, leptin serves an endocrine function to regulate body fat store. Peripheral and central administration of microgram doses of the recombinant leptin reduced food intake and body weight of ob/ob and diet-induced obese mice. The behavioral effects after brain administration suggest that leptin can act directly on neuronal networks that control feeding and energy balance.

However, there are some other kinds of obese mice and humans in which both the tissue mRNA and the plasma concentration of leptin is relative high and these mice fail to respond to injection of the recombinant leptin (Scott et al., 1996). This so-called "leptin

resistance" phenomena lead to another well-characterized recessive obesity mutation story -- the obese diabetic (db) mice. There was concern that the leptin might not have the right receptor. The autosomal recessive diabetes mutation (db) was first detected in progeny of a special strain mouse, and mapped in the middle of chromosome 4. The phenotype of db/db mice, includes severe, early-onset obesity, extreme insulin resistance, and strain-specific susceptibility to diabetes (Tartaglia et al., 1995; Barinaga et al., 1996; and Maffei et al., 1995.). The fatty (fa) gene in rats is thought to be a homolog of db, because fa/fa mutants have an identical behavioral and metabolic phenotype as db/db mutants and because fa and db map to syntonic chromosomal regions (Maffei et al., 1995; and Frederich et al., 1995). Since mice harboring mutations in the obese (ob) and diabetes (db) genes display similar phenotypes, fatty, it has been proposed that the ob and db genes might encode the ligand and receptor receptively, for a physiologic pathway that regulates body weight. Evidences for that hypotheses came from three groups at almost the same time. The work of Campfield et al. (1996) and Stephens et al. (1995) demonstrated that leptin introduced into the lateral or third brain ventricle is effective at low doses, arguing for a direct central affect of the leptin molecule. Tartaglia et al. (1995) first identified leptin-binding sites in the mouse choroid plexus. They built a cDNA expression library using mRNA isolated from this tissue and screened with a leptin-AP fusion protein to clone the leptin receptor (OB-R). OB-R is a single membrane-spanning receptor most related to the gp130 signal-transducing component of the interleukin L-6 receptor, and to the receptor for granulocyte colony-stimulating factor and leukaemia-inhibitory factor (Nakashima et al., 1997). Genetic mapping of this gene shows that it is within the 5.1 cm

interval of mouse chromosome 4. The db gene transcribes the OB-R, while the db/db mutant mice transcript an abnormal OB-R mRNAs in which the noncoding introns are spliced out of the RNA and interrupted to translate a short form of the OB-R without the cytoplasmic tail, which cannot transmit intracellular signals, and thus is a nonfunctional receptor. As for the fa/fa mutant mice, the OB-R gene is deleted, so it codes no receptor at all. OB-R is expressed in pancreatic islets, liver, kidney, spleen, lung, heart as well as in hypothalamus (Emilsson et al., 1997).

Bit by bit, the process of leptin signaling and the control of obesity is being pieced together. Intensive work now focuses on signal transduction pathway of ob receptor and the transport of leptin in blood.

Obesity is the most prevalent nutritional disorder in consumer societies. For instance, one-third of North American adults are more than 30% overweight (Pelleymounter et al. 1996, and Rohner et al., 1996). The condition is associated with other health problems such as non-insulin-dependent diabetes mellitus (NIDDM), hyperextension, hyperlipidaemia, coronary heart disease and musculoskeletal disorders. Both environmental and genetic factors contribute to these conditions, but in humans the genetic component is poorly defined.

Recent studies have suggested that obese humans and rodents (other than ob/ob mice) are not defective in their ability to produce leptin mRNA or protein and generally produce higher levels than lean individuals (Golden et al., 1997). This suggests that resistance to normal or elevated levels of leptin may be more important than inadequate leptin product in human obesity. Therefore, the underlying mechanism of leptin signal

reception is the main concern. The identification and expression cloning of the OB-R is undoubtedly "a major breakthrough" (Barinaga et al., 1996; Maffei et al., 1995; and He et al., 1995). It could help scientists understand how leptin works to control weight, and why overweight humans, who produce large amounts of leptin, appear insensitive to it. It may also lead scientists to design substances that might mimic or enhance leptin's effects and prove useful as anti-obesity drugs. "It opens a whole new opportunity for pharmacological research" says an obesity researcher (Rohner et al., 1996).

Researchers are now begining to use the recombinant leptin and OB-R to help unravel the biology of weight control and obesity (Pelleymounter et al., 1996; Scott et al., 1996; and Rentsch et al., 1996). The next questions to ask are whether obese people are insensitive to leptin because of defects in the receptor itself or in the signaling transduction pathways that the receptor turns on inside the cells. This kind of discovery will trigger a new race to find drugs that boost the activity of the receptor (Chua et al., 1996; Chehab et al., 1996; and Nisoli et al., 1996).

There is no guarantee such drugs will be found. Small organic compounds that can mimic the effects of protein hormones such as leptin have been notoriously elusive. For instance, "nobody has found or developed an organic compound that can mimic insulin, and we have had the insulin receptor in hand for years." (Chua et al., 1996). Whether a drug can be found which can mimic the effects of leptin, we will wait and see.

Little is known of the regulation of gene expression for the leptin at the level of transcription. He et al. (1995) have determined the genomic organization of the 5' end of the mouse obese gene, and identified a minimal promoter at -161 position which contains

the consensus sp1 and CCAAT/enhancer-binding protein (C/EBP) motifs (He et al., 1995). Cotransfection with C/EBPα caused 23-fold activation, which suggests that the leptin gene promoter is a natural target of C/EBPα (He et al., 1995; Miller et al., 1996; Vidal et al., 1996). However, the transcription mechanism involved is far from being understood, especially in other species.

There are reports that the ob gene expression is under hormonal control, which is expected for a key factor controlling body weight homeostasis and energy balance. The leptin gene expression is increased by feeding, insulin, and glucocorticoid (Miller et al., 1996; Vidal et al., 1996; Slieker et al., 1996; Kolaczynski et al., 1996; Widjaja et al., 1997; and Tuominen et al., 1997). The endotoxin and cytokines induce expression of leptin (Grunfeld et al., 1996). On the other hand, the antidiabetic thiazolicinedione, free fatty acid and agents that increase intracellular cAMP, such as beta-adrenergic agonists or Bt2cAMP down-regulate the expression of the leptin gene (Kallen et al., 1996; Zhang et al.,1996; and Rentsch et al., 1996; Deng et al., 1997). However, the mechanism and the DNA sequence responsible for this regulation of leptin gene expression warrants further examination.

Fat cell differentiation

Cell culture systems have been developed to study fat cell differentiation in vitro. These systems are either primary cultures of adipocyte precursor cells or preadipocyte cell lines, which have been isolated and cloned from a number of sources and can grow in culture through many passages. Many preadipocyte cell lines have been isolated: 3T3-L1 (Green and Kehinde, 1974), 3T3-F442A (Green and Kehinde, 1976), OB17 (Negrel et al., 1978), TA1 (Chapman et al., 1984), PFC6 (Ailhaud, 1982). The most extensively characterized preadipocyte cell lines are the 3T3-L1 and 3T3-F442A lines. These cell lines were derived from disaggregated mouse embryo cells and were selected for their propensity to accumulate triacylglycerol lipid droplets.

Adipocyte differentiation is a complex process involving a cascade of molecular events. The key features of the sequence of events in program of adipocyte development are illustrated in Figure 1 (cited from the review by MacDougald and Lane, 1995). Briefly, uncommitted multipotent stem cell of mesodermal origin (e.g.10T1/2 cell line) commits to become adipoblast (e.g.3T3L1 cells), then cell/cell contact at confluence and mitotic clone expansion to preadipocyte. The preadipocytes will finally be induced to terminally differentiate to immature and mature adipocyte (see review by Ailhaud et al., 1992; Sul et al., 1993; Cornelius et al., 1994; and MacDougald et al., 1995). Determination is the first step for cell development. MyoD family has been identified as a master control molecule (Edmondson et al., 1993), which can direct the fate of multipotent mesodermal cell lineages, repress proliferation and activate muscle cell differentiation. However, the homologue gene that control the commitment to adipocyte lineage have not been

identified yet. It has been estimated that at least 300 proteins are altered or expressed during adipogenesis (Sidhu, 1979; Sadowski et al., 1990). Many of these proteins have been identified , including those involved in lipogenesis and lipolysis [such as fatty acid synthase (Sul and Paolauskis, 1988), hormone sensitive lipase (Kawamura et al., 1981)], secretory proteins, such as leptin (Zhang et al., 1994) and adipsin (Spiegelman et al., 1983), and most importantly, the transcriptional factors C/EBP (Cao et al., 1991), PPARγ2 (Tontonoz et al., 1994b), which regulate adipocyte differentiation.

The expression of transcription factors must be rigidly controlled to facilitate the orderly progression of the differentiation program. A group of transcription factors have been identified as participants in the adipogenic pathway. These include the C/EBP family and the steroid/ thyroid hormone receptor superfamilies. The C/EBP family, including C/EBPα, β, δ and CHOP, are basic leucine zipper proteins which bind to specific DNA sequences as dimers (Cao et al., 1991). The CCAAT/enhancer-binding protein α (C/EBPα) is thought as a master regulator (Lane et al., 1996). C/EBPα is not only required, but is sufficient to trigger differentiation of 3T3-L1 preadipocytes (Lane et al., 1996). Overexpression of C/EBPα is sufficient to trigger differentiation of preadipocytes without exogenous hormonal inducers (Christy et al., 1991). Antisense C/EBPα RNA used to block expression of C/EBPα can prevent 3T3 L1 preadipocyte differentiation, even in the presence of differentiation inducer (Lin and Lane, 1992). Furthermore, the C/EBPα gene knockout mice died shortly after birth for unable to develop adipose tissue (Wang et al., 1995). The peroxisome proliferator-activated receptor (PPAR)γ is another important member of adipogenesis regulators (Schoonjans et al, 1996; Spiegelman, 1996).

PPARγ2 heterodimerizes with the retinoid X receptor (RXRα), forming ARF6, which binds DNA at an adipocyte regulatory element (ARE7) within the enhancer region to activate the adipocyte-specific gene aP2, which encodes an intracellular lipid binding protein (Tontonoz et al., 1994 a, b). PPARγ and C/EBPα act synergistically to induce adipogenesis (Tontonoz et al., 1994c).

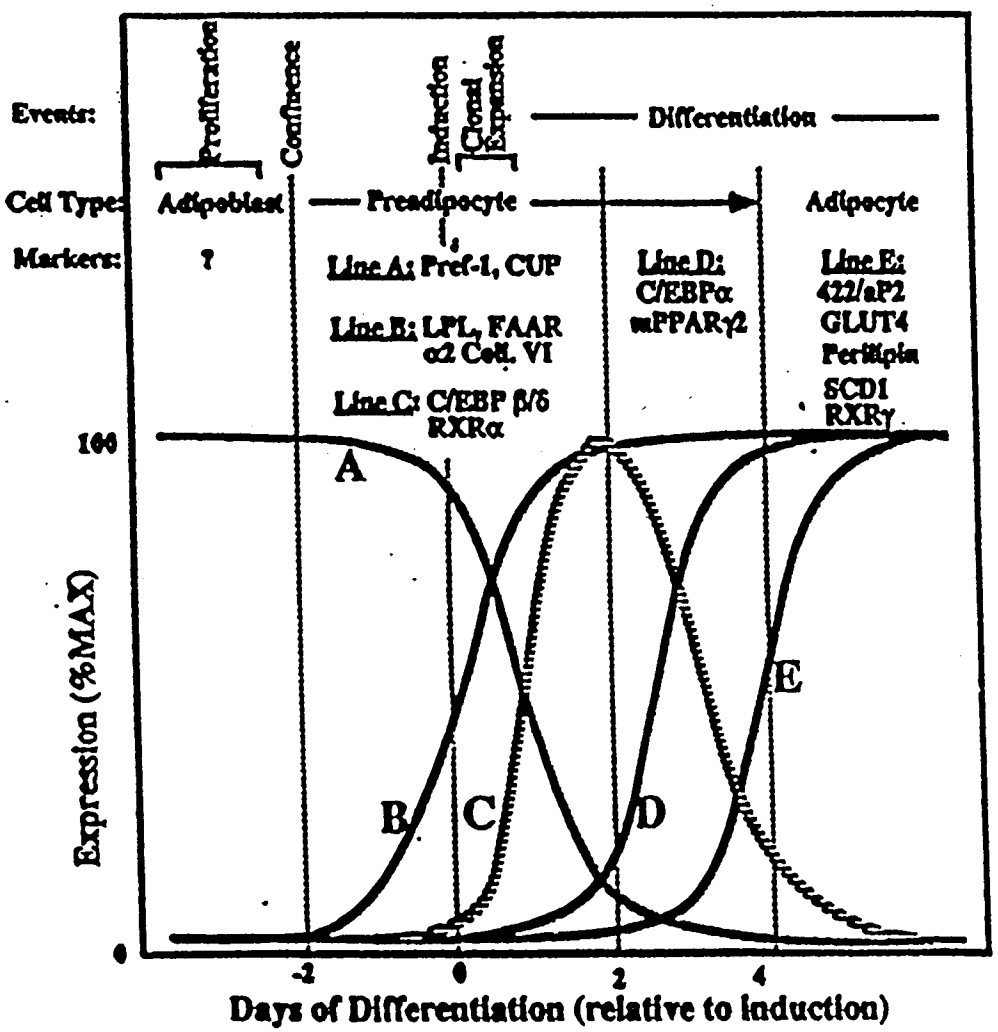


Figure 1. Stages in the adipocyte development program illustrating the events and temporal expression of various preadipocyte and adipocyte markers. (Cited from review by MacDougald and Lane, 1995)

The Steroid/thyroid hormone receptor superfamily is ligand-activated and binds to DNA hormone response elements (such as DR1) in target genes to regulate their expression and these include PPAR(see above), RXR (retinoic X receptor; Mangelsdorf et al., 1995), RAR (retinoic acid receptor; Schwarz et al.,1997), COUP-TF (Brodie et al., 1996b) and FAAR (fatty acid-activated receptor; Amri et al., 1995). Retinoic acid has been shown to regulate gene expression through both RAR and RXR (Xue et al., 1996). FAAR is expressed early in preadipocyte differentiation and has been shown to confer fatty acid response gene expression in fibroblast cells line which stably express the protein (Amri et al., 1995). In addition, many other transcription factors have been implicated in adipogenesis. These include preadipocyte repression element factor-1 (Pref-1) (Sul et al., 1993), ADD-1/SREBP1 (Kim and Spiegelman, 1996), C/EBP undifferented protein (CUP), lipoprotein lipase (LPL), stearoyl-Co desaturase I (SCD1), insulin-sensitive glucose transporter (GLUT4). (see review by Cornelius et al., 1994; and MacDougald et al., 1995). Some of the transcription factors can be positive regulators, some of them can be negative regulators in adipogenesis (Calkhoven et al., 1996 and Sul et al., 1993).

