GOVP1200132394

과제번호 2000-J-MM-01-13-36

인슐린 분비조절 관련 유전자의 전사활성을 조절하는 cis-acting element 및 trans-acting factor 검색

Analysis of polymorphism in the human GLUT2 promoter in NIDDM patients and its functional consequence to the promoter activity

연세대학교 의과대학

과학기술부

제 출 문

과학기술부 장관 귀하

본 보고서를 "인슐린 분비조절 관련 유전자의 전사활성을 조절하는 cis-acting element 및 trans-acting factor 검색" 과제의 보고서로 제출합니다.

2001 . 9 . 26

주관연구기관명 : 연세대학교

주관연구책임자 : 안 용 호

연 구 원 : 차 지 영

연 구 원 : 김 하 일

요 약 문

I. 제 목

인슐린 분비조절 관련 유전자의 전사활성을 조절하는 cis-acting element 및 trans-acting factor 검색

II. 연구개발의 목적 및 필요성

제 2형 당뇨병의 병인에 대한 연구가 현재까지 많이 이뤄지고 있으나, 일반적으로 인슐린 작용기전과 연관된 유전자들(insulin, insulin receptor, glucokinase, somatostatin exon 등) 또는 인슐린 분비과정에 관련된 생체분자 (GLUT2, Glucokinase, Na+-K+ ATPase, Ca2+ ATPase)의 coding region의 유전자 돌연변이와 이 들의 기능적인 이상에 대한 연구가 주류를 이루고 있다. 본 연구팀은 인슐린 분비과정에서 중요한 역할을 하는 제 2형 포도당운반체 (GLUT2) promoter에 결합하여 전사활성을 조절하는 trans-acting factor(전사조절인자)를 yeast one-hybrid system을 이용하여 screening하고자 한다. 이 연구를 통하여 제 2형 당뇨병의 병인과 연관된 전사인자를 발견하고, 이 들을 이용한 당뇨병 진단제 및 치료제 개발을 위한 기초연구를 실시하고자 한다. 본 연구팀은 과기처에서 지원하는 국책생명공학기술 개발사업 (현 분자의과학사업)에 참여하여 한국인 비인슐린 의존성 당뇨병 환자 20명을 대상으로 연구한 결과 약 60%의 비율로 GLUT2 promoter의 -44 부위에서 돌연변이(mutation)가 빈번하게 일어난다는 사실을 확인하였다. 특히 mutation을 확인하는 방법으로 사람의 말초혈액에서 lymphocyte를 분리한 다음 genomic DNA를 간단히 분리하여 중합효소 연쇄반응 (polymerase chain reaction, PCR)을 이용하는 분자생물학적인 기술을 이용함으로써 비인슐린 의존성 당뇨병을 손쉽게 진단하기 위한 system을 개발한 상태이다 (특허 등록 제 258190, 특정부위에서 돌연변이가 유발된 포도당운반체 GLUT2 유전자 및 이를 이용한 당뇨병 진단방법 및 진단시약, 2000년 3월 8일). 이러한 결과를 바탕으로 GLUT2 promoter의 돌연 변이가 실제 GLUT2 발현의 차이를 유발하는지 확인하고, 돌연변이발생 부위에 binding 하는 전사인자를 screening하고자 하며, 이들이 제 2형 당뇨병과 어떤 관계를 가지는지에 대한 연구를 하고자 한다. 이러한 연구를 통하여 이 단백질의 기능과 GLUT2 유전자의 표현이 당뇨병의 병인과 어떤 상관관계가 있는지에 대하여 연구하고, 이를 산업화하는데 최종목표를 둔다.

Ⅲ. 연구개발의 내용 및 범위

1. 인체 GLUT2 promoter activity에 대한 돌연변이의 효과

   : transient transfection assay, site directed mutagenesis

2. 인체 GLUT2 promoter 부위에 결합하는 단백질 검색 및 기능에 관한 연구

   (1) DNA-Protein interaction에 관한 연구(in vitro): Gel mobility shift assay (EMSA)

   (2) Transcriptional factors의 분리 및 특성 규명: Promoter activity assay 및 Mutation in transcriptional factors binding sites

   (3) DNA-Protein interaction에 관한 연구 (in vivo)(1) : Yeast one hybrid system

Ⅳ. 연구개발결과

1. 당뇨병 환자에서 발견되는 -44, +103 부위의 돌연변이 및 +107부위의 mutation이 GLUT2 유전자의 promoter activity를 억제함을 transient transfection으로 확인하였다.
2. EMSA 결과 transcription factor가 결합함을 알 수 있었고, 이러한 결합은 돌연변이에 의해 억제되었다.
3. -44 부위에 대한 Yeast one-hybrid 결과 60여개의 강한 결합을 할 수 있는 positive colonies를 얻었고, 이들 전사인자의 염기서열을 결정한 결과 그 중 6개의 단백질이 전사인자로 작용할 가능성이 있음을 알 수있었다.
4. +107 부위에 대한 one-hybrid assay 한 결과, 18개의 강한 결합을 하는 전사인자를 확인할 수 있었다.
5. 당뇨병환자에서 발견되는 높은 빈도의 돌연변이는 전사인자의 결합을 억제하여 GLUT2 유전자 발현의 감소에 기여할 가능성이 있으며, 그 대상 전사인자로는 아직 알려지지 않은 전사인자와 HNF1, HNF3 등이 관여할것으로 여겨진다.

