NFY represses the promoter activities of acyl CoA: diacylglycerol acyltransferase 2 gene by binding to CCAAT box in HepG2 cells

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Directed by Professor Yong-Ho Ahn

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ABSTRACT

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Acyl CoA: diacylglycerol acyltransferase (DGAT, E.C. 2.3.1.20) is an enzyme that catalyzes the final step of triacylglycerol (TG) synthesis and expressed in various tissues including liver and white adipose tissue. The enzyme is known to exist in two forms; DGAT1 and DGAT2. Mice lacking DGAT2 have a severe deficiency of TG synthesis with accompanying physical symptoms. However, little is known about the regulatory mechanisms controlling DGAT2 gene transcription. To understand transcriptional regulation of DGAT2 gene, we have determined transcription initiation sites by 5'-RACE. Serial deletion, transient transfection and luciferase assay of the 5'-flanking region of the human DGAT2 gene showed

that there is an inhibitory region at -570/-495 region upstream of the translation start site. Gel shift and supershift assays demonstrated that the transcription factor nuclear factor Y (NFY) binds to CCAAT box (-499/-495) of human DGAT2 promoter. A chromatin immunoprecipitation assay using HepG2 cells showed the binding of NFY to CCAAT box. Furthermore, over-expression of NFY repressed promoter activity of DGAT2 gene in a dose-dependent manner, suggesting that NFY is involved in negative transcriptional regulation of the human DGAT2 gene.

Key words : DGAT2, NFY, triglyceride, metabolism, transcriptional regulation

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I. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is being recognized by one of the component of obesity or type 2 diabetes mellitus (T2DM) which become world epidemics. NAFLD is the most common cause of liver dysfunction, ranging from simple fatty liver (hepatic steatosis), with benign prognosis, to a potentially advanced form, nonalcoholic steatohepatitis (NASH)[1,2,3]. Therefore, progression from NAFLD to NASH increases the risk for dysfunction liver. Hepatic steatosis is

characterized by the accumulation of triglyceride in hepatocytes, which is strongly associated with insulin resistance[1]. Triglycerides (triacylglycerols) are neutral lipids found in most eukaryotic cells, consisting of a glycerol backbone and three long-chain fatty acids[4]. They serve as a storage molecule of fatty acids (FAs) for energy utilization and membrane lipid biosynthesis in the adipose tissue and skeletal muscle. In addition, they are integral components of lipoprotein particles synthesized by the liver and small intestine[4]. Thus, understanding the molecular mechanisms of genes involved in triglyceride synthesis is crucial for elucidating the processes that may contribute to development of obesity, T2DM and other disorders of lipid metabolism.

Diacylglycerol, the obligate precursor to TG, is derived either from the glycerol-3-phosphate pathway or the monoacylglycerol pathway and esterified to TG by acyl CoA:diacylglycerol acyltransferase (DGAT)[5,6]. At present, two isoforms of DGAT; DGAT1 and DGAT2 are identified in mammalian tissues[6,7]. DGAT1 is highly expressed in human small intestine, colon, testis, and skeletal muscle, whereas DGAT2 is mainly expressed in adipose and liver in human[8]. Mice lacking DGAT2 die shortly after birth due to severe lipopenia with 90% of reduction in TG level and impaired skin barrier function[9,10]. These results indicate that DGAT2 plays a more crucial role than DGAT1 in whole body metabolism.

During the differentiation of NIH3T3-L1 preadipocytes into adipocytes, DGAT1 and DGAT2 mRNA are shown to be increased, suggesting the participation of C/EBPα and C/EBPβ[11]. Although DGATs are enzymes of lipid metabolism, sterol regulatory element binding element (SREBP) was not shown to affect their gene transcriptions[12,13]. In liver, X-box binding protein 1 (XBP1) is shown to induce DGAT2 gene expression[14]. In the primary cultured hepatocytes, DGAT1 and DGAT2 mRNA levels were decreased by MEK-ERK signaling pathway[15]. However, detailed regulatory mechanisms governing the transcription of DGAT2 gene is not well understood. Computer searches of the 5'-flanking sequence of DGAT2 gene showed that there are couples of CCAAT consensus sequences that may be responsible for the transcriptional regulation of the gene. Over the past 10 years, numerous transcriptional factors binding to the CCAAT box have been characterized[16]. To understand a transcriptional regulation of DGTA2 gene in liver, we have attempted to identify a trans-acting factor which binds to CCAAT box. In this study, we demonstrated that NFY binds to the CCAAT box of DGAT2 gene promoter and plays as a repressor of DGAT2 gene transcriptions.

Ⅱ. MATERIALS AND METHODS

1. Bioinformatics of the DGAT2 DNA sequence

The genomic organization of the human DGAT2 and the 5'-flanking region sequence of DGAT2 was obtained from the ENSEMBL human gene browser by BLAST researching (http://www.ensembl.org/index.html). Consensus sequences of potential binding sites for transcription factors were predicted by using Genomenet (http://www.genome.ad.jp/), TESS (Transcription Element Search System, available at http://www.cbil.upenn.edu/tess), Genomatrix (Genomatix Software GmbH, Munich, Germany, available at http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl) and TFSEARCH web sites (available at http://jaspar.cgb. ki.se/cgi-bin/jaspar_db.pl).

2. 5´-Rapid amplification of cDNA end (5'-RACE)

Total RNA was isolated from HepG2 cells (American Type Culture Collection number HB-8065), using TRIzol reagent (Invitrogen, Leek, Netherlands). Transcription initiation sites were determined by 5′-RACE-PCR using the 5´/3´RACE kit, 2nd Generation (Roche, Mannheim,Germany). Briefly, the first

strand of cDNA was synthesized from 2 μg total RNA using gene specific reverse primer hDGAT2 RACE1 (Table 1) and purified using MEGAquick-spin™ (Intron Biotechnology, Daejeon, Korea). Remaining RNA template was degraded by the RNase H. The first-strand cDNAs were tailed with poly[A] and amplified by PCR using the oligo dT-anchor primer and nested hDGAT2 RACE2 primer (Table 1). The cDNAs were second-amplified by PCR using the PCR anchor primer and nested hDGAT2 RACE3 primer (Table 1). PCRs were performed using *Pfu*-X DNA polymerase (Solgent, Daejeon, South Korea). To amplify DNA regions of high GC content, Band doctor, containing in *Pfu*-X DNA polymerase (Solgent) were used. PCR products were subjected to 1.2% agarose gel and DNA fragments were purified with MEGAquick-spin™ (Intron), subcloned into pTOP Blunt V2 using a TOPcloner blunt V2 kit (Enzynomics, Daejeon, South Korea). Nucleotide sequences were determined using an ABI3730XL DNA analyzer (Applied Biosystems, CA, USA).