The sequence of changes for some transcription factors during adipogenesis has been proposed (see review by Spiegelman and flier, 1996). The β and δ isoforms of the C/EBP reach a maximal level during the first 2 days of differentiation and then decline before the appearance of the α isoform. C/EBPα is thought to induce the expression of many other genes, including three adipocyte-specific structure genes (422/aP2, SCD1 and Glut 4). PPARγ2 increases in the cell at the same time as C/EBPα. PPARγ2 heterodimerizes with the RXRα and activates another adipocyte-specific gene aP2

expression, which is an intracellular lipid binding protein (Tontonoz et al., 1994).

Phosphorylation is a very important mechanism for transcription factors' activation or inactivation in gene expression regulation (Weigel, 1996; and Camp and Tafuri, 1997). It was found that 3T3-L1 preadipocytes transfected with a protein tyrosine phosphatase expression vector failed to differentiate and that treatment with vanadate, which is a phosphatase inhibitor, restored the capacity to differentiate (Liao and Lane, 1995). It was discovered that a tyrosine dephosphorylation event must occur during clonal expansion and initiation of adipocyte specific gene expression (Liao and Lane, 1995). Expression in NIH-3T3 cells of PPARγ with a nonphosphorylatable mutation at serine 112 increased the cell's sensitivity to ligand-induced adipogenesis and suppressed the ability of mitogens to inhibit differentiation (Hu et al., 1996).

COUP-TF

Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) was first identified as homodimer that binds to a direct repeat regulatory element in the chicken ovalbumin promoter (Pastorcic et al., 1986), but subsequently found in a number of other species (Qiu et al., 1994). The human COUP-TFI gene encode a 2461 bp mRNA encoding a 43-45 kd protein. The COUP-TFs compose a family of functionally relatated transcription factors (Wang et al., 1991). In mammalian species, there are two kinds of COUP-TFs, COUP-TFI and COUP-TFII, they are encoded by two distinct genes. COUP-TFI is identical to human erbA (EAR-3) (Miyajima et al., 1988), while COUP-TFII is identical to apolipoprotein AI-regulatory protein-1 (ARP-1) (Ladias and Karathanasis, 1991). The two COUP-TFs proteins are highly homologous, with the DNA binding differing by a single conservative amino acid change (T→S) and their C-terminal domain sharing 87% amino acid sequence identity (Wang et al., 1991). The COUP-TF family binds to an imperfect direct repeat of AGGTCA sequence, which also is the DNA half-site for several other nuclear receptors such as the estrogen receptor (ER), vitamin D3 receptor, thyroid hormone receptor, retinoic acid receptors and hepatic nuclear factor 4 (HNF4) (Cooney, 1992).

The homologs of COUP-TFs have been cloned from many different species (Qiu et al., 1995), f rom drosophila to zebrafish to human being. The sequences are higly conserved, which implies that COUP-TF plays important role during evolutionary (see review by Tsai, 1997). The sequence analysis revealed that COUP-TFs are member of the steroid/ thyroid superfamily, and because their ligands have not been identified, they

belong to the orphan receptor family (Wang et al., 1989). Based on the P-box sequence, COUP-TFs can be classified as members of estrogen receptor (ER)/TR subfamily which bind to a Pu-GGTCA repeat (Ladias et al., 1991).

Like other members in the steroid hormone receptor family, the DNA binding domain of COUP-TF has a highly conserved 66 amino acid sequence, which contains two conserved Zn-finger motifs. In COUP-TFs, all 20 invariant amino acids are conserved and 11 out of 12 conserved residues are identical except that a conserved lysine in the second finger is replaced by a glutamine (Wang et al., 1989). It is believed that the more N-terminal zinc finger determines the target gene or thyroid response element sequence specificity while the more C-terminal zinc finger contacts the sugar-phosphate backbone of the DNA helix (see review by Tsai and O'Malley, 1994). Point mutation at 7th cysteine of HNF-4 orphan receptor abolished its ability to bind HNF-4 response element, but did not hinder the heterodimerization with other nuclear receptors (Taylor et al., 1996). The C-terminal domain controls transcriptional activation (see review by Tsai and O'Malley, 1994). The putative ligand binding domain (LBD) contains a putative dimerization motif, which includes nine heptad repeats and the predicted 10 helical structures of the dimerziation interface. Crystal structure of the LBD of the human RXR reveals the helix 8, 9 and 10 formed by the nine heptad repeats are involved in the formation of an internal hydrophobic pocket which serves as dimerization interface (Bourguet et al., 1995). The most prominent interactions of the dimer interface comprise hydrophobic van der walts contacts between pairs of Leu 419, Pro423 (375 in COUP-TFI;) and Leu 430, as well as hydrophilic interactions involving Asp 379 and Glu 390 of one monomer with Arg 421

and Lys 417 plus Arg 358, respcectively, of the other monomer (Bourguet et al., 1995). With the exception of Leu 430 (382 in COUP-TFI which is replaced by serine), all these amino acid residues are conserved in the COUP-TFI. It is likely that COUP-TF utilizes the dimer interface to form homodimers. Mutation of one of the proline in that region has been shown to reduce the efficiency of RXR homodimerization (Zhang et al., 1994). A transrepression box between amino acids 398 to 408 at the C-terminal end was identified, deletion of the ten amino acids diminished or destablized the heterodimerization of COUP-TF with Gal-receptor resulting in the loss of transrepression (Leng et al., 1996).

As mentioned above, COUP-TFs were first identified by binding to an imperfect GTGTCAAAGGTCA direct repeat separated by one nucleotide in the ovalbumin promoter (Sagami et al., 1986). The effect of spacing and orientation of half-sites on the relative binding affinity of COUP-TFs has been systematically analyzed. The relative binding affinity for the direct repeats with different spacing is as follows: DR1, DR6, DR4, DR8, DR10, and DR11. COUP-TFs also bind to inverted and everted repeats of the consensus sequence (Cooney et al., 1992). Apparently, COUP-TFs display highest preference to bind DR1 elements. In fact, the most common COUP-TF-binding site found in natural promoters is DR1 consensus sequence, which is an AGGTCA direct repeat with nucleotide spacer. COUP-TFs have been shown to bind and repress DR1 consensus-regulatory elements in the promoter of many genes, including rat and human apolipoprotein CIII (Haddad et al., 1986; and Reue et al., 1988), chicken apolipoprotein VLDII (Wijnolds et al., 1991), mouse lactoferin (Liu and Teng, 1992, 1993) , mouse mammary tumor virus promoter(Kadowaki et al.,1992), and mouse OCT4 promoter

(Schoorlemmer et al., 1994). In addition to the DR1 element, COUP-TFs can also bind to the DR0 sequence in oxytocin (Burbach et al.,1994) and hemopexin promoters (Satih, et al.,1994), the DR2 element in the sea urchin actin III B gene (Chan et al., 1992), the DR6 of RIPE-1 element of the rat insulin 2 promoter (Hwung et al., 1988), the DR7 of arrestin gene (Lu et al., 1994), the DR9 of HIV-LTR (Cooney et al., 1991), and the everted repeats of eight- and 14-nucleotide spacings of the acyl-coA dehydrogenase gene promoter (Carter et al., 1994). It will be of great interest to analyze which transcription factors genes during fat cell differentiation contain the direct repeat sequence in their promoter region. It will definitely be helpful to identify the signal transduction pathway that COUP-TF is involved in during adipogenesis.

The binding versatility of COUP-TFs for the direct repeats has tremendous biological implications because many of these binding sites are also response elements for retinoid (DR2 and DR5), thyroid hormone (DR4), and vitamin D (DR3) receptors (Cooney et al., 1993; and Umesono et al., 1991). Retinoids, thyroid hormones, and vitamin D are well known hormones and morphogens for vertebrate development and differentiation. In addition, COUP-TFs have been proposed to play an important role in regulating organogenesis, neurogenesis, and cellular differentiation during embryonic development (Leng et al., 1996). COUP-TFs have been shown to antagonize the HNF-4 dependent transcriptional activation of many liver-specific genes (Sladek et al., 1990 and Mietus et al., 1992) and to suppress OCT3/4 expression during retinoid-induced differentiation of P19 embryonic carcinoma cells (Ben-Shushan et al., 1995). Overexpression of COUP-TF in transfected myogenic cells inhibit muscle cell

differentiation by suppressing the levels of myoD mRNA (Muscat et al., 1995). Misexpression of COUP-TFI dramatically affected the early xenpus development (Schuh et al., 1996). The homozygous newborn null mutants of COUP-TFI died perinatally between 8 and 36 hr after birth due to the defect in the neurons which affect the feeding behavior (Crowley et al., 1994 and Klein et al., 1993). Therefore, what is the molecular mechanism(s) by which COUP-TFs modulate the processes of cellular development and differentiation?

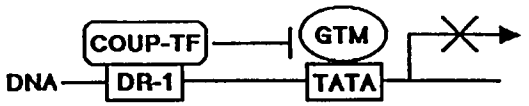
Five possible mechanisms are proposed, which are diagramed on the next page. These possibilities include:

1. COUP-TF competition with other nuclear receptors for common DNA binding sites (Cooney et al., 1993; Kliewer et al., 1992; Tran et al., 1992 and Jiang et al., 1997). Through direct competition with VDR, TR, RAR, ER and HNF-4, COUP-TFs have been shown to repress the hormonal induction of target genes of VDR, TR, RAR, ER and HNF-4.

2. Competition for RXR. It is well documented that RXR is a central heterodimeric partner for RAR, TR, VDR, PPAR and other orphan receptors (Direnzo et al., 1997). Through association with RXR, the heterodimeric receptors can then bind to the cognate response elements with high affinity and , thus, enhance the transactivation or transrepression. RXRs can potentially perform two functions: cooperative binding to hormone response elements and coordinate regulation of target genes by RXR ligands. It has been shown that PPAR, LXR, ER bind to a coactivator, either steroid receptor coactivator 1 (SRC-1) or CREB binding protein/ p300 (CBP/p300) and permit activation

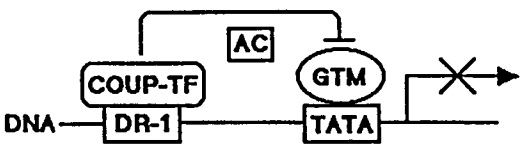
MECHANISMS OF TRANSCRIPTIONAL REPRESSION

1. Active Repression of Basal Transcription



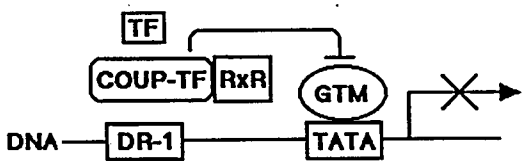
COUP-TF interacts with the General Transcription Machinery (GTM) reducing basal transcription

2. Competition of Common DNA Binding Elements



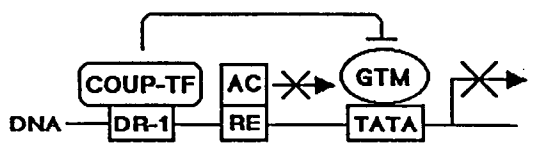
COUP-TF binding to DNA displaces the activating complex (AC) leading to repression

3. Competition of RXR



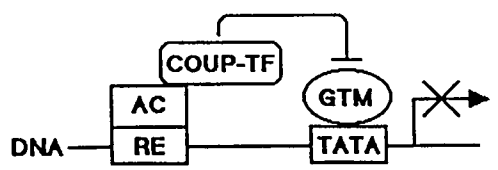
COUP-TF heterodimerization to RXR prevents the formation of AC (i.e. TF/RXR) and inhibits transcription with or without DNA binding

4. Active Repression of Transactivator-dependent Transcription



COUP-TF repression overrides the activating function of AC

5. Transrepression of Activated Transcription



COUP-TF tethers to AC and inhibits transcription without DNA binding

DR-1: Thyroid hormone/Retinoic acid receptor subfamily response element
TF: Other transcription factors
RE: DNA response element

by RXR-specific ligands, but RAR and TR prevent the binding of RXR ligand by recruiting the corepressor (either silencing mediator for retinoid and thyroid hormone receptors (SMRT) or nuclear receptors (N-CoR). The corepressor bind to the CoR box, a structural motif residing in the N-terminal region of the RAR and TR ligand binding domain. It will be of great interest to identify whether there is a N-CoR box in the COUP-TF like the TR, RAR.