V. 연구개발결과의 활용계획

당질 대사의 조절과 관련된 유전자의 promoter 부위의 mutation study를 이용한 당뇨병 조기 진단용 DNA chip 개발에 본 연구의 결과를 활용할 계획이다. 또한 본 연구 결과 돌연변이와 연관있는 전사인자를 protein chip의 probe으로 이용하여 실제 이들 단백질의 발현이 당뇨병과 어떠한 연관이 있는지 규명하는 연구를 계속 진행할 계획이다. 이와 같은 연구는 GLUT2의 PPRE를 이용한 제 2형 당뇨병 치료제 후보물질 탐색을 위한 대량 screening system 확립에 활용될수 있을것이다.

SUMMARY

Previously, we reported that the -44 region is frequently mutated from base A to G in type 2 diabetes mellitus and possibly related to decreased gene expression. Thus, we have designed an experiment to identify transacting factor(s) binding to this region, which may be responsible for the regulation of GLUT2 gene. To this end, we screened human liver cDNA library using yeast one-hybrid system. The cis-element binding the transactivation factors was constructed by synthesizing oligomers repeating the -46/-27 region three times and subcloned into pHisi, pHisi-1, and pLacZi vector. This sequence was incorporated into yeast exploiting homologous recombination phenomenon. In this study, we were able to obtain 80 positive clones and sequenced. Computer analysis of the clones revealed that 6 clones were possible protein factors regulating human GLUT2 promoter activity. The binding of these protein to yeast extracts were confirmed by gel retardation assay. Currently, detailed molecular characterization and biological significance of these proteins are under intensive study.

CONTENTS

I. Introduction 9

I.1. Purpose 9

I.2. Necessity for research 9

II. Status of Research and Development 11

III. Contents of R&D and Results 12

III.1. Experimental approach 12

III.1.1. Determination of mutation effects on the GLUT2 promoter activity 12

III.1.2. Screening and identification of proteins binding to human GLUT2 promoter 14

III.2. Results 16

III.2.1. Polymorphism in human GLUT2 promoter 16

III.2.2. Electrophoretic mobility shift assay 16

III.2.3. Site-directed mutagenesis of human GLUT2 promoter 17

III.2.4.. yeast one hybrid system 17

VI. Contribution of the research to science and industry 23

VI.1 Degree of achievements 23

VI.2. Contribution of science and industry 23

V. Applicability of the results of the project 24

VI. References 25

목 차

제 1 장 서론 9

제 1 절 연구의 목적 9

제 2 절 연구의 필요성 9

제 2 장 국내외 기술개발 현황 11

제 3 장 연구개발수행 내용 및 결과 12

제 1 절 연구개발수행 내용 12

1. 인체 GLUT2 promoter activity에 대한 돌연변이의 효과 12

2. 인체 GLUT2 promoter 부위에 결합하는 단백질 검색 및 기능에 관한 연구 14

제 2 절 연구개발수행 결과 16

1. Polymorphism in human GLUT2 promoter 16

2. Electrophoretic mobility shift assay 16

3. Site-directed mutagenesis of human GLUT2 promoter 17

4. Yeast one hybrid system 17

제 4 장 연구개발목표 달성도 및 대외 기여도 23

제 1 절 연구개발목표 달성도 23

제 2 절 대외 기여도 23

제 5 장 연구개발 결과의 활용 계획 24

제 6 장 참고문헌 25

제 1 장 서론

1. 최종목표

유전자의 표현에 가장 중요한 부위인 promoter부위에 변이가 발생한 경우 이 부위에 결합하는 단백질의 결합능력에 변화가 온 것으로 생각된다. 따라서 당뇨병에서 이 단백질이 어떤 기능을 하는 물질인지 확인할 수 있다면 이는 당뇨병의 근본 원인을 알아내는데 중요한 단서가 될 수 있으므로 그 성상을 확인하고자 한다. 이러한 연구를 통하여 이 단백질의 기능과 GLUT2 유전자의 표현이 당뇨병의 병인과 어떤 상관관계가 있는지에 대하여 연구하고, 이를 산업화하는데 최종목표를 둔다.