3. Plasmids construction.

The proximal promoter of human DGAT2 (-1340/-1) was amplified by PCR using HepG2 cell genomic DNA. The PCR products were subcloned into the *Mlu* I and *Hind* III site of the pGL3-basic luciferase reporter vector (Promega, Wis, USA).

Serial deletion constructs of DGAT2 promoter luciferase reporter (pHDGAT2L-1340) were prepared by using the Deletion Kit for Kilo-Sequencing (Takara Bio Inc., Otsu, Japan). The human NFYA, NFYB and NYFC cDNAs were prepared by PCR amplification using HepG2 cell cDNA. The human NFYA cDNA was subcloned into the pcDNA3-myc-His (Promega). The human NFYB and NFYC cDNAs were subcloned into the pcDNA3-myc2. Primers used for PCR are shown in Table 1.

4. Cell culture and transient transfection assay.

The HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS) and antibiotics including 100 U of penicillin/ml, 100 mg of streptomycin at 37℃ and 5% CO_2. Transient transfections were performed using FuGENE® HD (Roche, Mannheim, Germany). The day before the transfection, HepG2 cells were plated at a density of 3×10^5 cells per well in a 12-well plate in 1 ml of the medium with 10% FBS. Five hundred nanograms of reporter plasmid DNA, 100 ng of pCMV-β-galactosidase control vector, indicated amount of several expression plasmid DNAs and 3 μl of FuGENE® HD reagent were added to 100 ul medium without FBS and antibiotics, and incubated at room temperature for 15 min and then added to cells

that had reached 70–80 % confluence. After incubation for 5 hr, the medium was added with 800 μl of fresh medium containing 20 % FBS, and the cells were incubated for 24 hr. The cells were then washed twice with phosphate buffered saline (PBS), and lysed with 100 μl of reporter lysis buffer (Promega). Twenty μl from 100 μl of the cell lysate were assayed for luciferase activity using a luciferase assay kit (Promega) and a luminometer MicroLumatPlus LB96V (Berthod, Bundoora, Australia). The pGL3-Basic vectors were used as the negative controls. Transfection efficiency was determined by measuring β-galactosidase activity after co-transfection of both reporter construct and pCMV-β-galactosidase control vector, into the cells. Normalized luciferase activities were shown as the mean ± S.D. of three independent experiments in triplicate.

Table 1. Sequences of oligonucleotides used in this study

Name		Position	Sequence (5´→3´)	Experiment
hDGAT2-RACE1	Rev	Exon	GTTCCTGGTGGTCAGCAGGTTGT	5´-RACE
hDGAT2-RACE2	Rev	Exon	ACAGGACCCACTGGAGCACTGA	5´-RACE
hDGAT2-RACE3	Rev	Exon	GGAGTAGGCGGCTATGAGGGTC	5´-RACE
Oligo d[T]-anchor primer	For		GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV	5´-RACE
PCR anchor primer	For		GACCACGCGTATCGATGTCGAC	5´-RACE
hDGAT2-1340 (s) Mlu I	For	Promoter	CATACGCGTGGCACTCCCAAACCTCTCTCA	Cloning
hDGAT2-1 (as) HindIII	Rev	Promoter	CATAAGCTTGGCTGAAGCCCGCCCAGTCA	Cloning
hNFYA (s) Kpn I	For	Exon	CGATGGTACCATGGAGCAGTATACAGCAAACAG	Cloning
hNFYA (as) Xba I	Rev	Exon	CGATTCTAGAGGACACTCGGATGATCTGTGTCA	Cloning
hNFYB (s) Kpn I	For	Exon	CGATGGTACCATGACAATGGATGGTGACAGTTC	Cloning
hNFYB (as) Xba I	Rev	Exon	CGATTCTAGATCATGAAAACTGAATTTGCTGAAC	Cloning
hNFYC (s) Kpn I	For	Exon	CGATGGTACCATGTCCACAGAAGGAGGATTTGG	Cloning
hNFYC (as) Xba I	Rev	Exon	CGATTCTAGATCAGTCGCCGGTCACCTGG	Cloning
hDGAT2-514/-480 (s)	For	Promoter	GGCCGCCGGGCCGTACCAATCTCCGCGGGGGAGCG	EMSA
hDGAT2-514/-480 (as)	Rev	Promoter	CGCTCCCCCGCGGAGATTGGTACGGCCCGGCGGCC	EMSA
NFY (s)	For		AGACCGTACGTGATTGGTTAATCTCTT	EMSA
NFY (as)	Rev		AAGAGATTAACCAATCACGTACGGTCT	EMSA
CTF (s)	For		TTTTGGATTGAAGCCAATATGATAACT	EMSA
CTF (as)	Rev		AGTTATCATATTGGCTTCAATCCAAAA	EMSA
C/EBP (s)	For		TGCAGATTGCGCAATCTGCACT	EMSA
C/EBP (as)	Rev		AGTGCAGATTGCGCAATCTGCA	EMSA
GLUT4–LXRE (s)	For		CAGCCCCGGGTTACTTTGGGGCATTGCTCC	EMSA
GLUT4–LXRE (as)	Rev		GGAGCAATGCCCCAAAGTAACCCGGGGCTG	EMSA
hDGAT2-608/-587 (s)	For	Promoter	GCCAAAAGACAGCCCCTCCTAA	ChIP
hDGAT2-415/-394 (as)	Rev	Promoter	GAATGCGTGAGAGGAGAGCAGC	ChIP
hDGAT2+5002/+5023 (s)	For	Intron	AGGGCCTCTGAGTGACCAACTG	ChIP
hDGAT2+5195/+5216(as)	Rev	Intron	AATCAGCCAGCAGCTCCATACC	ChIP