3. Active repression. COUP-TFs have been shown to repress basal transcriptional activity of a number of thymidine kinase reporters containing DR3, DR4, or DR5 hormone response elements (Cooney et al., 1992 and 1993). This silencing of basal transcriptional activity is response element specific and is unlikely due to squelching of TFIIB (Leng et al., 1996). Perhaps it is more likely that COUP-TFs interact with a common target, a putative corepressor that mediates the repression (Chen and Evans, 1995 and Horlein et al., 1995). Whether SMRT or N-CoR is such a common target mediating the active silencing has yet to be defined.

4. Transrepression. It has been demonstrated that COUP-TFs can be tethered to DNA in the absence of their cognate response elements via LBD-LBD interactions with other receptors such as TR, RAR, and RXR to transrepress the ligand-dependent transactivation of the above nuclear receptors (Leng et al., 1996). A region between amino acids 398 to 408 at the C-terminal of COUP-TFI was found to play an important role in the transcriptional repression function of COUP-TFI (Leng et al., 1996). Deletion of these ten amino acids diminished or destabilized the heterodimerization of COUP-TF with Gal4-receptor, resulting in the loss of transrepression in a CAT reporter system.

5. Active repression of transactivator-dependent transcription. It has been demonstrated that COUP-TF repression can override the activating function of acidic, proline-rich, glutamine-rich and Ser/The-rich transactivator-medicated transactivation (Leng et al., 1996).

In most cases, COUP-TFs function as negative regulators in transient transfection assays, but COUP-TF was initially found as an activator of chicken ovalbumin gene expression. COUP-TF has been shown to stimulate the transcription of transferrin promoter in Hep3B cell, but not in Sertoli cells (Schaeffer et al., 1993). In addition, it has been demonstrated that COUP-TF and HNF-4 bind to the AF-1 element in the PEPCK gene and serve as an accessory factor to augment glucocorticoid response in activation of the PEPCK gene expression (Hall et al., 1995). The expression of COUP-TF genes are regulated by retinoids (all trans- and 9-cis retinoic acid) (Jonk et al., 1994) and the well-known morphogens sonic hedgehog (Shh; Lutz et al., 1994).

COUP-TF in adipogenesis

Previous study in this lab found that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inhibits fat cell differentiation by affecting the differentiation -dependent trans-acting factors both in vitro and in vivo (Chen et al., 1996; and Brodie et al., 1996a). TCDD treatment decreased the mRNA levels of C/EBPα and PPARγ2, did not effect the mRNA levels of RXRα and RARα in 3T3-L1 cell line. TCDD did not change the mRNA or protein levels of C/EBPβ. The time-dependent inhibition of cell differentiation by TCDD was correlated with the levels of aryl hydrocarbon receptor (AhR). In vivo treatment of rats with TCDD inhibits in vitro adipogenesis of primary cells isolated from epididymal or inguinal fat pads and reflects the results seen in 3T3-L1 cells. The TCDD treatment in vivo inhibited the increase of mRNAs for glycerol-3 phosphate dehydrogenase (GPDH), lipoprotein lipase (LPL), PPARγ2, ap2 and C/EBPα during differentiation. C/EBPβ and CHOP mRNAs were unaffected (Brodie et al., 1996c).

Furthermore, this laboratory found TCDD or retinoic acid (RA) induces COUP-TF binding to a PPAR/RXR (ARF6) binding sequence (ARE7), which is a DNA response element of the adipocyte-specific aP2 gene promoter (Brodie et al., 1996b). Within 15 min of treating 3T3-L1 cells with TCDD or RA, the AhR is present within the cell nucleus, and increased binding of COUP-TF to ARE7 oligomer occurs. In untreated preadipocytes, COUP-TF mRNA increased at confluence and then decreased after induction. TCDD did not alter COUP-TF mRNA changes. Dephosphorylating the nuclear extracts from TCDD and RA treated cells eliminated binding of COUP-TF to ARE7. COUP-TFI mRNA is presented early stage in adipogenesis period and is down regulated,

but not completely inhibited after induction of 3T3-L1 cells to differentiate.

Transient transfection assay has proved that overexpression of COUP-TF inhibit adipogenesis (data not published). These data indicate that COUP-TF may play a negative role in preadipocyte differentiation and that COUP-TF binding to DNA is correlated with TCDD and RA-induced phosphorylation.

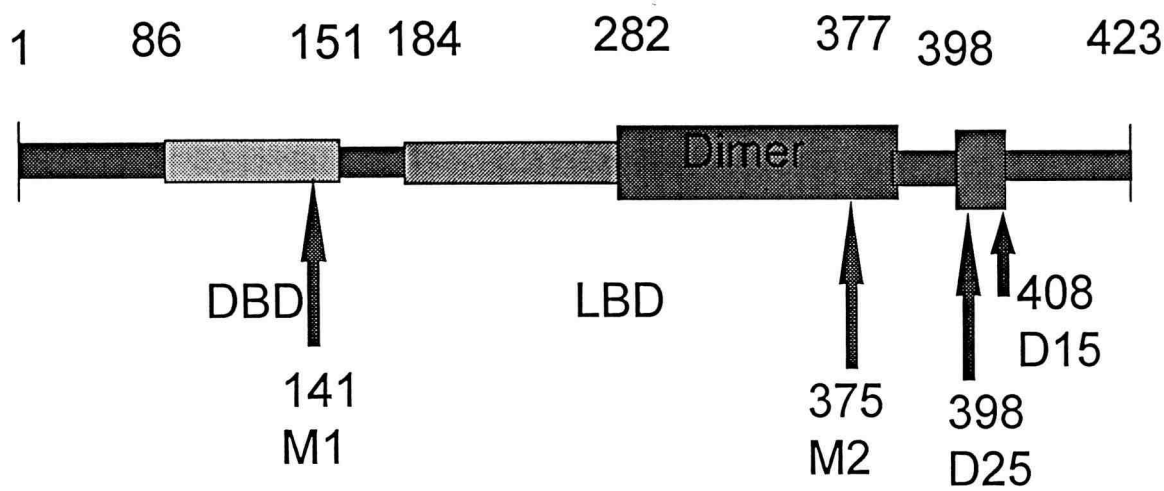


Figure 2. Sites of mutagenesis. Arrows show the sites of mutagenesis. M1 is mutanted at position of DNA binding domain (change the Cys to Arg at the amino acid position 141). M2 is mutated at position of dimerization domain (change the Pro to Thr at the amino acid position 375). D15 is deleted the last 15 amino acids (408-423) at the C-terminal. D25 is deleted the last 25 amino acids (398-423) at the C-terminal.

But the molecular repression mechanism (s) of COUP-TF during adipogenesis is not known. Does the DNA binding require for the COUP-TF's function? If the dimerization is required for the COUP-TF's function? If there is a transrepression motif on the COUP-TF? Therefore, the objective of this study is to supply a base to answer these

questions. Four mutations have been generated by oligo-directed mutagenesis. Among these mutations, one is mutated at position of DNA binding domain (change the Cys to Arg at the amino acid position 141), one is mutated at position of dimerization domain (change the Pro to Thr at the amino acid position 375), one is deleted the last 15 amino acids (408-423) at the C-terminal, and the last one is deleted the last 25 amino acids (398-423) at the C-terminal (Figure 2). By transfecting into preadipocyte cell line, these mutations provide a solid base to study the molecular mechanism(s) of COUP-TF-mediated repression during adipogenesis and the signal transduction passway involved.

MATERIALS AND METHODS

Cloning the coding fragment of human COUP-TFI by PCR

The cDNA plasmid (pRSCOUP-TFI) of human COUP-TFI was kindly supplied by Dr. Ming-Jer Tsai, Baylor College of Medicine. The coding fragment (1272 bp) was cloned by polymerase chain reaction (PCR). The oligonucleotide primers were synthesized by the Central Services Lab, Center for Gene Research and Biotechnology, Oregon State University. The forward primer used was 5'tggcGGATCCatggcaatggtagttagcagctggcga3' and the reverse primer used was 5'cccGAATTCgtaggagcactggatggacatgta3'. BamHI and EcoRI restriction enzyme sites were engineered as indicated by capital letters. Experiments to optimize conditions for the PCR assay are all the same except the concentration of $MgCl_2$, which were 1.5 mM, 3.0 mM and 4.5 mM $MgCl_2$ respectively. The best condition for the PCR in a 50 µl reaction was 25 ng template DNA, 25 pmole each primer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1X reaction buffer, 1 X Q-solution and 1.25 units Taq DNA polymerase (Qiagen, Chatswirth, CA). The PCR reaction was first preheated at 94°C for 2 min., then carried out for 32 cycles: denatured at 94°C for 1 min, annealed at 60°C f or 1 min., elongated at 72°C for 1 min., finally extended at 72°C, for 5 min.. To confirm that the PCR product was human COUP-TFI specific, some of the PCR product was digested with restriction enzyme Pvu II or Pst I (Promega, Madison, WI), the digestion products with standard molecular weight marker (1 kb DNA ladder from Gibco BRL, Gaithersburg, MD) were then separated by electrophoresis on 1% agarose gel (0.35 g agarose in 35 ml 1X TBE buffer; heat in microwave until dissolved; add 1.5 µl of 5% ethidium bromide; pour and insert comb).

Restriction digestion of phagemid pBluescript II SK (+) and the PCR product

The phagemid pBluescript II SK (+) (Stratagene, Inc., La Jolla, CA) and PCR product was digested with BamHI and EcoRI (New England Biolabs, Inc.), isolated by

electrophoresis on 1% agarose gel, the desired DNA fragment was recovered and purified from the agarose gel. Briefly, 2 μg of vector (pBluescript II SK (+)) DNA or PCR products were mixed with 2 μl of 10 X restriction buffer (NE buffer for BamHI), distilled water to give 18 μl total volume, 1 μl (5 to 10 units) each of EcoRI and BamHI, incubated at 37^{0}C for 2 hours, Reactions were stopped by heating at 68^{0}C for 10 min. The reactions and the 1kb MW ladder were loaded on a 1% agarose gel. Electrophoretic separation was achieved using 100 V for 1 hour on a BioRad sub-cell apparatus (BioRad, Hercules, CA). Purification of DNA fragments from agarose was performed according to DNA purification kit instruction of manufacturer (Bio-Rad). The protocol used can be describled as the following: cut the desired DNA agarose gel as small as possible under UV-light, add 100 μl binding buffer to dissolve, add 5 μl of Pre-A-Gene Matrix, incubate 5-10 min., gently agitate, centrifuge 30 seconds at 14,00rpm, add 125 μl binding buffer to rinse the pellet, wash twice with 125 μl washing buffer, air dry 5 min. to remove the tracer of liquid, add 30 μl of elution buffer, incubate for 5 min. at 37-50^{0}C, centrifuge 1 min., transfer the supernatant.

Ligation of restriction fragment and vector

About 500 ng of COUP-TFI PCR product DNA digested with EcoRI and BamHI were ligated into 100 ng of (linear) pBluscript II SK (+) vector DNA digested with EcoRI and BamHI. The ligation condition was 10 pM rATP (PH 7.0), 1 x ligase buffer and 2 units T4 DNA ligase (Stratagene), ligated overnight at 4 ^{0}C. Heat at 68 ^{0}C for 15 min. to stop reaction.

Transformation of E. Coli. with the ligated phagemid

The above ligated DNA was transformed to E.coli, XL1 Blue strain cell. The transformation of E.coli with the ligated plasmid was performed as following. Prechill test tubes (15 ml Falcon 2059 polyprolene tube) on ice, combine 10 μl of ligation DNA with 0.1 ml XL1 Blue competent cells (brought from Stratagene), incubate on ice 1 hour, heat shock at 42 ^{0}C for 1 min,chill on ice for 2 min., add prewarmed LB medium 0.9 ml, shake

at 37 ^{0}C for 1 hour, spread 200 μl on agar plates with Ampicillin and IPTG and Xgal, incubate plate overnight at 37 ^{0}C.

Small-scale preparation of plasmid DNA from broth cultures

Two single white colonies were picked and inoculated into 2 ml of LB medium (contain 100 μg /ml ampicillin), incubated the 2 ml cultures with aeration at 37 ^{0}C overnight. The small-scale plasmid DNA from the 2 ml culture were prepared by the alkaline lysis method. The miniprep kit were brought from Qiamen. Briefly, centrifuge 1.5 ml of overnight culture for 1 min. At 14,000 rpm, discard supernatant and resuspend cells in 200 μl Buffer I; add 200 μl buffer II to lysis cells, mix genetly; add 200 μl buffer III, mix gently by inverting 8 times; Centrifuge 3 min.; transfer 500 μl supernatant to a fresh tube and mix with 1 ml 100% ethanol, hold for 10 minutes at room temperature, centrifuge 3 min at full speed, wash the pellet with cold 70% ethanol twice; air dry for 5 min., dissolve the pellet in 25 μl TE buffer. Measure the O.D., and calculate the DNA concentration.

Restriction analysis and sequencing of DNA to confirm insert

1 μg of the above COUP-TFI plasmid DNA was taken to digest with PvuII and Pst I separately, the digestion reaction with a DNA ladder were then loaded on a 1% agarose gel to check the digestion pattern. Furthermore, the right plasmid DNA sequence was confirmed using an ABI 373A automated sequencer and dye terminator chemistry. The primers used for sequencing were T3 primer: aattaaccctcactaaaggg, T7 primer: taatacgactcactataggg, and coup-5s: ccaacaggaactgtcccatcga, for the first about 450 bp forward sequence, the last about 450 bp reverse sequence, and the mid 450 bp forward sequence. The human COUP-TFI pBluescript II SK (+) phagemid was therefore named Phagemid pBCOUP-TFI-M(aster). Sequence was analyzed using the Genetics Computer Group (GCG) software developed at the University of Wisconsin (1987).