2. 연구의 필요성

당뇨병의 95%를 차지하는 제 2형 당뇨병(type 2 diabetes)은 세계적으로 그 발병률이 증가하고 있다. 따라서 당뇨병의 발병원인을 찾아내는 연구는 건강 및 생산성 제고에도 중요한 일이라 하겠다. 최근에 미국에서 45세 이상의 성인을 대상으로 혈당검사를 의무화하도록 권장하는 것도 이러한 이유 때문이다.

제 2형 당뇨병의 병인에 대한 연구가 현재까지 많이 이뤄지고 있으나, 일반적으로 인슐린 작용기전과 연관된 유전자들(insulin, insulin receptor, glucokinase, somatostatin exon 등) 또는 인슐린 분비과정에 관련된 생체분자 (GLUT2, Glucokinase, Na+-K+ ATPase, Ca2+ ATPase)의 coding region의 유전자 돌연변이와 이 들의 기능적인 이상에 대한 연구가 주류를 이루고 있다. 본 연구팀은 인슐린 분비과정에서 중요한 역할을 하는 제 2형 포도당운반체 (GLUT2) promoter에 결합하여 전사활성을 조절하는 trans-acting factor(전사조절인자)를 yeast one-hybrid system을 이용하여 screening하고자 한다. 이 연구를 통하여 제 2형 당뇨병의 병인과 연관된 전사인자를 발견하고, 이 들을 이용한 당뇨병 진단제 및 치료제 개발을 위한 기초연구를 실시하고자 한다. 본 연구팀은 과기처에서 지원하는 국책생명공학기술 개발사업 (현 분자의과학사업)에 참여하여 한국인 비인슐린 의존성 당뇨병 환자 20명을 대상으로 연구한 결과 약 60%의 비율로 GLUT2 promoter의 -44 부위에서 돌연변이(mutation)가 빈번하게 일어난다는 사실을 확인하였다. 특히 mutation을 확인하는 방법으로 사람의 말초혈액에서 lymphocyte를 분리한 다음 genomic DNA를 간단히 분리하여 중합효소 연쇄반응 (polymerase chain reaction, PCR)을 이용하는 분자생물학적인 기술을 이용함으로써 비인슐린 의존성 당뇨병을 손쉽게 진단하기 위한 system을 개발한 상태이다 (**특허 등록 제 258190, 특정부위에서 돌연변이가 유발된 포**

도당운반체 glut2 유전자 및 이를 이용한 당뇨병 진단방법 및 진단시약, 2000년 3월 8일). 이러한 결과를 바탕으로 GLUT2 promoter에 binding 하는 전사인자를 screening하고자 하며, 이들이 제 2형 당뇨병과 어떤 관계를 가지는지에 대한 연구를 하고자 한다.

대한민국국회도서관장서

제 2 장 국내외 기술개발 현황

당뇨병에서 함수탄소 대사와 관련된 여러 유전자의 표현 조절 장애와 연관된 연구는 아직 미미한 상태이다. Non-insulin dependent diabetes mellitus (NIDDM) 환자나 그 가계연구에 의해 관련 유전자로 glucokinase, GLUT2 등의 함수탄소 대사 관련 유전자와 이들 유전자의 발현을 조절하는 전사인자, HNF1, HNF3, HNF4등의 돌연 변이가 보고되어 있다. 이들 유전자들의 발현과 당뇨병과의 연관성을 찾기 위해 동물모델을 이용한 실험이 활발히 진행되어 오고 있으며, 그 결과로서 당뇨병 모델에서 GLUT2 유전자의 발현이 감소한다는 사실이 밝혀졌다. 그러나 실제적으로 promoter의 어느 부위에 변이가 잇을지를 확인하는 일이 간단하지 않고 변이가 있더라도 기능적인 확인 과정이 쉽지 않기 때문에 아직 연구가 이루어져 있지 않은 상태이다. 본 연구팀은 한국인을 대상으로 GLUT2 유전자의 돌연변이를 확인하고, 그 돌연변이에 의해 유전자의 전사조절이 이루어지는지 확인하고자 하였다. 또한 유전자 발현이 감소한다면 그러한 발현 감소가 전사인자의 결합이 억제 되기 때문인지 확인하고자 하였다.

제 3 장 연구개발수행 내용 및 결과

1. 연구개발수행 내용

가. 인체 GLUT2 promoter activity에 대한 돌연변이의 효과

(1) amplification of human GLUT2 promoter region

In order to amplify the promoter region of human GLUT2 gene, hGT2-5 primer (-294 ~ -275, sense, 5'-TGCTT AAGCT TATAC TCCCC-3'), hGT2-7 primer (+301 ~ +282, antisense, 5'- GGAGT CCTGT GAATT CCAGG-3'), hGT2-8(-122 ~ -101, sense, 5'-TCCAT GCTCC AGAGC ACAGC-3') were used. G at -286 position (within sense primer) and C at +291 (within antisense primer) were replaced with C and G to introduce Hind III and EcoR I site for subcloning, respectively.