Rev, Reverse; For,Forward; V=A,C or G

5. Nuclear extracts and in vitro translated protein preparation.

The HepG2 cells were washed with ice-cold PBS and resupended in ice-cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and complete protease inhibitor Cocktail (Roche)]. The cells were put on ice for 10 min and incubated for additional 15 min in the buffer A containing 0.5% Nonidet P-4. The pellet was harvested by centrifuging at 6,000 × *g* for 1 min, resuspended in extract buffer [20 mM HEPES (pH 7.9), 400 mM KCl, 4.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1mM PMSF, 1 mM $NaNO_3$, 1 mM NaF and complete protease inhibitor cocktail (Roche)] and incubated on ice for 1 hr. The cell lysate centrifuged at 10,000 × *g* for 10 min. Equal amount of glycerol (15%) was added to the supernatant and stored at -80°C until use. *In vitro* translated proteins of hNFYA-myc-His, hNFYB-myc2 and hNFYC-myc2 were prepared by using a coupled transcription/translation kit (Promega). Briefly, each NFY was *in vitro* translated by adding 2 µg of expression vector to an aliquot of the TNT® Quick Master Mix and incubated in a 50µl reaction volume for 60–90 minutes at 30°C. The amount of translated proteins was confirmed by western blot analysis using anti-Myc antibody (Cell Signaling, MA, USA).

6. Electrophoresis mobility shift assay (EMSA).

The oligodeoxynucleotide probes (Table 1) for the gel shift assays are shown in Table 1. Complementary oligonucleotides were annealed to each other to generate double-stranded DNA by incubating at 95 ^{o}C for 5 min and gradually cooled down to 37 ^{o}C. The double-stranded DNA probes were labeled with [α-^{32}P] (Amersham Biosciences, UK) using T4 polynucleotide kinase (New England Biolabs Inc., MA, USA). The labeled double-stranded oligonucleotides were purified using Sephadex G25 spin column (Pharmacia, NJ, USA). The reaction mixture (20 μl), containing 5 μg of nuclear extract from HepG2 cells or 3 μl of *in vitro* translated proteins, 10 mM Hepes (pH 7.9, KOH), 1 mM $MgCl_2$, 30 mM KCl, 1 mM EDTA , 5% glycerol and 1 μg of poly (dI-dC) were preincubated on ice in the absence of the probe for 20 min and incubated for additional 20 min on ice with the 50,000 cpm of radiolabeled probe (approximately 0.02 pmol). The reaction mixtures were then subjected to electrophoresis with a native 5% polyacrylamide gel at 150 V for 90 min in 0.5 × TBE buffer (45 mM Tris-borate, pH 8.0, 45 mM boric acid, 1 mM EDTA). For competition experiments, excessive unlabeled competitor oligonucleotides were added to the binding reaction mixtures before the addition of the radiolabeled oligonucleotide probe. For the supershift experiments, 2 μg of each antibody was added to the reaction mixture before the addition of the radiolabeled

oligonucleotide probe. The labeled probe was then added to the reaction mixture and was incubated at 4 ^{o}C for additional 30 min, and subjected to polyacrylamide gel (5%) for electrophoresis. The anti-NFYA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gels were dried and exposed using a FLA7000 image analyzer (Fuji Photo Film, Tokyo, Japan).

7. Chromatin immunoprecipitation (ChIP) assay.

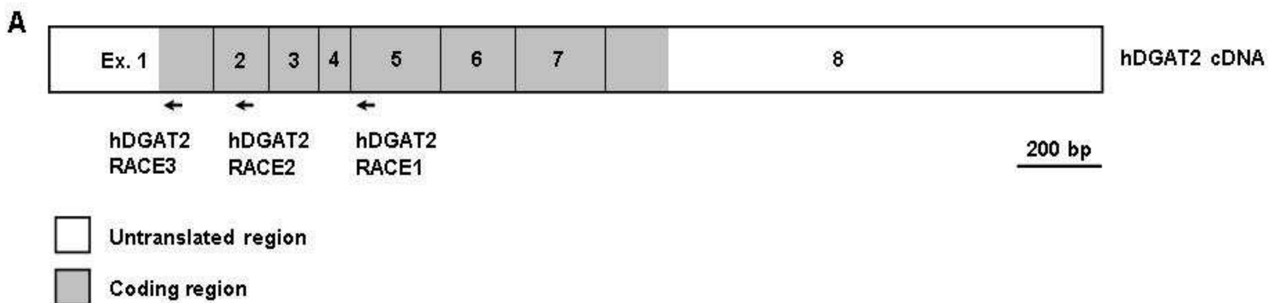
ChIP assay was performed using ChIP Assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's instructions. Briefly, approximately 10^6 cells were treated with 1% formaldehyde (final concentration, v/v) in cell culture medium for 10 min at 37 ^{o}C to cross-link proteins to DNA. The cells were washed twice with PBS, pH 7.4 containing protease inhibitor cocktail (Roche), harvested by centrifugation, resuspended in 200 μl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), and protease inhibitor cocktail) and incubated on ice for 10 min. After incubation, supernatant was obtained by sonication followed by brief centrifugation at 10,000 × *g* for 10 min. The supernatant was divided into aliquots (100 μl) and diluted with a 10-fold volume of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl) and pre-cleared using a 50% slurry of