Preparation of helper phage M13KO7 stock

The helper phage M13K07 stock was prepared according to the "Current Protocols in Molecular Biology" by Ausubel et al., 1987. Briefly, a fresh overnight culture of a male (F') E. coli strain (JM101) was grown; Inoculate 2 ml LB with 20 μl of JM 101 overnight culture and incubate with aeration of 1 hour at 37 ^{0}C; add one plaque of M13KO7 phage and incubate 1 hour at 37 ^{0}C; Add kanamycin to 50 μg/ml, incubate with aeration overnight at 37 ^{0}C; Titer phage stock on male strain.

Preparation of single-stranded, uracil-containing DNA template

The phagemid pBCOUP-TFI-M was transformed into the dut$^-$ung$^-$ E.coli strain cells (CJ236). The method of transformation is the same as described in the previous section: Transformation of E.coli. with the ligated phagemid. The dut$^-$ mutation inactivates dUTPase, thereby increasing the cellular dUTP pool, which results in increased incorporation of uracil (in place of thymine) during DNA synthesis. The ung$^-$ mutation inactivates uracil N-glycosylase, a DNA repair enzyme which removes uracil bases, this mutation prevents removal of uracil from the DNA. The uracil-containing phagemid DNA (pBCOUP-TFI-M) served as the template for in vitro DNA synthesis primed by the mutagenic oligonucleotide. The single-stranded DNA template containing uracil was prepared according to Molecular Biology Technique note, Oregon State University and the instruction manual for Muta-Gene phagemid in vitro mutagenesis kit of the manufacturer (BioRad). Briefly, Grow CJ 236 (containing pBCOUP-TFI-M) overnight at 37^{0}C with aeration in LB medium with 50 μg / ml ampicillin; inoculate 2 ml of L broth with 25 μl of overnight culture from front step, Incubate at 37^{0}C with aeration for 1 hour; Add ampicillin to 25 μg / ml, incubate for another 30 minutes at 37 ^{0}C; Add 40 μl of M13KO7 helper phage; incubate at 37 ^{0}C for 1 to 2 hours with gentle shaking; Add entire 2 ml of infected culture to 8 ml of L broth + 0.001% thiamine + 25 μg / ml μg ampicillin + 50 μg / ml kanamycin; Incubate with aeration for 16 hours at 37 ^{0}C. Centrifuge the cultured cells at 10,000 rpm for 10 minutes; Mix 6 ml supernatant with 720 μl 5 M NaCl + 960 μl 30% PEG 8000; hold at 4 ^{0}C. Centrifuge at 10,000 rpm for 10 minutes at 4 ^{0}C,

discard supernatant and save small beige pellet; Resuspend pellet in 0.5 ml of TE buffer; Extract twice with one volume of a 1:1 mixture of phenol : chloroform and then with a 24:1 mixture of chloroform : isoamyl alcohol; Add 2 volumes of ethanol and 0.5 volume of 7.5 M ammonium acetate to precipitate the DNA; Measure OD at 260 nm and calculate the concentration of the single-stranded DNA; Examine DNA on an 1% agarose gel to look recovery of single-stranded DNA. Run a size standard (1 kb ladder) for comparison.

Phosphorylation of oligonucleotide

The oligonucleotide primers were synthesized by the Central Services Lab, Center for Gene Research and Biotechnology, Oregon State University. The oligonucleotides used were as follows:

COUP-MR1: 5'gcacttcttgaggcggcGgtattggcactg3' (pBMR1);

COUP-MR2: 5'tgcgcagcgaggTcagtcgcagcagca3' (pBMR2);

COUP-DR3: 5'gcttgatatcgaattccta---cagtaacatatcgcggat3' (pBDR1);

COUP-DR4: 5'gcttgatatcgaattccta---gggggttttacctacca3' (pBDR2).

The capital letters and dash marks denote changes or deletion from wild type antisense sequence. The names of mutant plasmids derived from these oligonucleotide in the later steps are given in parentheses. The oligonucleotides were phosphorylated as following: Mix: 10 X polynucleotide kinase buffer 3 µl, 1 mM ATP 13 µl, mutagenic oligonucleotide 200 pmol, add distilled water to 30 µl; Add 4.5 units of T4 polynucleotide kinase; incubate at 37 ^{0}C for 45 minutes; heat at 65^{0}C for 10 minutes; Store at -20 ^{0}C.

Annealing mutant oligonucleotide to template

The phosphorylated mutant oligonucleotides were annealed to the uracil-containing single-stranded DNA as follows: on ice, mix 1 µg of single -stranded template DNA (pBCOUP-TFI-M containing uracil DNA), 1 µl phosphorylated oligonucleotide primer, 2 µl 5X sequenase buffer (U.S. Biochemical), 6 µl distilled water. Incubate the tube at 70 ^{0}C for 1 minute; Allow the reactions to cool to room temperature at a rate of approximately 1 ^{0}C per minute over a 2 hours period; Place the reactions on the ice.

In vitro DNA synthesis by primer extension

Using the uracil-containing single-stranded pBCOUP-TFI-M DNA as template, and the phosphorylated mutant oligonucleotides as primer, the new DNAs were synthesized by addition of sequenase DNA polymerase and dNTPs. At the end of the synthesis, DNA ligase ligated the newly synthesized strand of the now-double-stranded plasmid, preventing further synthesis which would replace the mutagenic oligonucleotide. The experiments of DNA synthesis by primer extension in vitro were performed as follows. To each 10 μl annealing reaction, add 2 μl of 5 X Sequenase buffer, 1.5 μl of 10mM ATP, 1 μl of 0.1 M DTT, 3.2 μl of 2.5 mM (Sigma), 1 μl of T4 DNA ligase (Stratagene), 1 μl of Sequenase DNA polymerase (U.S. Biochemical); incubate 5 minutes at room temperature, then 75 minutes at 37 ^{0}C. Examine 2 μl of the synthesis product by 1% agarose gel electrophoresis. Run a size standard (1 kb ladder) and a double-stranded plasmid for comparison.

Transformation of synthesis reactions into E.coli and recovery of clones

Two μl of each the synthesis reactions were transformed to E. Coli, XL1 Blue cell. The method of transformation used was the same as described in the "transformation of E. Coli. With the ligand phagemid"section. The nonmutant template DNA strand contains uracil, whereas the mutant DNA strand synthesized in vitro does not. Uracil repair, which occurs as the plasmid begins replication, causes selective loss of the parental nonmutant strand, thereby increasing the chances of isolating the oligonucleotide-directed mutation.

Small-scale preparation of plasmid DNA from putative mutants

Two single white colonies from each four mutant plates (pBMR1, pBMR2, pBDR1 and pBDR2) were picked and inoculated into 2 ml of LB medium (contain 100 μg/ml ampicillin), cultured with aeration at 37 ^{0}C overnight. The small-scale preparations of plasmid DNA from the four mutants were done by the same method as described in the "small-scale preparation of plasmid DNA from broth cultures" section.

Confirmation of mutants by restriction analysis and sequencing of plasmid DNA

One μg of each mutant plasmid DNA (pBMR1, pBMR2, pBDR1 and pBDR2) were digested with PvuII and PstI, and the digestion patterns were examined by gel electrophoresis. Furthermore, the generated mutant plasmid DNA sequence was confirmed using an ABI 373A automated sequencer and dye terminator chemistry by Sanger's dideoxy DNA sequencing method. The primer used for sequencing pBMR1 and pBMR2 DNA was coup-5s: ccaacaggaactgtcccatcga, the primer used for sequencing pBDR1 and pBDR2 was T7 primer: taatacgactcactataggg. Sequences were analyzed using the Genetics Computer Group (GCG) software developed at the University of Wisconsin (1987).

RESULTS

Cloning the encoding fragment of human COUP-TFI by PCR

The human COUP-TFI encoding fragment (1272 bp) was cloned by polymerase chain reaction (PCR). The forward primer used was 5'tggcGGATCCatggcaatggtagttagcagctggcga3' and the reverse primer used was 5'cccGAATTCgtaggagcactggatggacatgta3'. The BamHI and EcoRI restriction enzyme sites were engineered as indicated by capital letter. The best experimental condition for the PCR assay in a 50 µl reaction was 25 ng template DNA, 25 pmole each primer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1X reaction buffer, 1 X Q-solution and 1.25 units Taq DNA polymerase (Qiagen) (Figure 3).

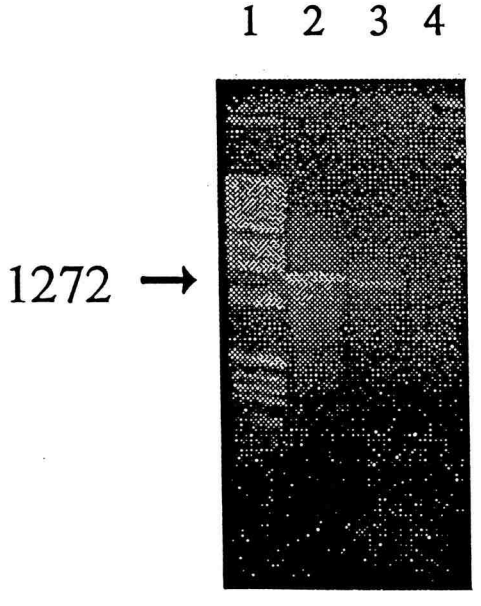


Figure 3. Optimization of the PCR condition. Experiments to optimize conditions for the PCR assay are all the same except the concentration of $MgCl_2$. The best condition for the PCR in a 50 µl reaction was 25 ng template DNA, 25 pmole each primer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1X reaction buffer, 1 X Q-solution and 1.25 units Taq DNA polymerase (Qiagen). The PCR reaction was first preheated at 94°C for 2 min., then carried out for 32 cycles: denatured 94°C, 1 min, annealed 60°C, 1 min., elongated 72°C, 1 min., finally extended 72°C, 5 min. Arrows show the position and molecular weight in bp.

Lane 1, 1 kb DNA ladder. Lane 2, at 1.5 mM of $MgCl_2$. Lane 3, at 3.0 mM of $MgCl_2$. Lane 4, at 4.5 mM of $MgCl_2$.

To confirm that the PCR product was human COUP-TFI, some of the PCR product was digested with restriction enzyme PvuII or PstI, the digestion products were then separated by electrophoresis on 1% agarose gel (figure 4). As expected by calculation from the mapsort of human COUP-TFI, the restriction enzyme PvuII yields a 950 pb fragment, a 300 bp fragment, and a 35 bp fragment (size too small to be seen). The restriction enzyme PstI yields a 800 bp fragment, a 300 bp and a 240 bp fragment. These data confirm the PCR product was human COUP-TFI.

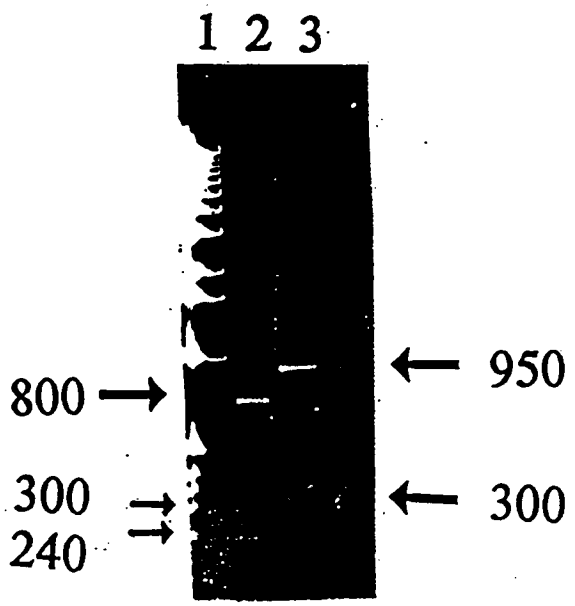


Figure 4. Restriction digestion analysis of the PCR product. Arrows show position and molecular weight in bp. Lane 1, 1 kb DNA ladder. Lane 2, digested by restriction enzyme PstI. Lane 3, digested by restriction enzyme PvuII.

Subcloning the PCR product into phagemid pBluescript II SK (+)

To insert the human COUP-TFI PCR product into the phagemid pBluescript II SK (+), the phagemid pBluescript II SK (+) (Stratagene, Inc., La Jolla, CA) and PCR product was digested with BamHI and EcoRI (New England Biolabs, Inc.), isolated by

electrophoresis on 1% agarose gel (Figure 5), the desired DNA fragment were recovered and purified from the agarose gel, then the COUP-TFI PCR product DNA digested with EcoRI and BamHI were ligated into pBluscript II SK (+) vector DNA digested with EcoRI and BamHI.