PCR amplification was performed in 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 15 pmoles of each primer, 100 mM of dNTP mix, 0.01% gelatin and 1.5 units of ExTaq DNA polymerase (Takara, Japan). The reaction mixture was denatured for 3 min at 94 ℃, and went through 30 cycles of denaturation (94 ℃ for 30 sec), annealing (55 ℃ for 30 sec) and extension (72 ℃ for 30 sec). At the final stage of PCR, the extension reaction was carried out at 72 ℃ for 10 min.

(2) Subcloning and pCAT3-H(-294) construction

The general procedure for subcloning was carried out by standard methods described by Sambrook *et al*. Amplified hGLUT2 DNAs (595 bp) from patient samples were isolated from agarose gel and purified using Gene Clean II kit (Bio 101, Vista, CA, USA). The amplified DNAs were then subcloned into pT7BlueT-vector (Novagen, Madison, WI, USA), and the insert DNA was excised out using EcoR I and Hind III and made the end blunt using dNTPs and Klenow enzyme. The DNA was transferred to Sma I site of pCAT3 basic vector (Promega, Madison, WI, USA) in order to measure promoter strength. The orientation of the insert DNA was confirmed by DNA sequencing using T7 DNA sequencing kit (Pharmacia, Sweden) with a primer (Promega, Madison, WI, USA) annealing to the multiple cloning site of pCAT3 basic vector. The resulting recombinant DNA was named *pCAT3-H(-294)* and used for transfection study.

(3) DNA sequencing

Direct PCR sequencing was performed according to the manufacturers protocol (Thermal Cycling kit, Perkin Elmer, USA), and run in 8% denaturing polyacrylamide gel. For direct sequencing, hGT2-5, hGT2-8, and hGT2-9 (sense, +49 ~ +68, 5′-CCTAG TGGAA CAAAG GTATT-3′) were used as primers. The mutated sequences were confimed by automatic sequence analyser, ALFwin Sequence Analyser 2.00 (Amersham pharmacia biotech).

(4) Site specific mutagenesis of human GLUT2 promoter region

Wild type human GLUT2 promoter [H(-294)] was subjected to mutation using Quickchange mutagenesis kit (Stratagene, USA). The oligonucleotides used for introducing mutations at the specific sites of human GLUT2 promoter are summarized in Table 1. -44 and -269 in the parentheses denote the position of mutation at the promoter. The mutagenesis reaction was performed as described by manufacturers protocol. Briefly, the reaction was performed in 50 µl containing 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 0.1% Triton X-100, 0.1 mg/ml nuclease free bovine serum albumin, 10 ng of pCAT-H(-294), 125 ng of primer sets, dNTP mix, and 2.5 units of Pfu DNA polymerase. The reaction was carried out in a Thermal Cycler (Perkin Elmer) by denaturing at 95 ℃ for 30 min. Subsequent 12 reaction cycles included denaturation at 95 ℃ for 30 sec, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 12 min. After reaction, 10 units of Dpn I were added to the reaction mixture to digest methylated template DNA and used for the transformation of E. coli XL1-Blue (Stratagene, USA). The DNA sequences of mutants generated by Quickchange kit were confirmed by DNA sequencing.

(5) Cell culture and transient transfection

HIT-T15 cells, a pancreatic β-cell line, were cultured in Hams F-12K media supplemented with 10% fetal calf serum, 2.5% dialyzed horse serum and antibiotics (penicillin 100 units/ml, streptomycin 0.25 g/ml, amphotericin B 0.0085%). Cells were maintained as monolayer cultures and grown in appropriate media. Plasmid DNAs were purified on Qiagen Midiprep kit columns (Qiagen) three times independently. For transfection, 20 µl of lipofectin (GIBCO, BRL, USA) and DNA mixture (5 µg of

pCAT3-H(-294), or site-specific mutants and 1 μg of CMV-β-galactosidase) were used. Transfection was performed according to manufacturers instruction. After 16 h-incubation period, the remaining lipofectin-DNA complexes in the culture fluid were removed and replaced with fresh Hams F-12K media and cultured another 72 h.

(6) β-galactosidase activity assay, protein measurements, and CAT activity assay

Cells were washed with phosphate buffered saline (PBS), scraped, resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8), and disrupted by freezing and thawing. The suspension of disrupted cells were centrifuged at 12,000*g* for 5 min and the supernatants were collected. Aliquots of 30 μl were used for measuring β-galactosidase activity and the remainders were heated at 65 ℃ for 10 min to inactivate deacetylase. Insoluble materials were removed by centrifugation at 12,000*g* for 5 min and the supernatants were used for CAT assay. The protein concentration was measured by the method of Bradford. The CAT activities were normalized with respect to β-galactosidase activity and protein concentration. The CAT activities were measured by phosphoimaging system (BAS2500, Fuji Photo Film Co, Tokyo, Japan) and the % conversion to acetylated chloramphenicol was calculated.