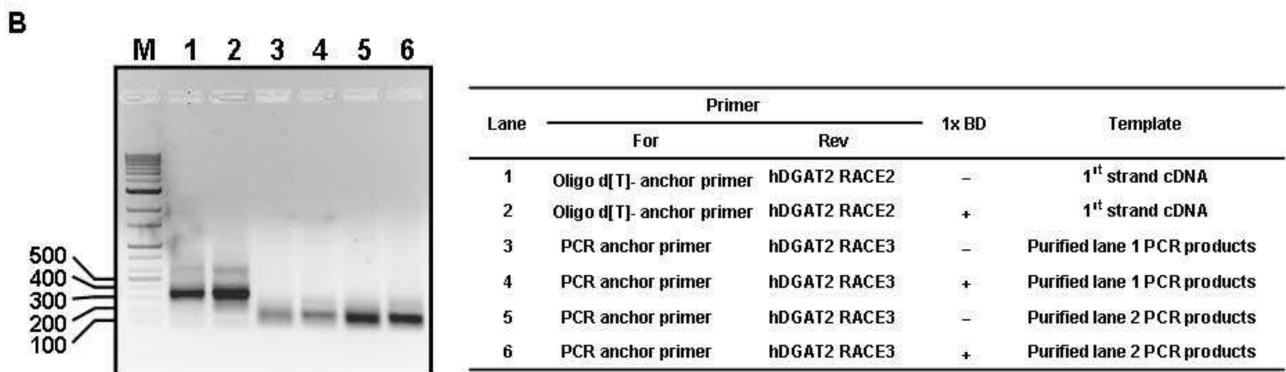
protein A-agarose/herring sperm DNA for 1 h. For immunoprecipitations, 4 μg of NFYA antibody, without antibody or normal rabbit IgG was added and agitated overnight at 4 ^{o}C. The complexes were collected with protein A-agarose/herring sperm DNA and eluted from the beads. The eluted DNA was amplified by PCR using primers for each set (Table 1). The PCR products were resolved 1.5% agarose gel and visualized with ethidium bromide staining.

III. RESULTS

1. Determination of transcription start sites of the human DGAT2 gene

To determine transcription initiation site, 5'-RACE was performed. First stranded DNA was synthesized from total RNA of HepG2 cells using hDGAT2 RACE1 primer (Fig. 1A, Table 1) and amplified by PCR using the first strand cDNA as template and the oligo dT-anchor primer (Fig. 1A, B). For the verification of PCR products, nested PCR was performed using hDGAT2 RACE 2 and RACE3 and PCR anchor primers (Fig. 1A, B). These DNAs were subcloned into pTOP Blunt V2 and sequenced to determine putative transcription initiation site(s). As shown in the box (Fig. 2), -51, -60 and -64bp (base G) from translation initiation site (+1) were assumed a potential transcription initiation site(s). The region upstream of these bases was used as a promoter of DGAT2 gene (Fig. 2) and nested hDGAT2 RACE2 primer resulted in the amplification of the major one.





Lane	Primer For	Primer Rev	1x BD	Template
1	Oligo d[T]- anchor primer	hDGAT2 RACE2	–	1st strand cDNA
2	Oligo d[T]- anchor primer	hDGAT2 RACE2	+	1st strand cDNA
3	PCR anchor primer	hDGAT2 RACE3	–	Purified lane 1 PCR products
4	PCR anchor primer	hDGAT2 RACE3	+	Purified lane 1 PCR products
5	PCR anchor primer	hDGAT2 RACE3	–	Purified lane 2 PCR products
6	PCR anchor primer	hDGAT2 RACE3	+	Purified lane 2 PCR products

Fig. 1. Analysis of transcription start site of human DGAT2 geneby 5′-RACEPCR. *A*. Structure of human DGAT2 cDNA. Arrowsindicated localization of three DGAT2 specific primers using in5′-RACE analysis. *B*. 5′-RACE-PCR using HepG2 cDNA and human DGAT2 specific primers resulted in the amplification of approximately 300 bp size DNA fragments (lane 1 and 2) and 100 bp size DNA fragments (lane 3-6). *M* is the DNA marker. *BD*, Band doctor; additive buffer of PCR reaction mixture for amplifying GC-rich region in the template.

```
-1340 gtggcactcc caaacctctc tcagttcaag cacccgcgcc tctcacttca gcctcatttt
-1280 ccacagttgc ctgggctcca gccactccag ggcccccagc cctgccccaa acatgctggc
          E-Box                                              IRS
-1220 ttttctaaca ctttatgcct atctttctgc tttgccctca caccttccat ctgcaaaatt
                                                             E-Box
-1160 cctattcatt aatgggcaaa ccattctgga atgctttcta tgtgccagac actacctagg
-1100 ctctttttat accttatctc atttacttct caaagtacct cacaaaaata ataattatta
                                                          IRS
-1040 ttcccatttt acagatatgg aaactgaggc tcaagagagg cagggcaaga actggactcc
 -980 tagtctatct gattccaaac ctgctgtgca ccaacctctc tgggaagtgc accagcgccc
 -920 ccactccccc agcccagggc agagtcttct ctactctgtg gttccatagc cccctggttt
 -860 ctcgcttgtc ccggccgttg tcaccagtga ctgtgtgcgg ctgtctctgc tgccccccag
 -800 cgtgagcatc ccaagggcag aacctgggct gatctggctg ggtccccagc acccagcagg
 -740 gtataggtgc tctgtgaggt ttcttaataa agagatggaa agccagaagc agtttgggtg
                                                                  Sp1
 -680 gttgagatac ccagccctaa cctaactaat cctgatacca gtgaccaaaa gcgggacctt
                                 HNF4             USF1
 -620 cgcatctttg ctgccaaaag acagcccctc ctaaagagta aaggcccgac cccctgcacg
                                                                  Sp1
 -560 ccctctgcct gcaccgcacg tgcttggttt tccccgcccg ggtactggcc gccgggccgt
                            E-Box,            Sp1
 -500 accaatctcc gcgggggagc gcccggggtc ggactgaggg agcgagggga ataaccgggc
        NFY, CTF, C/EBP
 -440 gcgccccttg gaagcagggc tcagagctgc tctcctctca cgcattcccc ggatccgcgc
 -380 ggagcaggct gctggccagc cccgggcccg cgccaagcag agcctcaggt gcggttcccc
                                                        E-Box
 -320 cacaagcaag tggcgcgggc ggcggcttag aacggcccgc cccgcccgcc gcgtcggcgc
            E-Box
 -260 ctgccccgtt gtgaggtgat aaagtgttgc gCTCCGGGAC GCCAGCGCCG CGGCTGCCGC
                   SREBP-1
 -200 CTCTGCTGGG GTCTAGGCTG TTTCTCTCGC GCCACCACTG GCCGCCGGCC GCAGCTCCAG
                                                                 E-box
 -140 GTGTCCTAGC CGCCCAGCCT CGACGCCGTC CCGGGACCCC TGTGCTCTGC GCGAAGCCCT
  -80 GGCCCCGGGG GCCGGGGCAT GGGCCAGGGG CGCGGGGTGA AGCGGCTTCC CGCGGGGCCG
  -20 TGACTGGGCG GGCTTCAGCC  ATGAAGACCC TCATAGCCGC CTACTCCGGG GTCCTGCGCG
                           +1
```