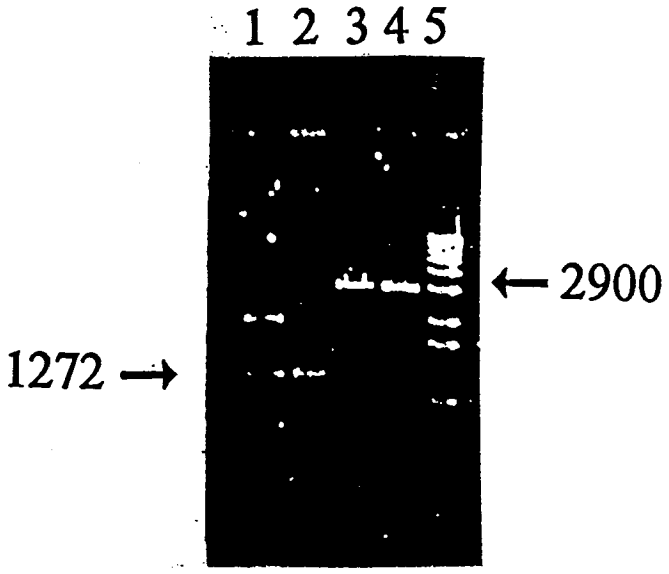


Figure 5. Digestion of the PCR product and the phagemid pBluescript II SK (+) with restriction enzymes BamHI and EcoRI. Arrows show the position and values of molecular weight in bp. Lane 1, DNA mass Ladder. Lane 2, digestion of PCR product with restriction enzymes BamHI and EcoRI. Lane 3 , digestion of phagemid pBluescript II SK (+) with restriction enzymes BamHI and EcoRI. Lane 4, digestion of pBluescript II SK (+) with restriction enzymes EcoRI only. Lane 5, 1 kb DNA ladder.

Restriction analysis and sequencing of plasmid DNA to confirm insert

The ligated DNA was transformed to E.coli, XL1 Blue strain cell, and plated on an agar plate. About 50 white colonies were obtained. Two single white colonies were picked for small-scale DNA preparation. To verify that the DNA is the desired human COUP-TFI, 1 μg of the prepared COUP-TFI plasmid DNA was used for do restriction

analysis with PvuII and Pst I to separately (Figure 6). As expected, digestion with restriction enzymes EcoRI and BamHI yields 2 fragments of 1271 bp and 2900 bp, digestion with restriction enzyme PvuII yields four relative small fragments of 2540 bp, 950 bp, 555 bp, and 191 bp while digestion with restriction enzyme PstI yields a large fragment of 4000 bp and a small fragment of 300 bp. Furthermore, the correct plasmid DNA sequence was confirmed using an ABI 373A automated sequencer and dye terminator chemistry. The human COUP-TFI pBluescript II SK (+) phagemid was used to make single strand DNA, which was used as template for site directed mutagenesis, therefore, it was named Phagemid pBCOUP-TFI-M(aster).

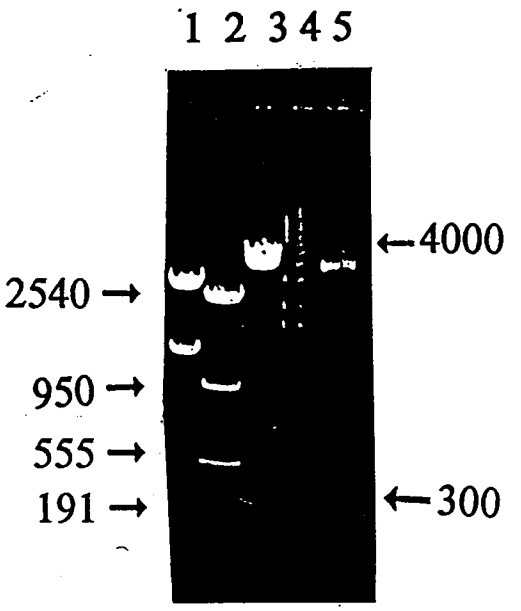


Figure 6. Restriction digestion analysis of the phagemid pBCOUP-TFI-M. Arrows show position and molecular weight in bp. Lane 1, digested with restriction enzyme EcoRI and BamHI. Lane 2, digested by restriction enzyme PvuII. Lane 3, digested by restriction enzyme PstI. Lane 4, 1kb DNA ladder. Lane 5, uncut pBCOUP-TFI-M.

Preparation of single-stranded, uracil-containing DNA template

The phagemid pBCOUP-TFI-M was transformed into the dut$^-$ung$^-$ E.coli strain cells (CJ236). The dut$^-$ mutation inactivates dUTPase, thereby increasing the cellular dUTP pool, which results in increased incorporation of uracil (in place of thymine) during

DNA synthesis. The ung$^-$ mutation inactivates uracil N-glycosylase, a DNA repair enzyme which removes uracil bases, this mutation prevents removal of uracil from the DNA. The uracil-containing phagemid DNA (pBCOUP-TFI-M) served as the template for in vitro DNA synthesis primed by the mutagenic oligonucleotide. The single-stranded DNA template containing uracil was prepared according to Molecular Biology Technique Note, Oregon State University and the instruction manual for Muta-Gene phagemid in vitro mutagenesis kit of the manufacturer (BioRad). The single-stranded DNA was examined on an 1% agarose gel with a size standard (1 kb ladder) and a double-stranded pBCOUP-TFI-M DNA for comparison. The single-stranded DNA runs faster than double strand DNA (Figure 7).

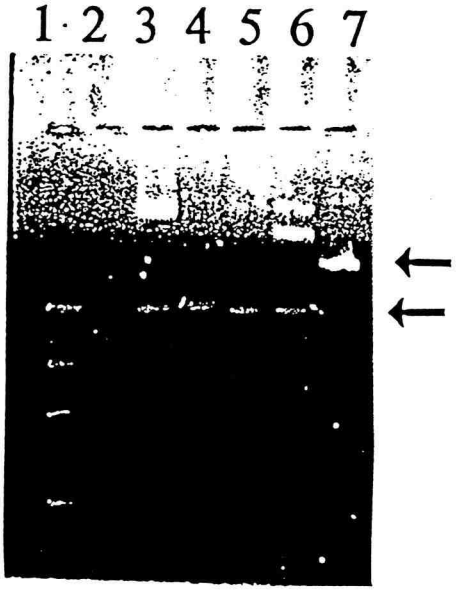


Figure 7. Verification of single-stranded pBCOUP-TFI-M DNA. Arrows show the position of supercoiled form DNA Lane 1, DNA Mass ladder. Lanes 3, 4, 5, 6 are single-stranded pBCOUP-TFI-M DNA containing uracil. Lane 7 is double-stranded pBCOUP-TFI-M DNA for comparison.

Site-directed mutagenesis

The oligonucleotides used for site-directed mutagenesis were as follows:

COUP-MR1: 5'gcacttcttgaggcggcGgtattggcactg3' (pBMR1);

COUP-MR2: 5'tgcgcagcgaggTcagtcgcagcagca3' (pBMR2);

COUP-DR3: 5'gcttgatatcgaattccta---cagtaacatatcgcggat3' (pBDR1);

COUP-DR4: 5'gcttgatatcgaattccta---gggggttttacctacca3' (pBDR2).

The capital letters and dash marks denote changes or deletion from wild type antisense sequence. The names of mutant plasmids derived from these oligonucleotide in the later steps are given in parentheses. The oligonucleotides were phosphorylated. The phosphorylated mutant oligonucleotides were annealed to the single-stranded DNA containing uracil. Using the single-stranded uracil containing pBCOUP-TFI-M DNA as template, the phosphorylated mutant oligonucleotide as primer, new DNA were synthesized by adding Sequenase DNA polymerase and dNTPs. At the end of the synthesis, the newly-synthesized strand of the double-stranded plasmids were ligated to the phosphorylated 5'-end of primer, preventing further synthesis which would replace the mutagenic oligonucleotide. The synthesis product was examined by 1% agarose gel electrophoresis (Figure 8).

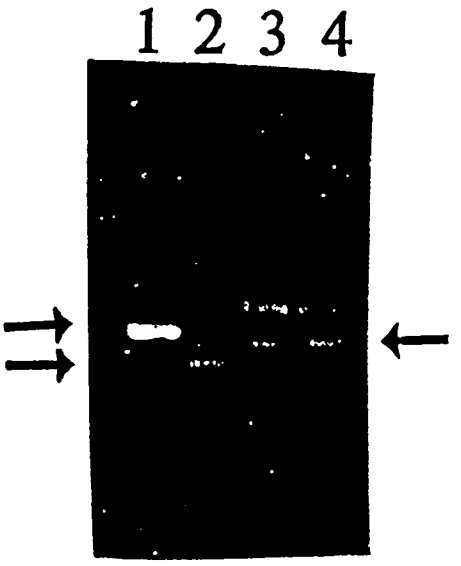


Figure 8. DNA synthesis in vitro. The oligonucleotides were phosphorylated, annealed to the single-stranded DNA containing uracil. Using the single-stranded pBCOUP-TFI-M DNA containing uracil as template, the phosphorylated mutant oligonucleotides as primer,

the new DNA were synthesized by adding Sequenase DNA polymerase and dNTPs. At the end of the synthesis, the newly-synthesized strand of the now-double-stranded plasmid were ligated to the phosphorylated 5'-end of primer. The synthesis product was examined by 1% agarose gel electrophoresis. Arrows show position of supercoiled form of DNA. Lane 1, double strand pBCOUP-TFI-M DNA as positive control. Lane 2, single strand pBCOUP-TFI-M DNA. Lane 3 and 4, in vitro newly synthesized mutant double strand pBCOUP-TFI DNA.

Restriction analysis and sequencing of plasmid DNA to confirm mutants

The in vitro DNA synthesis reactions were transformed to E. Coli, XL1 Blue cell and plated on four agar plates. About 60 colonies were obtained for each mutant plate. The nonmutant template DNA strand contains uracil, whereas the mutant DNA strand synthesized in vitro does not. Uracil repair, which occurs as the plasmid begins replication, causes selective loss of the parental nonmutant strand, thereby increasing the chances of isolating the oligonucleotide-directed mutation. To screen the mutants, two single white colonies from each four mutant plates (pBMR1, pBMR2, pBDR1 and pBDR2) were picked to do small-scale preparations of plasmid DNA. 1 μg of each above 4 putative mutants plasmid DNA (pBMR1, pBMR2, pBDR1 and pBDR2) were digested with PvuII and examined the digestion pattern by gel electrophoresis (Figure 9). As expected, digesting the four mutants with restriction enzyme PvuII yield four relative small fragments of 2540 bp, 950 bp, 555 bp, and 191 bp. Furthermore, the generated mutant plasmid DNA sequences were confirmed using an ABI 373A automated sequencer and dye terminator chemistry by Sanger's dideoxy DNA sequencing method (see appendix 6,7, 8, 9).

In summary, the coding fragment (1272 bp) of human COUP-TFI was cloned by PCR. The PCR product was digested with BamHI and EcoRI, isolated by electrophoresis on 1% agarose gel, ligated into the pBluscript II SK (+) vector (Stratagene), and named pBCOUP-TFI-M. The sequence of the cloned fragment was confirmed by Sanger's dideoxy DNA sequencing method. The oligo-directed mutagenesis was performed according to Molecular Biology Technique note, Oregon State University and the instruction manual for Muta-Gene phagemid in vitro mutagenesis kit of the manufacturer

(BioRad). Briefly, the phagemid vector pBCOUP-TFI-M was transformed into dut$^-$ung$^-$ E.coli strain CJ236, which then was infected with helper phage M13KO7 to make the uracil-containing single-stranded DNA. Using the SS DNA as template and mutated oligo as primer, double strand DNA was synthesized and transformed into normal E.coli strain XL1Blue cells, in which the uracil-containing single-stranded DNA would be destroyed and the newly synthesized mutated DNA would be replicated. All the mutants generated by the site-directed mutagenesis were verified by Sanger's dideoxy DNA sequencing (see appendies).

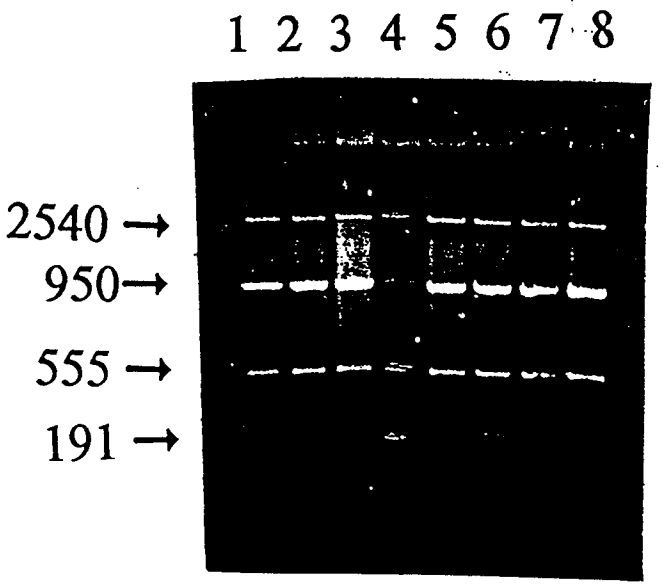


Figure 9. Restriction digestion of the four mutants of pBCOUP-TFI with restriction enzyme PvuII. Two colonies of each mutants were picked for DNA mini-preparation. Arrows show position and molecular weight in bp. Lane 1 and 2, digestion of mutant pBMR1 DNA. Lane 3 and 4, digestion of mutant pBMR2 DNA. Lane 5 and 6, digestion of mutant pBDR1 DNA. Lane 7 and 8, digestion of mutant pBDR2 DNA.

DISCUSSION

The C-terminal LBD of nuclear receptors is a complex multiple functional module containing ligand-binding, dimerization, transcriptional repression, and activation functions. The Chicken Upstream Transcription Factor (COUP-TF), an orphan member of the steroid/thyroid nuclear receptor superfamily, has been proposed to play an important role in regulating organogenesis, neurogenesis, and cellular differentiation during embryonic development, but mainly a negative role (Leng et al., 1996). Recently, our laboratory has demonstrated that COUP-TF is involved and play a negative role in the adipogenesis (Brodie et al., 1996 a, b; 1997). It will be of great interesting to study the molecular mechanism(s) by which COUP-TF modulate the process of cellular development and differentiation.