나. 인체 GLUT2 promoter 부위에 결합하는 단백질 검색 및 기능에 관한 연구

(1) DNA-protein interaction에 관한 연구 (in vitro) : Electrophoretic mobility shift assays(EMSAs)

Competitive EMSA was performed(Schreiber et al, 1989) using oligonucleotides of wild type and G mutations (sense ATTT CTCT TTTC ACCA(G)GCT CCCAATTACTG, antisense CAGTAA TTGG GAGC T(C)GGTGAAAAGAGAA AT). Briefly,50,000 cpm of [a-32P] labled probe, 5~15 ug of nuclear extracts were incubated in 10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, pH 8.0, 1 mM DTT, 7% glycerol, and 0.1 mg of poly (dI-dC) on ice for 30 min. Non-denaturing polyacrylamide gel(4%) containing 2.5% glycerol, and 0.25X TBE were run for 2-3 hr at 200 volts before sample loading. The sample was separated in the gel at 200 volts for 2 hr. Gel was dried and exposed to X-ray film.

(2) *DNA-protein interaction에 관한 연구 (in vivo) : yeast one hybrid system*

(가). Construction of target element and target-reporter constructs

The repeating sequences(60mer) of sense and antisense oligonucleotides covering (-46 ~ -27) of human GLUT2 promoter region were synthesized and annealed. The annealed double stranded oligonucleotides were subcloned into yeast expression vectors, pHisi, pHisi-1, and pLacZi. The DNAs were amplified by transforming into E. coli and sequenced to confirm the orientation of the inserts. The recombinants carrying the oligonucleotides were named pHisi/GT2, pHISi-1/GT2, and pLacZi/GT2, respectively. These recombinants were digested with variuos restriction enzymes and incorporated into yeast strain, which is Ura-, His- strain YM4271(Genotype: MATa, Ura3-52, His3-200, Ade2-101, Lys2-801, Leu2-3, 112, Trpl-903, Tyrl-501) using homologous recombinant phenomenon. The resulting yeast strain(YM4271AC, Genotype: MATa, Ura3-52, His3-200, Ade2-101, Lys2-801, Leu2-3, 112, Trpl-903, Tyrl-501) was obtained which incorporated pHisi and pLacZi. These strains can be used as markers for both Histidine selection and β-galactosidase.

(나). Screening an AD(activation domain) fusion library for DNA-BP(binding protein) genes

Basic strategy for screening transcription factors interacting with DNA elements(-46 ~ -27) were described previously(Kwon and Hur, 1997). The cDNA library fused to Gal4 activation domain was purchased from Clontech (Palo Alto, CA, USA) to find out cDNA(s) encoding 44 region of human GLUT2 promoter region. The cDNA library was titerated and amplfied once before experiment.

Competent yeast cell was transformed by AD/cDNA library and plated on the SD/-Leu/-His/30 mM 3-AT and incubated for 3~5 days at 30 °C. 30 mM 3- amino triazole (3-AT) was used to suppress nonspeific HIS3 gene expression in yeast YM4271 strain. The resulting clones were incubated on SD/-Leu/-His/30 mM 3-AT plates for 3 days, and β-galactosidase activity was measured.

(다). β-galactosidase filter assay

Transformed colonies were transferred to Whatman 572 filter paper and soaked

in the liquid mitrogen for 30 sec and thawed at room temperature. The filters were soaked into X-gal/Z buffer solution (60mM Na2HPO4, 40mM NaH2PO4, 10mM KCl, 1mM MgSO4, pH7.0, 50mM β-mercaptoethanol, 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and incubated at 30 ℃. The expression of β-galactosidase was measured by incubating for 30min to 24 hrs at 30 ℃ incubator.

2. 연구개발수행 결과

가. *Polymorphisms in human GLUT2 promoter*

Direct PCR sequencing of the human GLUT2 promoter region from 100 normal Korean subjects and 100 NIDDM patients showed sequence differences at positions -149(A), -122(C) which were reported as C and T, respectively. In addition, the nucleotide reported G at -44 region was A in majority of the samples. It was also reported that the nucleotide -44 was A on human genome project (www.ensembl.org, Ensembl gene ID: ENSG00000114308, sequences on AC068853). Taken together, we regarded the sequence -44A as wild type.

The examination of the sequence in the promoter region (294~+301) showed the polymorphisms at positions at -269, -44, and +103 regions, where most variations were observed at -44 region in the mixed form of A/G. Table 2 summarizes the frequencies of polymorphisms observed in normal subjects and NIDDM patients. At -44 region, the normal base A is present as A/G mixed form in 35 out of 100 NIDDM patients. Also, the G homozygote was found in 10 out of 100 samples. Interestingly, one of the NIDDM patients who had A/G mixed bases also had mutation at -269 and +103 position, where base A and A were changed to C and G, respectively. In normal subjects, 14.0% and 9.0% showed A/G and G mutation, respectively.