Fig. 2. Nucleotide sequence of the 5′-flanking region of the human DGAT2 gene. The transcription initiation site(s) were determined by sequencing of PCR products (Fig. 1). Boxed Gs at –51, -60 and -64 denote transcription initiation sites. +1 denotes translation initiation site. The sequence of 5'-flanking region of DGAT2 gene was obtained from pHDGAT2L-1340. Putative binding sites for trans-acting factors are shown.

2. Identification of trans-acting factors responsible for regulating DGTA2 gene promoter

In an attempt to understand a mechanism of human DGAT2 gene expression, we first cloned human DGAT2 promoter. To identify the regulatory regions of DGAT2 gene, we prepared 5' serial deletion constructs generated by PCR. Nine human DGAT2 gene promoter-luciferase reporter constructs (-159/-1, -249/-1, -333/-1, -495/-1, -570/-1, -695/-1, -851/-1, -1022/-1 and -1340/-1) were subjected to transient transfection using HepG2 cell line. TRANSFAC search suggested that there are several consensus sequences which bind transcription factors. The mRNA level of mouse DGAT2 was shown to be affected by insulin in adipocytes or dietary status in adipocytes and liver[17]. These data suggest that transcription factors involved in the insulin-regulated gene regulation, such as SREBP-1, USF1, USF2, NFY, and Sp1 may be possible regulators of DGAT2 gene expression. To confirm the possibility, we performed luciferase assay with expression vectors for these trans-acting factors and hDGAT2 (-1340/-1) reporter construct in the HepG2 cells. Of all the transcription factors tested, only NFY decreased the promoter activity by 50% (data not shown). Furthermore, over-expression of NFY repressed the promoter activity of DGAT2 gene in a dose-dependent manner (Fig. 3). Serial deletion revealed that -570/-495bp region is responsible for the decreased promoter

activities. These results indicate that -570/-495bp contains negative-response elements, of which sequence is a conserved CCAAT box (5'-CCAAT-3') (Fig. 4).

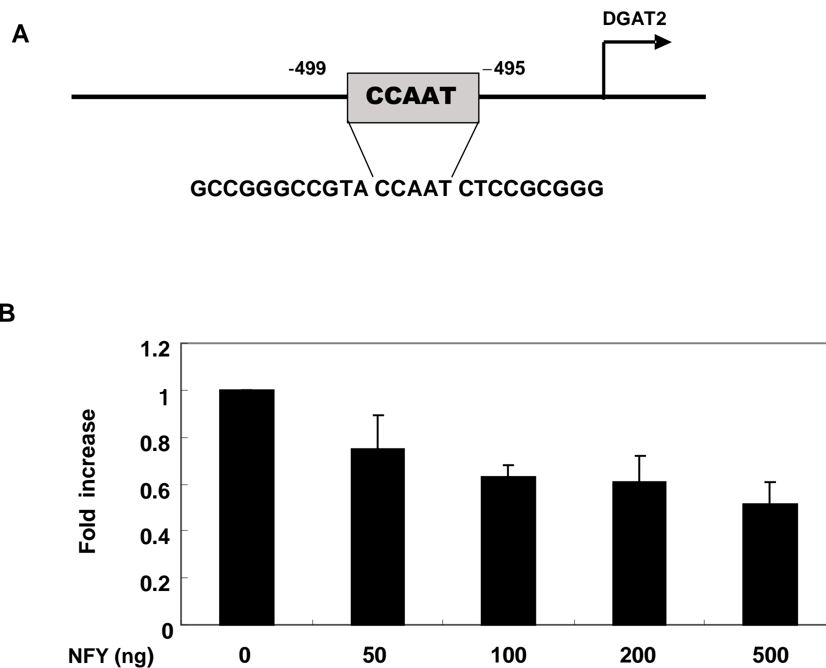


Fig. 3. DGAT2 promoter activities are repressed by NFY. A schematic representation of putative CCAAT box in the human DGAT2 promoter (a). Human DGAT2 promoter is repressed by NFY in a dose-dependent manner. Expression plasmids of NFY or the control plasmid (pcDNA3) was cotransfected with DGAT2 promoter reporter construct (pHDGAT2L-1340) into HepG2 cells (b). Normalized luciferase activities are shown as mean $\pm$ S.D of three independent experiments in triplicate and are expressed as fold increase relative to the basal activity.

Fig. 4. Determination of NFY binding site in the human DGAT2 promoter. Five hundred μg of NFY expression vectors was transfected with deletion constructs of DGAT2 promoter (500 μg) into HepG2 cells. The effect of NFY (black bar) on the promoter activity was compared with that transfected with pcDNA (grey bar). Normalized luciferase activities are shown as mean ± S.D of three independent experiments in triplicate and are expressed as fold increase relative to the basal activity.