The COUP-TF family binds to the AGGTCA repeat sequence, which also is the DNA half-site for several other nuclear receptors such as the estrogen receptor (ER), vitamin D3 receptor, thyroid hormone receptor, retinoic acid receptors and hepatic nuclear factor 4 (HNF4) (Cooney et al., 1993; Kliewer et al., 1992; Tran et al., 1992; and Jinag et al., 1997). Like other members in the steroid hormone receptors family COUP-TF has a highly conserved 66 amino acid sequence that constitutes the DNA-binding domain, in which all 20 invariant amino acids are conserved and 11 out of 12 conserved residues are identical. This region contains nine cysteines, eight of which form two zinc fingers. It is believed that the more C-terminal zinc finger determines the target gene or steroid response element sequence specificity while the more C-terminal zinc finger contacts the sugar-phosphate backbone of the DNA helix. Point mutation at 7th cysteine of HNF-4 orphan receptor abolished its ability to bind HNF-4 response element, but did not hinder the heterodimerization with other nuclear receptors (Taylor et al., 1996). The C-terminal domain controls transcriptional activation. The putative ligand binding domain (LBD) contains a putative dimerization motif. Mutation of one of the proline in that region has been shown to reduce the efficience of RXR homodimerization (Zhang et al., 1994). A transrepression box between amino acids 398 to 408 at the C-terminal end was identified,

deletion of the ten amino acids diminished or destablized the heterodimerization of COUP-TF with Gal-receptor resulting in the loss of transrepression (Leng et al., 1996).

But the molecular transcriptional repression mechanism(s) of COUP-TF during adipogenesis is not known. Does the DNA binding require for the COUP-TF's function? If the dimerization is required for the COUP-TF's function? If there is a transrepression motif on the COUP-TF? In order to answer these questions, making mutants on the DNA binding domain, the ligand binding domain and on the repression box, then transfecting into 3T3L1 cell to study their effect on adipogenesis would be a good research strategy. Four mutations have been generated by oligo-directed mutagenesis. Of these mutations, one is mutated at position of DNA binding domain (change the Cys to Arg at the amino acid position 141), one is mutated at position of dimerization domain (change the Pro to Thr at the amino acid position 375), one is deleted the last 15 amino acids (408-423) at the C-terminal, and the last one is deleted the last 25 amino acids (398-423) at the C-terminal (Figure 2).

Important variables that can influence the outcome of PCR include the $MgCl_2$ concentration and the cycling temperatures. Additives that promote polymerase stability and processivity or increase hybridization stringency, and strategies that reduce nonspecific primer-template interactions, especially prior to the critical first cycle, can greatly improve sensity, specificity, and yield. So it is very important to optimize the reaction components and conditions for a successful PCR. For the COUP-TFI, and the primers we used as mentioned in the Material and Methods, the best concentration of $MgCl_2$ is 1.5 mM (Figure 2). For oligonucleotide-directed mutagenesis, the orientation of the target sequence relative to the vector is critical, because the mutagenic oligonucleotide must be the complement of the strand that becomes packaged when the phagemid replicates as a single-stranded phase. For the phagemid pBluescript II SK(+/-), the lacZ coding (or sense) strand is also the phage M13 plus (or packaged) strand. I has inserted the EcoRI-BamHI fragment from the COUP-TFI product in the same orientation relative to lacZ: the coding strand of COUP-TFI is in the plus strand. Therefore, the packaged pBCOUP-TFI-M single stranded DNA that I would use as template for the mutagenesis

would contain the coding (or sense) strand of COUP-TFI, and the mutagenic oligonucleotide I would use should corresponds to the antisense strand of COUP-TFI, otherwise, they would not anneal each other.

If the DNA binding and heterodimerlization or homodimerlization is required for the COUP-TFI's function in adipogenesis repression, then overexpression of mutant COUP-TFI at DNA binding site or ligand binding site will not inhibits adipogenesis. Similarly, if there is a transrepression motif exist for COUP-TFI, then overexpression of mutant COUP-TFI at the transrepression motif will not inhibit (or reduce the inhibition ability) adipogenesis. Therefore, by transfecting into preadipocyte cells and establish stable line, these mutations will provide a solid fundament to study the molecular mechanism(s) of COUP-TF-mediated repression during adipogenesis and the signal transduction passway involved.

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Appendices

Appendix A. The nucleotide and amino acid sequence of the COUP-TFI

```
                                                                                                F1 →
                                                                                CTC CCC GGG CCC AAA GAT ATG GCA ATG GTA GTT      15
                                                                                                MET ALA MET VAL VAL       5

AGC AGC TGG CGA GAT CCG CAG GAC GAC GTG GCC GGG GGC AAC CCC GGC GGC CCC AAC CCC GCA GCG CAG GCG GCC CGC GGC GGC GGC GGC      105
SER SER TRP ARG ASP PRO GLN ASP ASP VAL ALA GLY GLY ASN PRO GLY GLY PRO ASN PRO ALA ALA GLN ALA ALA ARG GLY GLY GLY GLY       35

GGC GCC GGC GAG CAG CAG CAG CAG GCG GGC TCG GGC GCG CCG CAC ACG CCG CAG ACC CCG GGC CAG CCC GGA GCG CCC GCC ACC CCC GGC      195
GLY ALA GLY GLU GLN GLN GLN GLN ALA GLY SER GLY ALA PRO HIS THR PRO GLN THR PRO GLY GLN PRO GLY ALA PRO ALA THR PRO GLY       65

ACG GCG GGG GAC AAG GGC CAG GGC CCG CCC GGT TCG GGC CAG AGC CAG CAG CAC ATC GAG TGC GTG GTG TGC GGG GAC AAG TCG AGC GGC      285
THR ALA GLY ASP LYS GLY GLN GLY PRO PRO GLY SER GLY GLN SER GLN GLN HIS ILE GLU CYS VAL VAL CYS GLY ASP LYS SER SER GLY       95

AAG CAC TAC GGC CAA TTC ACC TGC GAG GGC TGC AAA AGT TTC TTC AAG AGG AGC GTC CGC AGG AAC TTA ACT TAC ACA TGC CGT GCC AAC      375
LYS HIS TYR GLY GLN PHE THR CYS GLU GLY CYS LYS SER PHE PHE LYS ARG SER VAL ARG ARG ASN LEU THR TYR THR CYS ARG ALA ASN      125
                                                  ← M1
AGG AAC TGT CCC ATC GAC CAG CAC CAC CGC AAC CAG TGC CAA TAC TGC CGC CTC AAG AAG TGC CTC AAA GTG GGC ATG AGG CGG GAA GCG      465
ARG ASN CYS PRO ILE ASP GLN HIS HIS ARG ASN GLN CYS GLN TYR CYS ARG LEU LYS LYS CYS LEU LYS VAL GLY MET ARG ARG GLU ALA      155

GTT CAG CGA GGA AGA ATG CCT CCA ACC CAG CCC AAT CCA GGC CAG TAC GCA CTC ACC AAC GGG GAC CCC CTC AAC GGC CAC TGC TAC CTG      555
VAL GLN ARG GLY ARG MET PRO PRO THR GLN PRO ASN PRO GLY GLN TYR ALA LEU THR ASN GLY ASP PRO LEU ASN GLY HIS CYS TYR LEU      185

TCC GGC TAC ATC TCG CTG CTG CTG CGC GCC GAG CCC TAC CCC ACG TCG CGC TAC GGC AGC CAG TGC ATG CAG CCC AAC AAC ATT ATG GGC      645
SER GLY TYR ILE SER LEU LEU LEU ARG ALA GLU PRO TYR PRO THR SER ARG TYR GLY SER GLN CYS MET GLN PRO ASN ASN ILE MET GLY      215

ATC GAG AAC ATC TGC GAG CTG GCC GCG CGC CTG CTC TTC AGC GCC GTC GAG TGG GCC CGC AAC ATC CCC TTC TTC CCG GAT CTG CAG ATC      735
ILE GLU ASN ILE CYS GLU LEU ALA ALA ARG LEU LEU PHE SER ALA VAL GLU TRP ALA ARG ASN ILE PRO PHE PHE PRO ASP LEU GLN ILE      245

ACC GAC CAG GTG TCC CTG CTA CGC CTC ACC TGG AGC GAG CTG TTC GTG CTC AAC GCG GCC CAG TGC TCT ATG CCG CTG CAC GTG GCG CCG      825
THR ASP GLN VAL SER LEU LEU ARG LEU THR TRP SER GLU LEU PHE VAL LEU ASN ALA ALA GLN CYS SER MET PRO LEU HIS VAL ALA PRO      275

TTG CTG GCC GCC GCC GGC CTG CAT GCC TCG CCC ATG TCT GCC GAC CGC GTC GTG GCC TCC ATG GAC CAC ATC CGC ATC TTC CAG GAG CAG      915
LEU LEU ALA ALA ALA GLY LEU HIS ALA SER PRO MET SER ALA ASP ARG VAL VAL ALA PHE MET ASP HIS ILE ARG ILE PHE GLN GLU GLN      305

GTG GAG AAG CTC AAG GCG CTA CAC GTC GAC TCA GCC GAG TAC AGC TGC CTC AAA GCC ATC GTG CTG TTC ACG TCA GAC GCC TGT GGC CTG     1005
VAL GLU LYS LEU LYS ALA LEU HIS VAL ASP SER ALA GLU TYR SER CYS LEU LYS ALA ILE VAL LEU PHE THR SER ASP ALA CYS GLY LEU      335

TCG GAT GCG GCC CAC ATC GAG AGC CTG CAG GAG AAG TCG CAG TGC GCA CTG GAG GAG TAC GTG AGG AGC CAG TAC CCC AAC CAG CCC AGC     1095
SER ASP ALA ALA HIS ILE GLU SER LEU GLN GLU LYS SER GLN CYS ALA LEU GLU GLU TYR VAL ARG SER GLN TYR PRO ASN GLN PRO SER      365
                        M2 →                                                                                  ← R3
CGT TTT GGC AAA CTG CTG CTG CGA CTG CCC TCG CTG CGC ACC GTG TCC TCC TCC GTC ATC GAG CAG CTC TTC TTC GTC CGT TTG GTA GGT     1185
ARG PHE GLY LYS LEU LEU LEU ARG LEU PRO SER LEU ARG THR VAL SER SER SER VAL ILE GLU GLN LEU PHE PHE VAL ARG LEU VAL GLY      395
                              ← R2                                                                  ← R1
AAA ACC CCC ATC GAA ACT CTC ATC CGC GAT ATG TTA CTG TCT GGG AGC AGC TTC AAC TGG CCT TAC ATG TCC ATC CAG TGC TCC TAG ACC     1275
LYS THR PRO ILE GLU THR LEU ILE ARG ASP MET LEU LEU SER GLY SER SER PHE ASN TRP PRO TYR MET SER ILE GLN CYS SER ***          423

TTG GGC GCT TCC CAC CTG CCC CGT CCC CCT AGA GAC TCA GAG GAC CCA CCT GGG CCA AGG ACT CCA AAG CCG CGG GGA CAC CGG GAA GTG     1365

CAG CGG GCC AGG CAG GCT GGG TGG GAG GGA GGA GGG CCG AGA CAG GAG CAG CCC ACC CAG CAG AAA TAC AAT CCG AGC TAC AAA GCA TGG     1455
```

Appendix B. The pBluescript II SK (+/-) phagemid maps

The pBluescript® II SK (+/-) phagemid is a 2961-bp phagemid derived from pUC19. The SK designation indicates the polylinker is oriented such that *lacZ* transcription proceeds from *Sac* I to *Kpn* I.

f1 (+) origin: (3-459 bp) f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

f1 (-) origin: (3-459 bp) f1 filamentous phage origin of replication allowing recovery of the antisense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

ColE1 origin: (1032-1972 bp) Plasmid origin of replication used in the absence of helper phage.