나. *Electrophoretic mobility shift assay*

Considering high incidence of polymorphism on -44 region in NIDDM patients, it was tempting to speculate that this region could play an important role for the regulation of transcription. To examine whether transcription factors bind to this

region, we next performed electrophoretic mobility shift assay using double stranded oligonucleotide containing -44A as a probe. As shown in Fig. 1, a clear shifted band of DNA-binding protein was found, suggesting that this region could participate in regulating transcription *in vivo*. Next, we determined the effect of mutations on the binding affinity of the protein with the probe (Fig. 1, lane 6 ~ 9). The DNA-protein complex was competed by unlabeled 44A oligonucleotide whereas slightly competed by unlabeled 44G oligonucleotide. However, M3 (CAG→AGT) showed no competition, suggesting that the protein binding to 44A oligonucleotide is specific and the binding affinity is higher in -44A than others. In the case of +103 region, we already published that HNF1 and HNF3 could bind this region and regulate expression of the GLUT2 gene.

다. *Site-specific mutagenesis of human GLUT2 promoter*

Although the mutations in exon regions of GLUT2 gene were well known in relation to NIDDM, the functional consequence of the promoter mutations in various diabetes states was not established. In order to study the effect of mutation on the promoter activities, we carried out site-specific mutagenesis and transient transfection experiment. As shown in Fig. 2, mutant GLUT2 promoter showed remarkable reductions in CAT activity in HIT-T15 cells. The promoter activities of the mutants [A(-44)C, A(-44)T, A(-44)G] were reduced by 52.3%, 63.8%, or 63.6% respectively, when compared to those exerted by wild type ($p<0.05$). The mutation at -269 region also showed reduction in CAT activity. The CAT activities shown by A(-269)C, and A(-269)G mutants were reduced by 62.3%, and 44.8% respectively, when compared to those of wild type ($p<0.05$). We previously reported that the G mutation of the +103 region reduced GLUT2 promoter activity to 78% of wild type. Triple mutant (#32-3), that contains mutations at -269, -44, and +103, showed a marked reduction (75.7%) in CAT activity when compared to wild type.

라. *yeast one hybrid system*

The consensus sequences in the promoter region are sites for multiple protein binding. Thus, it is necessary to search for protein(s) regulating the GLUT2 gene expression by binding these region. To this end, yeast one-hybrid system is very

powerful tool. In this study, triple repeat of 44 region was synthesized and subcloned into yeast expression vector. The yeast clones were selected in His- media and β-galactosidase assay was performed for the cloned grown on SD/-Leu/-His/30 mM 3-AT solid media. The resulting 80 clones were considered positive clones expressing proteins binding to 44 region. To confirm the presence of inserts in expression vectors harboring in the yeast, PCR was performed using 5-AD insert screening amplimer and 3-AD insert screening amplimer. These insert DNAs were prepared by min-prep. method and sequenced to compare previsouly reported cDNAs of known function. Extensive computer search for 80 clones revealed that most cDNAs were proved to be pancreatic enzymes related to exocrine function, such as lipase, β-amylase, chymotrypsin. It is not know how the pancreatic enzymes appeared as (a) transcription factor(s) binding to 44 region. We have excluded these clones for further screening. 6 clones, named 2, 6, 9, K3, K12, 30 were possible candidates of transcription factors. Computer search suggested that insert from clone 2 (about 1 kb in size) may be serum calcium decreasing factor). Other factors as suggested by computer analysis were summarized in Table 3.

Table 1. Oligonucleotides used for site-specific mutagenesis

Nucleotide names	Sense Primer(5'→3')	Antisense primer(5'→3')
A(-44)T*	ATTTCTCTTTTCACCTGCTCCCAATTACTG	CAGTAATTGGGAGCAGGTGAAAAGAGAAAT
A(-44)C	ATTTCTCTTTTCACCCGCTCCCAATTACTG	CAGTAATTGGGAGCGGGTGAAAAGAGAAAT
A(-44)G	ATTTCTCTTTTCACCGGCTCCCAATTACTG	CAGTAATTGGGAGCCGGTGAAAAGAGAAAT
A(-269)C	TTATACTCCCCAGTACAATGTTGAGATTCA	TGAATCTCAACATTGTACTGGGGAGTATAA
A(-269)G	TTATACTCCCCAGTAGAATGTTGAGATTCA	TGAATCTCAACATTCTACTGGGGAGTATAA

*Numbers in the parenthesis refer to the sites of mutation where normal A was changed to T

Table 2. Analysis of the mutations in the hGLUT2 promoter

Location	Normal (n=100)		NIDDM (n=100)	
-44	A	77	A	55
	A/G	14	A/G	35*
	G	9	G	10

One of this group had additional mutations at -269, +103, where A, and A were changed to C, and G, respectively. Fischer s exact test (the numbers of G mutation were included in A/G group, $p<0.05$)