3. Identification of NFY binding sites in the human DGAT2 promoter

In order to identify cis-element(s) binding NFY, HepG2 nuclear extracts or *in vitro* translated NFYA, NFYB and NFYC were incubated with radiolabeled -514/-480bp fragment and EMSA were performed (Fig. 5). The addition of a 200-fold molar excess of unlabeled NFY probe completely abolished the band formed by DNA-protein interaction (Fig. 5A, lane 3), but unlabeled CTF, C/EBP and LXRE probes failed to compete the binding of NFY complexes (Fig. 5A, lane 4-6). These results indicate the specific binding of NFY complex to the CCAAT box within human DGAT2 promoter. Moreover, addition of anti-NFYA antibody caused the supershift of the NFY band (Fig. 5A, lane 7). EMSA using *in vitro* translated NFY showed similar results. Incubation with NFYA-myc-His, NFYB-myc2 and NFYC-myc2 with the probe formed strong protein-DNA complex (Fig. 5B, lane 7, indicated by arrow) and the band was weakened by self or authentic NFY consensus sequences (Fig. 5B, lane 6,7). The band was supershifted by anti-Myc antibody (Fig. 5B, lane 10). These data suggest that NFY bind to the CCAAT box (-499/-495) of DGAT2 promoter.

Fig. 5. NFY binds to CCAAT box of DGAT2 promoter. Radiolabeled -514/-480 bp (50,000 cpm) fragment was incubated with HepG2 nuclear extract (5 μg) (a), or recombinant NFYA-myc-His, NFYB-myc2 and NFYC-myc2 mixtures (3 μl) (b), which were prepared by *in vitro* translation. Fifty thousand cpm of NFY, CEBP, CTF, and LXRE consensus sequences were labeled and used as competitors. For competition assay, 200-fold molar excess of cold probe was added. For supershift assay, 2 μg of anti-NFYA and anti-Myc antibody was used. The reaction mixtures were subjected to 5% (w/v) non-denaturing polyacrylamide gel. NFY; nuclear factor Y, CEBP; ccaat/enhancer binding protein, CTF; ccaat-binding transcription factor**,** LXRE; liver X receptor element.

4. NFY subunits bind to CCAAT box in the DGAT2 promoter

To identify NFY binding to DGAT2 promoter at the cellular level, chromatin was extracted from HepG2 cell line and precipitated DNA with anti-NFYA antibody or IgG antibody. The immunoprecipitated DNA by anti-NFYA antibody was amplified using hDGAT2 primers (Fig. 6A). As shown in Fig. 6B, NFY form a complex with the DGAT2 promoter in HepG2 cells through binding to the CCAAT sequence of -499 /-495bp region.

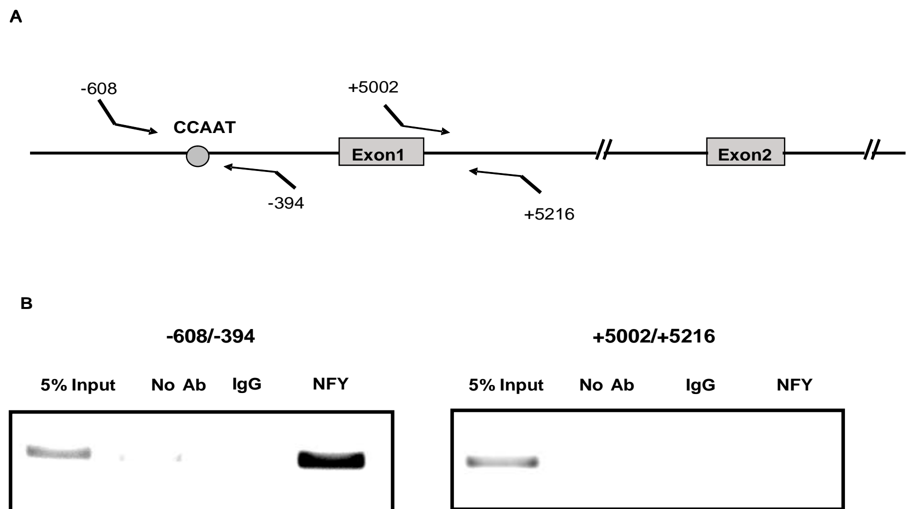


Fig. 6. Chromatin immunoprecipitation (ChIP) assay. Schematics of the genomic DNA structure of DGAT2 gene. The region between -608/-394bp and the first intron (+5002/+5216) in the DGAT2 gene were amplified (a). HepG2 cells were treated with formaldehyde to cross-link endogenous proteins and DNA. Samples of sonicated chromatin were immunoprecipited by preimmune IgG (2 μg) and anti-NFYA antibody (2 μg). DNAs in the presence or the absence of antiserum were immunoprecipitated and PCR amplified using primer pairs specific to the -608/-394bp region. To confirm the same amount of chromatins used in immunoprecifitation between groups, input chromatin (5% input) and chromatin

were incubated in the absence of antiserum (No Ab.) The amplified PCR fragments were resolved in 1.5% agarose gel.

Ⅳ. DISCCUSION

Transcriptional regulations occured by the integration of cis-elements generally exist in the promoter regions of genes and transacting factors acting at a distance. Computer searches of human DGAT2 gene promoter suggested a couple of *cis*-elements that may contribute to the regulation of DGAT2 gene, including SREBP-1, C/EBP and NFY. Transient transfections of these factors to HepG2 cells revealed that NFY showed repression of DGAT2 promoter activity. NFY is a transcription factor ubiquitously expressed and functions in the form of trimers of subunits A, B and C[17-20]. In the formation of trimeric NFY, tight association of NFYB and NFYC subunits is necessary before binding subunit A[17]. The trimers confer the specificity and affinity for specific DNA sequence[20]. The N-terminal part of the NFYA is responsible for NFYB and NFYC association and the C-terminal is required for DNA binding[17]. Primary structures of NFY subunits deduced from cDNA sequences are well conserved among species[21-23]. Bioinformatics of the 5'-flanking region of the human DGAT2 gene suggested the presence of a couple of E-boxes. Because the promoter region of human DGAT2 gene is not defined yet, we have determined the transcription initiation site by 5'-RACE. Serial deletion study, EMSA and ChIP assay of the promoter suggested that NFY binds to -499/-495bp region.