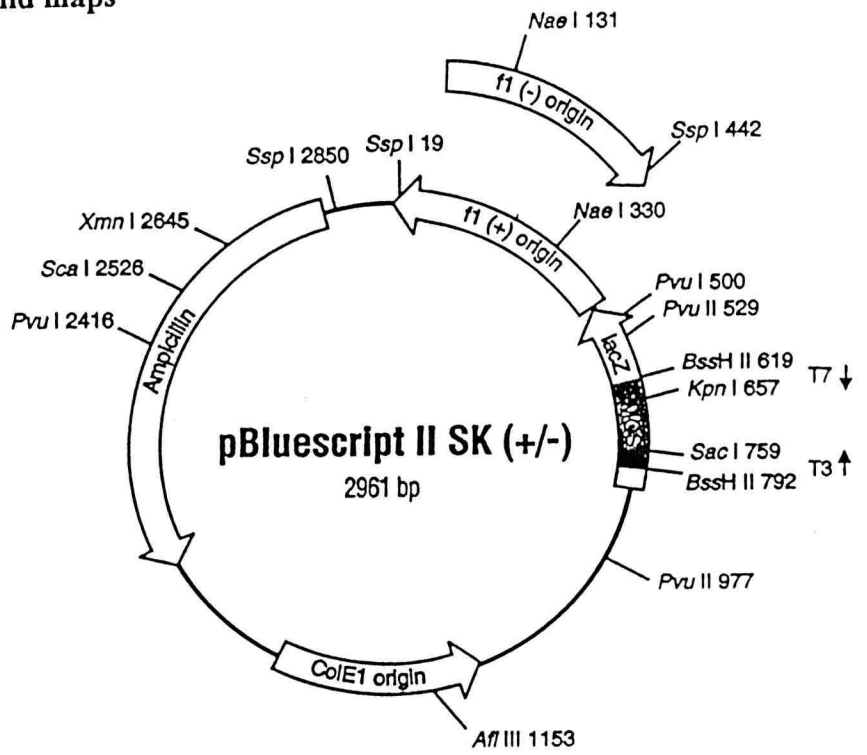
***lacZ* gene:** (*lac* promoter: 816-938 bp) This portion of the *lacZ* gene provides α-complementation for blue/white color selection of recombinant phagemids. An inducible *lac* promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

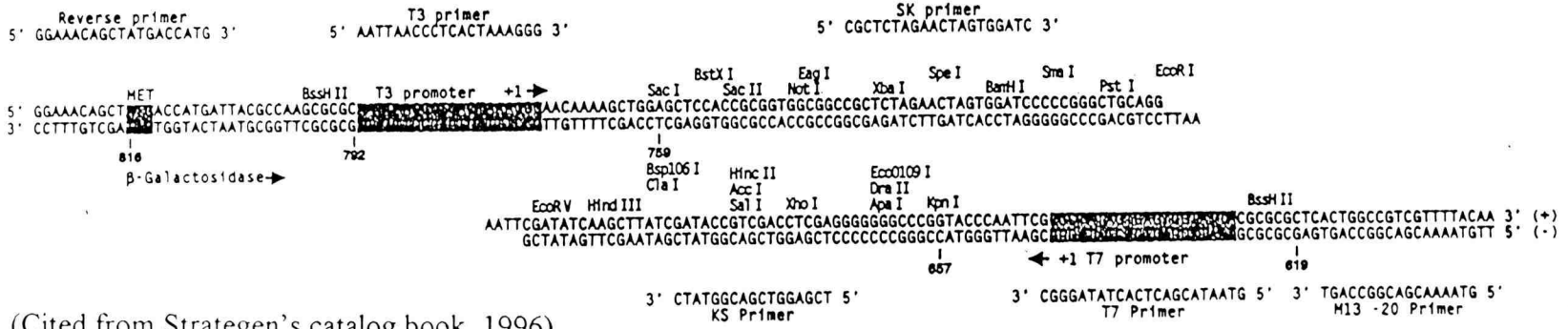
MCS: (657-759 bp) Multiple cloning site flanked by T3 and T7 RNA promoters (please see the polylinker sequence below).

Ampicillin: (1975-2832 bp) Ampicillin-resistance gene (Amp^r) for antibiotic selection of the phagemid vector.

Please Note: The upper strand is designated the (+) strand and the lower strand is designated the (-) strand.

GenBank® # X52328 [SK(+)] & # X52330 [SK(-)]





(Cited from Strategen's catalog book, 1996)

Appendix C. The pBluescript II SK (+/-) phagemid restriction sites

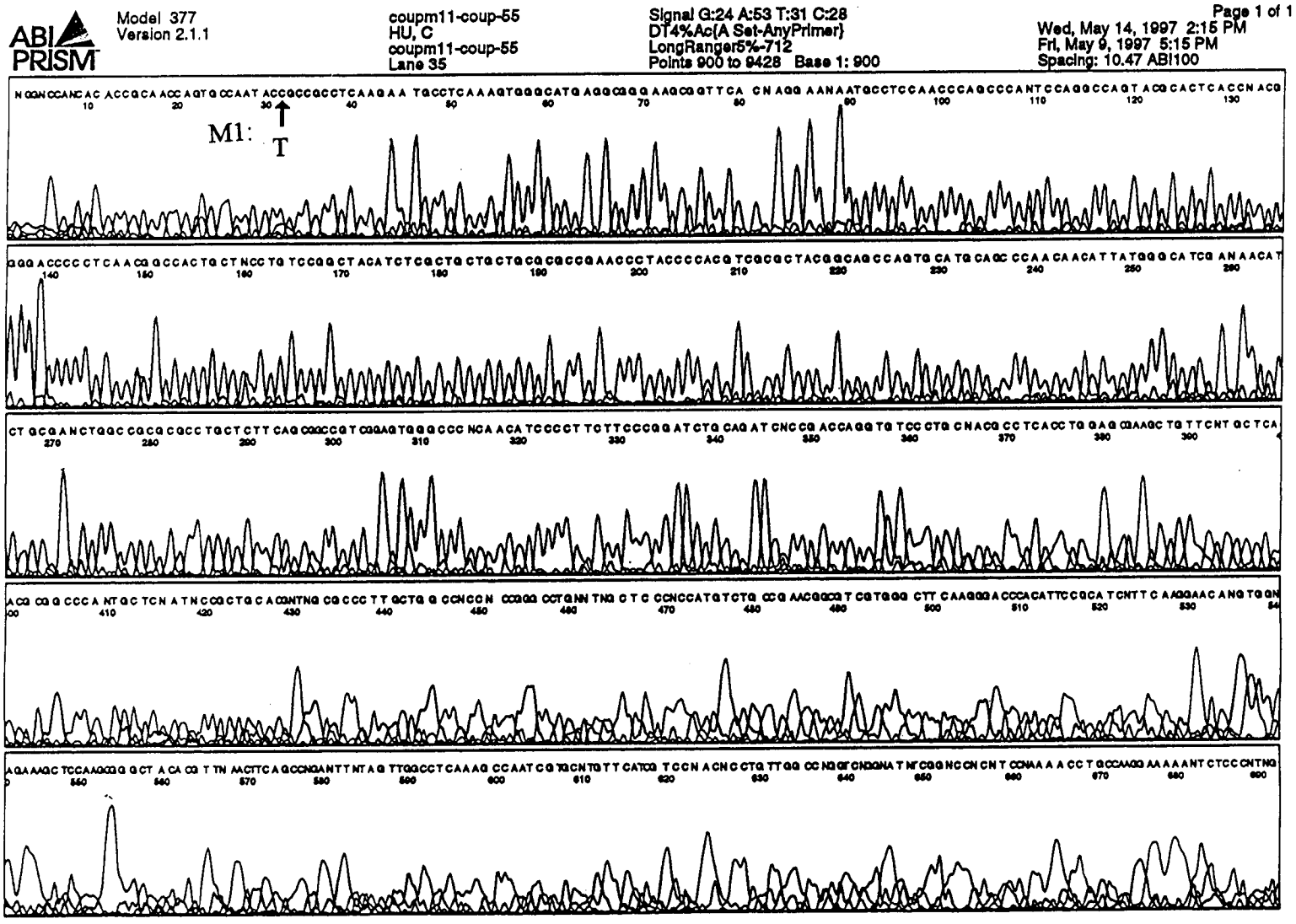
Acc I	1	675
Hinl I	1	2583
Afl III	1	1153
Aha II	1	2583
AlwN I	1	1569
Apa I	1	663
BamH I	1	719
Bsa I	1	2123
Bsp106 I	1	684
BstX I	1	751
Dra III	1	(+: 227), (-: 237)
Esg I	1	738
EcoO109 I	1	660
EcoR I	1	701
EcoR V	1	697
HgiE II	1	1739
Hind II	1	676
Hind III	1	689
Kpn I	1	657
Nae I	1	(+: 330), (-: 131)
Not I	1	738
Pst I	1	711
Sac I	1	759
Sac II	1	750
Sal I	1	674
Sca I	1	2526
Sma I	1	715
Spe I	1	725
Xba I	1	731
Xho I	1	668
Xma I	1	2645
Aos I	2	479, 2268
ApaL I	2	1467, 2713
Ava I	2	668, 713
Ava II	2	2184, 2406
Bgl I	2	472, 2166
BsmA I	2	2107, 2883
BspH I	2	1873, 2881
BsrH II	2	619, 792
Cfr 101	2	(+328), (-:129), 2126
Pvu I	2	500, 2416
Pvu II	2	529, 977
Rsa I	2	655, 2526
Rsp I	2	500, 2416
Ssp I	2	(+: 19), (-: 442), 2850
Tth 111 II	2	1743, 1782
Ban II	3	(+: 302), (-: 163), 663, 759
Dra I	3	1912, 1931, 2623
Mme I	3	(+: 204), (-: 259) 1368, 1552
Vsp I	3	924, 983, 2218
Alw I	4	727, 1802, 1899, 2679
Ban I	4	(+: 264), (-: 193), 653, 897, 1994
Dde I	4	1428, 1837, 2003, 2543
Eae I	4	608, 737, 991, 2433
Fok I	4	555, 2012, 2193, 2480
Hae II	4	(+: 378, 386), (-: 79, 87), 1031, 1401
Hga I	4	(+: 454), (-: 12), 1264, 1842, 2582
Hgi A I	4	759, 1471, 2632, 2717
Hgi CI	4	(+: 264), (-: 193), 653, 897, 1994
SfaN I	4	1250, 2302, 2493, 2742
BstN I	5	576, 893, 1181, 1302, 1315
EcoR II	5	574, 891, 1179, 1300, 1313
Acc II	6	(+: 302), (-: 163), 663, 730, 1472, 2631, 2718
Hph I	6	(+: 228), (-: 234), 1889, 2116, 2532, 2738, 2773
Mae I	6	(+: 378), (-: 81), 726, 732, 1648, 1901, 2236
Nci I	6	658, 714, 715, 1533, 2229, 2580
Sdu I	6	(+: 302), (-: 163), 663, 759, 1471, 2632, 2717
Taq I	7	(+: 260), (-: 199), 669, 675, 684, 699, 1253, 2697
Xho II	7	719, 1794, 1805, 1891, 1903, 2671, 2688
Hinf I	8	(+: 154, 176), (-: 282, 304), 632, 968, 1053, 1128, 1524, 2041
Mae II	8	(+: 171, 183, 226, 336), (-: 123, 233, 276, 288), 595, 1856, 2272, 2645
Mbo II	8	(+: 367), (-: 95), 504, 1024, 1815, 1886, 2641, 2719, 2828
Nla III	8	811, 1157, 1877, 2368, 2378, 2456, 2492, 2885
Sau96 I	8	(+: 218), (-: 240), 507, 659, 660, 1088, 2167, 2184, 2406
ScrF I	11	576, 658, 714, 715, 893, 1181, 1302, 1315, 1533, 2229, 2580
Mae III	12	(+: 400, 412), (-: 44, 56), 568, 588, 1509, 1572, 1688, 1971, 2302, 2360, 2513, 2701
Bbv I	13	(+: 393), (-: 64), 462, 535, 721, 964, 1045, 1063, 1482, 1572, 1575, 1781, 2084, 2475
Hpa II	13	(+: 329), (-: 130), 657, 714, 871, 1360, 1507, 1533, 1723, 2127, 2161, 2228, 2338, 2580
Mnl I	14	(+: 254), (-: 207), 520, 663, 677, 773, 1001, 1052, 1260, 1335, 1584, 1984, 2065, 2213, 2419
Pal I	14	(+: 77, 219), (-: 242,384), 509, 611, 661, 740, 994, 1168, 1179, 1197, 1631, 2089, 2169, 2436
Bsp50 I	15	(+: 35, 411, 431, 455), (-: 6, 30, 50, 426), 621, 623, 749, 794, 1000, 1002, 1200, 1781, 2111, 2604, 2936
Dpn I	15	499, 721, 1721, 1796, 1807, 1815, 1893, 1905, 2010, 2351, 2369, 2415, 2673, 2690, 2726
Sau3A I	15	497, 719, 1719, 1794, 1805, 1813, 1891, 1903, 2008, 2349, 2367, 2413, 2671, 2688, 2724
Nla IV	15	(+: 266, 287, 299), (-: 162, 174, 195), 655, 661, 662, 721, 899, 1185, 1224, 1996, 2091, 2131, 2342, 2932
Alu I	17	(+: 57, 314) (-: 147, 404), 529, 691, 757, 763, 818, 913, 977, 1095, 1321, 1411, 1457, 1714, 2235, 2335, 2398
Mae I	19	(+: 15, 26, 38, 49, 66, 164, 435), (-: 24, 295, 393, 410, 421, 433, 444),

Enzymes that do not cut :