44A ATTTCTCTTTTCACCAGCTCCCAATTACTG
M3 ATTTCTCTTTTCACAGTCTCCCAATTACTG
44G ATTTCTCTTTTCACCGGCTCCCAATTACTG

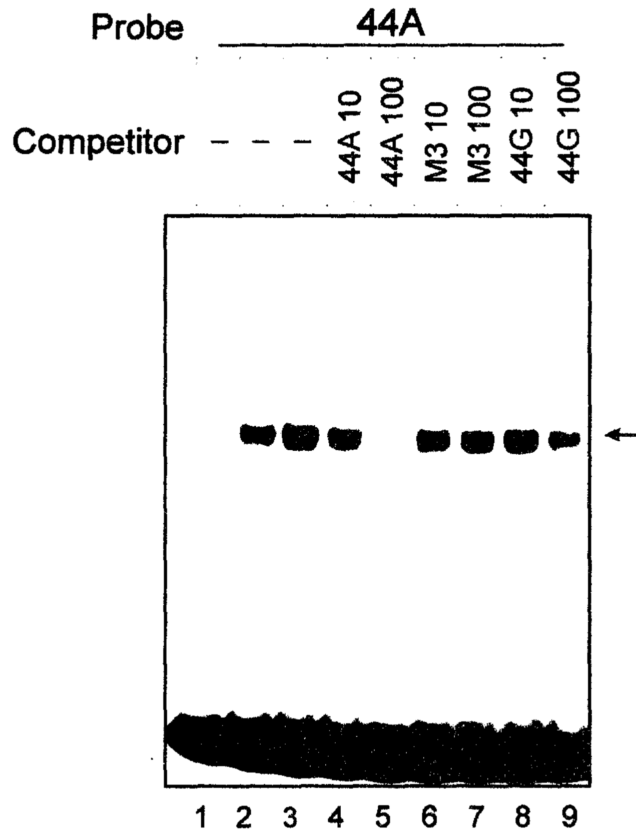

Fig. 1. EMSA of 44A oligonucleotides. The sequences of the fragment used for EMSAs are shown. 32P-labeled 44A was incubated with rat liver nuclear extracts (5 or 10 μg) (*lane* 2 and 3). The probe was incubated with 10 μg nuclear extracts in the presence of a 10- or 100-fold molar excess of the indicated cold competitor: 44A (*lane* 4 and 5), M3 (*lane* 6 and 7), 44G (*lane* 8 and 9).

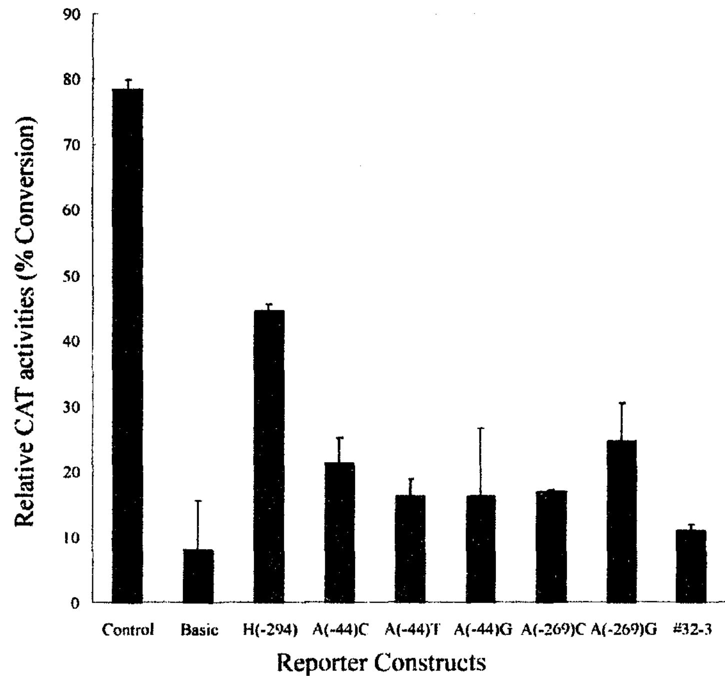

Fig. 2. Effect of site specific mutations of the human GLUT2 promoter on the CAT activity. The wild type *pCAT3*-H-294 was subjected to site specific mutation. The resulting mutant promoter constructs were used for transient CAT activity assay in HIT-T15 cells. A(-44)C, A(-44)T, A(-44)G, A(-269)C, A(-269)G represent the base changes introduced at the promoter region (numbers in the parenthesis). #32-3 represent a promoter which has triple mutations at 269, -44, +103 region. CAT activity was assayed by exposing TLC plates to phosphoimaging enhancer plate for 2 h. Data shown are average $\pm$ SD of the means of three separate experiments in triplicates.

Table 3. Summary of potential transcriptional factors obstained from yeast one-hybrid system.