NFY serves as either an activator or as a repressor. NFY is reported to bind to SOX3 and GAS6 gene promoter and activate their gene expressions[24,25]. Furthermore, NFY exerts its transcriptional effect by interacting with partner trans-acting factors such as Sp1[26,27], C/EBPα [28] and SREBP[29,30,31,32,33]. In particular, in connection with SREBP, NFY plays a key role in the regulations of genes involved in lipid metabolism, such as 3-hydroxy-3-methylglutaryl-CoA reductase[30], farnesyl diphosphate synthase[31], acetyl-CoA carboxylase[32] and fatty acid synthase [33]. In contrast, NFY was shown to repress PNRC1 and SCGB3A1 gene expression by direct binding to the CCAAT box of their promoters[34,35]. The activating effect of NFY is shown to be mediated by interaction with p300[36] or by recruiting TFIID to the promoters[37,38]. The negative role of NFY on its target genes is known to be mediated by recruiting histone deacetyalses[39].

In this study, we demonstrated that the promoter activity of DGAT2 gene is repressed by the transfection of NFY to HepG2 cells. The promoter activity of DGAT2 was decreased in a dose dependent manner. We have identified and characterized the CCAAT box located at -495/-570bp of human DGAT2 gene promoter, which binds NFY. In this study, DGAT2 gene expression is repressed by NFY, however, the significance of NFY-mediated DGAT2 gene regulation in terms of lipid metabolism, obesity, and type 2 diabetes needs further study.

V. CONCLUSION

1. By using 5' RACE PCR, we obtained three of different transcription start sites at -51, -60 and -64bp in the DGAT2 promoter, which is considered to be crucial.

2. NFY inhibit the expression of the DGAT2 gene via direct binding on the CCAAT box in the human DGAT2 promoter.

3. We reported the transcriptional regulation of human DGAT2 gene for the first time in this study.

REFERENCES

1. Postic, C., and Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest. 2008;118:829–38.
2. Abdelmalek, M.F., and Diehl, A.M. Nonalcoholic fatty liver disease as a complication of insulin resistance. Med. Clin. North Am. 2007;91:1125–49.
3. Charlton, M. Nonalcoholic fatty liver disease: a review of current understanding and future impact. Clin. Gastroenterol. Hepatol. 2004;2:1048–58.
4. Coleman, R. A., and D. P. Lee. Enzymes of triacylglycerol synthesis and their regulation. Prog. Lipid Res. 2004;43:134–76.
5. Cases, S., S. J. Smith, Y-W. Zheng, H. M. Myers, S. R. Lear, E. Sande, S. Novak, C. Collins, C. B. Welch, and A. J. Lusis, et al. Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc. Natl. Acad. Sci. USA. 1998;95:13018–23.
6. Oelkers, P., A. Behari, D. Cromley, J. T. Billheimer, and S. L. Sturley. Characterization of two human genes encoding acyl coenzyme A:cholesterol acyltransferase-related enzymes. J. Biol. Chem. 1998;273:26765–71.
7. Cases, S., S. J. Stone, P. Zhou, E. Yen, B. Tow, K. D. Lardizabal, T. Voelker, and R. V. Farese, Jr. Cloning of DGAT2, a second mammalian diacylglycerol

acyltransferase, and related family members. J. Biol. Chem. 2001;276:38870–6.

8. Meegalla, R. L., J. T. Billheimer, and D. Cheng. Concerted elevation of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity through independent stimulation of mRNA expression of DGAT1 and DGAT2 by carbohydrate and insulin. Biochem. Biophys. Res. Commun. 2002;298:317–3.

9. Smith, S. J., S. Cases, D. R. Jensen, H. C. Chen, E. Sande, B. Tow, D. A. Sanan, J. Raber, R. H. Eckel, and R. V. Farese, Jr. Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking DGAT. Nat. Genet. 2000;25: 87–90.

10. Stone, S. J., H. Myers, B. E. Brown, S. M. Watkins, K. R. Feingold, P. M. Elias, and R. V. Farese, Jr. Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. J. Biol. Chem. 2004;279:11767–76.

11. Payne, V., W. Au, S. Gray, E. Nora, S. Rahman, R. Sanders et al. Sequential regulation of diacylglycerol acyltransferase 2 expression by CAAT/enhancer-binding protein beta (C/EBPbeta) and C/ EBPalpha during adipogenesis. J. Biol. Chem. 2007;282:21005–14

12. Horton, J., N. Shah, J. Warrington, N. Anderson, S. Park, M. Brown, and J. Goldstein. Combined analysis of oligonucleotides microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc. Natl. Acad. Sci. USA. 2003;100:12027–32.

13. Chi-Liang Eric Yen, Scot J. Stone, Suneil Koliwad and Robert Farese, Jr. Thematic Review Series: Glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. Journal of Lipid Research. 2008; 49:2283-301.

14. Lee, A., E. Scapa, D. Cohen, and L. Glimcher. Regulation of hepatic lipogenesis by the transcription factor XBP1. Science. 2008;320:1492–6.

15. Tsai, J., W. Qiu, R. Kohen-Avramoglu, and K. Adeli. MEKERK inhibition corrects the defect in VLDL assembly in HepG2 cells: potential role of ERK in VLDL-ApoB100 particle assembly.Arterioscler. Thromb. Vasc. Biol. 2007;27:211–8.

16. Mantovani, R. A survey of 178 NF-Y binding CCAAT boxes. Nucleic Acids Res. 1998;26:1135-43.

17. Mantovani, R. The molecular biology of the CCAAT-binding factor NF-Y. Gene. 1999;239;15-27.

18. Sankar N. Maity, Satrajit Sinha, E. Cristy Ruteshouser, and Benoit de Crombruggh. Three different polypeptides are necessary for DNA binding of the mammalian heteromeric CCAAT binding factor. J Biol Chem. 1992;267:16574-80.

19. Kim IS, Sinha S, de Crombrugghe B, Maity SN. Determination of functional domains in the C subunit of the CCAAT-binding factor (CBF) necessary for formation of a CBF-DNA complex: CBF-B interacts simultaneously with both

the CBF-A and CBF-C subunits to form a heterotrimeric CBF molecule. Mol Cell Biol. 1996;16:4003-13.

20. Sinha S, Lu J, Maity SN, de Crombrugghe B. Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. Proc Natl Acad Sci U S A. 1995 ;92:1624-8.