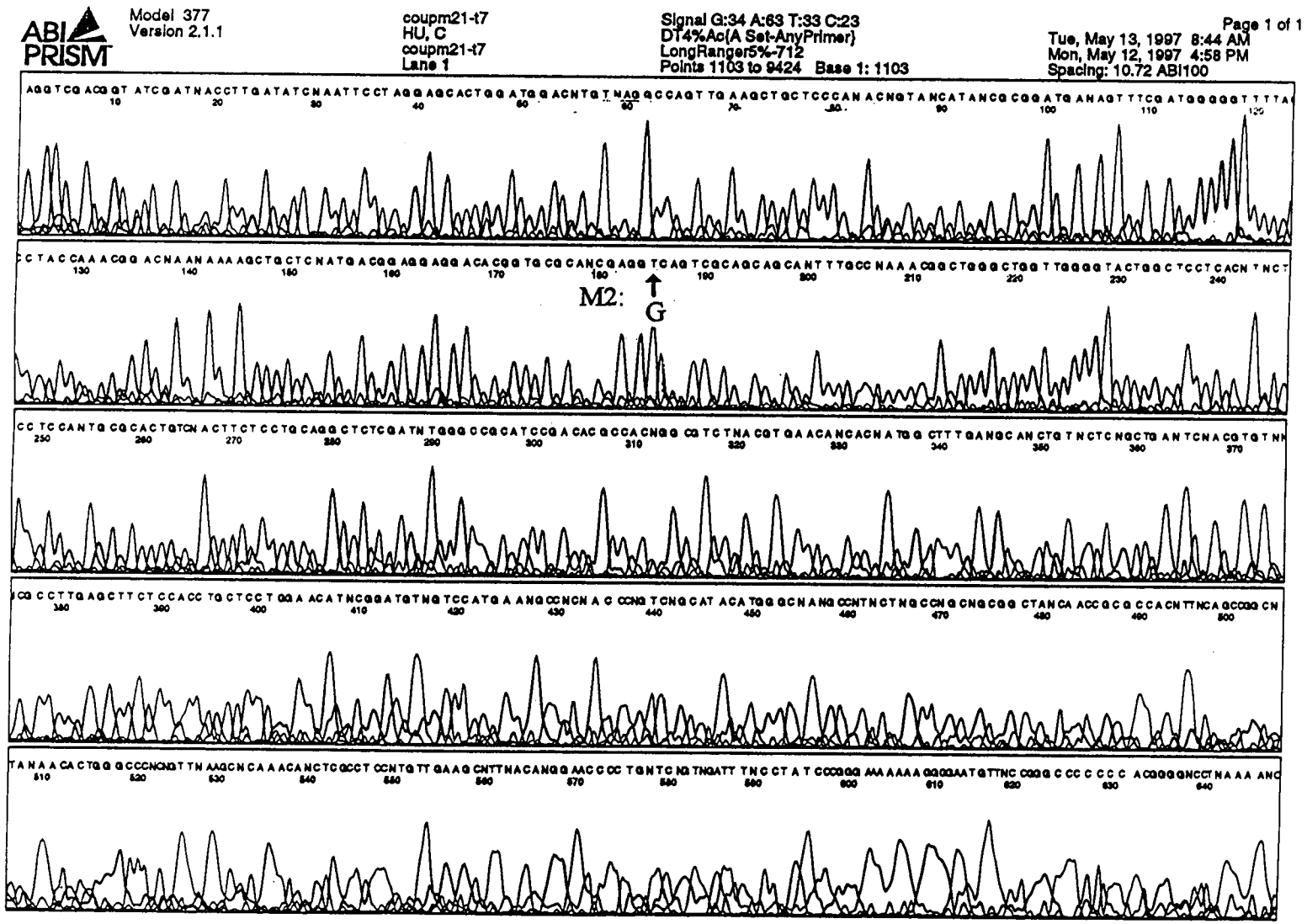
Ase II, Afl II, Aoc I, Asu II, Ava III, Avr II, Bal I, Bcl I, Bgl II, BsiC I, Bsm I, BspM I, BspM II, BstE II, Eco47 III, Eco72 I, EcoB, EcoN I, Esp I, Hpa I, Mfe I, Mlu I, Mst II, Mun I, Nar I, Nco I, Nde I, Nhe I, Nru I, Nsi I, PflM I, PmaC I, PpuM I , Rsr II, Sau I, Sfi I, Sna I, SnaB I, Sph I, Scf I, Stu I, Sty I, Tth111 I, Xca I

(Cited from Strategen's catalog book, 1996)

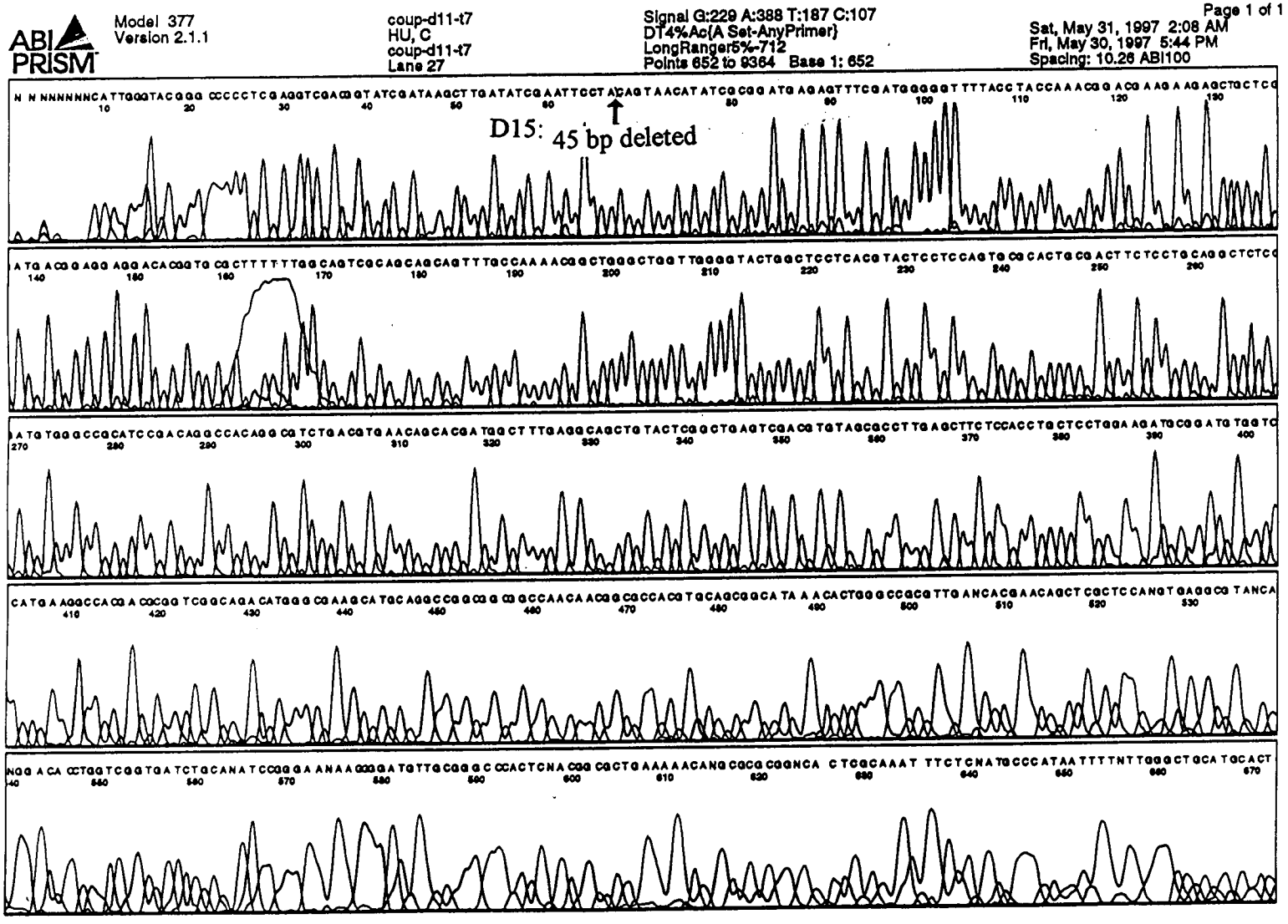
Appendix D. Verification of the nucleotide sequene of the mutant pBMR1



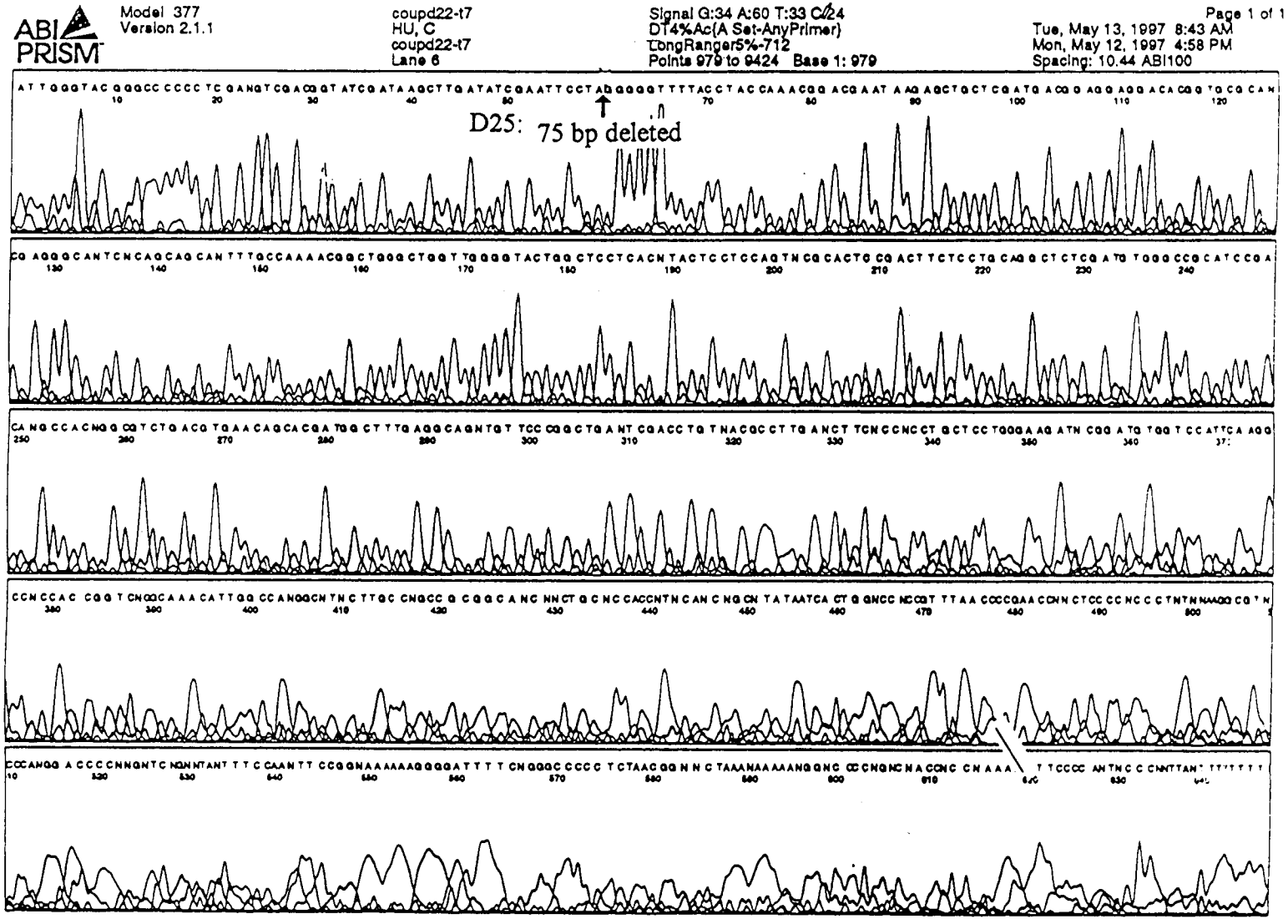
Appendix E. Verification of the nucleotide sequene of the mutant pBMR2



Appendix F. Verification of the nucleotide sequene of the mutant pBDR1

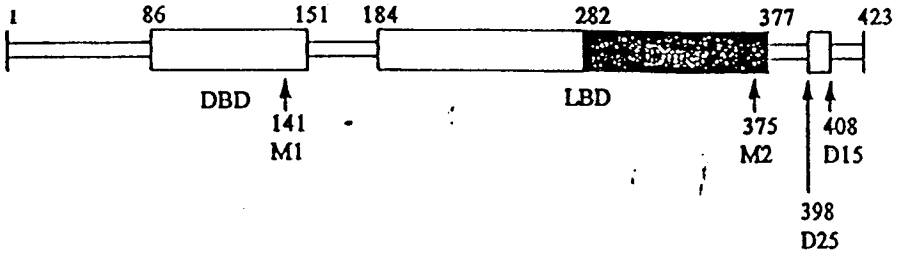


Appendix G. Verification of the nucleotide sequene of the mutant pBDR2

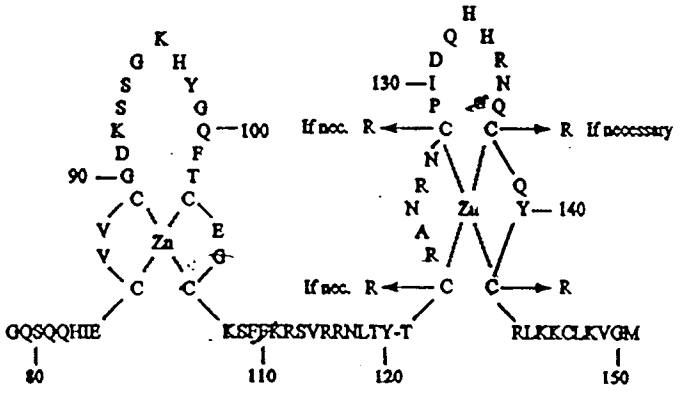


Appendix H. Structure of the COUP-TFI

Schematic representation of the COUP-TFI. The DNA-binding (DBD), putative ligand-binding (LBD) and putative transrepression domains are boxed. Dimerization motif is shown by shaded area in the LBD. Arrows indicate where mutation or deletion will be introduced



Amino acid sequence of the COUP-TFI DNA-binding domain. N-terminal Zn finger is responsible for DNA-binding. Cysteine 141 in this Zn finger will be mutated to arginine. If this mutation fails to disrupt DNA-binding, additional mutations at cysteines 138, 128 and 122 will be introduced.



Partial amino acid sequence of the COUP-TFI dimerization motif. Helix 9, helix 10, and the 6th, 7th, 8th and 9th heptad repeats are marked. Leucine 371 and proline 375 and are the major hydrophobic contacts, and aspartic acid 331 and glutamic acid 342 are the major hydrophilic contacts. Proline 375 will be mutated to threonine. If necessary, aspartic acid 331 to asparagine, glutamic acid 342 to glutamine, and leucine 371 will be mutated to alanine.