21. R Hooft van Huijsduijnen, X Y Li, D Black, H Matthes, C Benoist, and D Mathis. Co-evolution from yeast to mouse: cDNA cloning of the two NF-Y (CP-1/CBF) subunits. EMBO J. 1990;9:3119–27.

22. Vuorio T, Maity SN, de Crombrugghe B. Purification and molecular cloning of the "A" chain of a rat heteromeric CCAAT-binding protein. Sequence identity with the yeast HAP3 transcription factor. J Biol Chem. 1990;265:22480–6

23. D M Becker, J D Fikes, and L Guarente. A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. Proc Natl Acad Sci U S A. 1991;88:1968-72.

24. Krstic A, Mojsin M, Stevanovic M. Krstic A, Mojsin M, Stevanovic M. Regulation of SOX3 gene expression is driven by multiple NF-Y binding elements. Arch Biochem Biophys. 2007;467:163-73.

25. Wang J, Qiao Y, Sun F, Wan Y, Huang T, Ye T, Yu Y. Identification and characterization of mouse Gas6 promoter. Biochem Biophys Res Commun.

2008;371:567-72.

26. Kenneth L. Wright, Terry L. Moore, Barbara J. Vilen , Adrienne M. Brown,, Jenny P.-Y. Ting . Major histocompatibility complex class II-associated invariant chain gene expression is up-regulated by cooperative interactions of Sp1 and NF-Y. J Biol Chem. 1995;270:20978-86.

27. Shunbin Xiong, Subrahmanyam S. Chirala, and Salih J. Wakil. Sterol regulation of human fatty acid synthase promoter I requires nuclear factor-Y- and Sp-1-binding sites. Proc Natl Acad Sci U S A. 2000;97:3948–53.

28. Park SK, Oh SY, Lee MY, Yoon S, Kim KS, Kim JW. CCAAT/enhancer binding protein and nuclear factor-Y regulate adiponectin gene expression in adipose tissue. Diabetes. 2004;53:2757-66.

29. Simon M. Jackson, Johan Ericsson, Timothy F. Osborne, Peter A. Edwards. NF-Y has a novel role in sterol-dependent transcription of two cholesterogenic genes. J Biol Chem. 1995;270:21445-8.

30. Dooley KA, Millinder S, Osborne TF. Sterol regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y. J Biol Chem. 1998;273:1349-56.

31. Johan Ericsson, Simon M. Jackson, Peter A. Edwards. Synergistic binding of sterol regulatory element-binding protein and NF-Y to the farnesyl diphosphate

synthase promoter is critical for sterol-regulated expression of the gene. J Biol Chem. 1996;271:24359-6.

32. Lopez JM, Bennett MK, Sanchez HB, Rosenfeld JM, Osborne TF. Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. Proc Natl Acad Sci U S A. 1996;93:1049-53.

33. Mary K. Bennett, Jose M. Lopez, Hugo B. Sanchez, Timothy F. Osborne. Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. J Biol Chem. 1995;270:25578-83.

34. Zhang Y, Chen B, Li Y, Chen J, Lou G, Chen M, Zhou D. Transcriptional regulation of the human PNRC promoter by NFY in HepG2 cells. J Biochem. 2008;143:675-83.

35. Tomita T, Kimura S. Regulation of mouse Scgb3a1 gene expression by NF-Y and association of CpG methylation with its tissue-specific expression. BMC Mol Biol. 2008;9:5.

36. Q Li, M Herrler, N Landsberger, N Kaludov, V V Ogryzko, Y Nakatani, and A P Wolffe. Xenopus NF-Y pre-sets chromatin to potentiate p300 and acetylation-responsive transcription from the Xenopus hsp70 promoter in vivo. EMBO J. 1998;17:6300–15.

37. Frontini M, Imbriano C, diSilvio A, Bell B, Bogni A, Romier C, Moras D, Tora L, Davidson I, Mantovani R. NF-Y recruitment of TFIID, multiple interactions

with histone fold TAF(II)s. J Biol Chem. 2002;277:5841-8.

38. F Coustry, S Sinha, S N Maity, and B Crombrugghe. The two activation domains of the CCAAT-binding factor CBF interact with the dTAFII110 component of the Drosophila TFIID complex. Biochem J. 1998;331(Pt 1):291-7.

39. Peng Y, Jahroudi N. The NFY transcription factor inhibits von Willebrand factor promoter activation in non-endothelial cells through recruitment of histone deacetylases. J Biol Chem. 2003;278:8385-94.

Abstract (in Korean)

간암 세포 주에서 NFY에 의한 인간 DGAT2의 전자 조절

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박영신

Acyl CoA: diacylglycerol acyltransferase는 중성 지방 합성의 마지막 단계에 관여하고, 간과 백색 지방과 같은 여러 조직에서 발현되는 효소이며, DGAT1와 DGAT2의 두 형태로 존재한다고 알려져 있다. DGAT2가 결여된 실험 쥐에서 중성 지방 부족으로 인한 심각한 체내 증상이 나타났고, 이것은 포유류의 중성 지방 합성에서 DGAT2가 중요한 역할을 하는 것을 알려준다. 본 연구에서는, 인간 DGAT2 유전자의 발현에 관련되는 분자적인 기전을 규명하기 위하여, Promoter의 염기서열을 순차적으로 제거하고, Transient transfection과 Luciferase assay 방법을 통하여 -499에서 -495bp 부위에 CCAAT 박스가 존재하며, NFY에 의해 억제되는 현상을 확인할 수 있었다. 또한, NFY를 순차적인

농도로 과 발현시켰을 때, DGAT2의 Promoter 활성이 저해되는 것을 확인하였다. EMSA와 ChIP Assay를 수행한 결과 전사인자인 NFY가 DGAT2 Promoter상의 CCAAT 서열에 결합하는 것을 확인하였으며, 이것은 NFY가 인간 DGAT2 유전자의 전사를 억제하는 기전에 관련되어 있다는 것을 증명해 준다.

핵심 되는 말: DGAT2, NFY, triglyceride, metabolism, transcriptional regulation