-44 region에 결합하는 단백질

Clone No.	Size (kb)	Putative identification	DNA sequence
2	1 kb	Caldecrin (serum calcium-decreasing factor)	T(21)CCCGAGGAGAAGGA
6	1 kb	KIAA0242	GGATCCGAATTCGCGGCC
9	0.8 kb	Tissue inhibitor of metallo-proteinases3 (very low homology)	TGGCTACCTAAGGTTAAC
K3	0.6 kb	Chromosome 4 clone	CTAATGATTCACTTTTA
K12	1 kb	Ribosomal protein L9	AGAGGAACCCTGCGGAGG
30	1.8 kb	Hhuman regenerating protein(reg)	GGATCCGAATTCGCGGCC

+107 region에 결합하는 단백질

No.	Clone No.	Insert Size (kb)	Putative identification	DNA sequence
1	7	2.5	actin-related protein 1B, contractin beta	GCCTGCTGGGCAGGG
2	9	1.55	zinc finger protein, Ets-related transcription factor	GCGGCAGCTG
3	12	0.75	colipase preproprotein	GAGAAGATCCTGA
4	15	0.8	Unknown	TCGAGGGATATTA
5	19	0.85	pancreatic lipase	AATTTATTTGGTA
6	20	0.98	caldecrin (serum calcium decreasing factor)	CATTTTCTCGTTG
7	21	2.2	Unknown	TTTTTTTTTTTGT
8	22	0.8	Unknown	AACTTTGTCTATT
9	23	0.6	alcohol dehydrogenase	TTTTTTTTTTTTT
10	24	1	pancreatic elastase	AAAACTCATGATG
11	29	1.8	Unknown	TTCCAGACAGTGT
12	41	4	carboxypeptidase B1	ACAATGTTGGCAC
13	43	1	trypsinogen	ATAACATGTCTGT
14	45	0.4	carboxypeptidase A1	GAGAGGCCAGGGC
15	47	0.55	ribonuclease A	AGGTCACCACCTA
16	49	0.8	Unknown	CCAACTTTACCTA
17	52	0.5	carboxypeptidase A1	GAGTGTCCTGTTG
18	56	1.7	Unknown	CATAGTTGTCACT

제 4장 연구개발목표 달성도 및 대외기여도

제 1 절 연구개발목표 달성도

번호	세부연구개발목표 (선정시 계획서상의 연구목표)	달성내용	달성도 (%)
1	GLUT2 promoter 전사활성 조절인자 screening	Yeast one hybrid를 비롯한 여러 가지 기술로 trans-acting factor를 검색	100
2	전사조절인자들이 결합하는 cis-element 확인	EMSA, supershift assay를 이용하여 promoter에 결합하는 trans-acting factor 확인	100
3	전사조절인자들의 특성규명	trans-acting factor들이 유전자의 활성에 미치는 영향에 대해 연구	100

제 2 절 대외 기여도

GLUT2 promoter 부위의 mutation을 발견하여 당뇨병 진단 KIT를 개발하여 특허를 등록하였다. Human GLUT2 promoter의 -44 부위의 돌연변이에 의해 유전자 발현의 감소를 가져오고 이러한 활성감소는 당뇨병인의 하나로 작용할 가능성이 있음으로 이를 정리하여 논문 투고할 예정이다. +103 부위의 돌연변이에 의해 HNF1 또는 HNF3의 결합이 약해지고 이로인해 promoter activity가 감소됨을 밝혀 Journal of Biological Chemistry에 발표하였다. HNF1은 이미 다른 연구단에 의해 당뇨병과 관련된 전사인자임이 밝혀져 있으며, 돌연변이 부위에 이 단백질이 결합한다는 사실은 전사인자가 정상적으로 발현되고 기능할수 있어도 그 대상 유전자의 결합부위의 변이에 의해 유전자 발현이 조절될 수 있음을 나타낸다. 따라서 HNF1 등과 같은 전사인자의 돌연변이와 당뇨병의 연관성이 없는 집단인 경우 그 대상 단백질의 유전자 돌연변이도 고려해 보아야 한다. 본 연구의 결과는 GLUT2의 유전적 변이에 의한 발현 감소가 당뇨병과 상관관계가 있음을 시사하며, 본 연구에 사용된 방법은 다른 유전적 변이를 찾는것에 이용될 수 있고, 나아가 그러한 돌연변이 부위에 결합하는 전사인자가 당뇨병에 연관될수 있음을 제시한다.

제 5 장 연구개발결과의 활용계획

본 연구의 결과는 유전자의 promoter 부위의 mutation study를 이용한 당뇨병 조기 진단용 DNA chip의 개발에 활용할 계획이다. 비인슐린 의존성 당뇨병의 발병기전에 있어 여러 유전자의 돌연변이가 관련됨은 이미 알려져 있으나, 체계적으로 분석한경우가 없음으로 이들 유전자의 돌연변이와 당뇨병과의 상관관계를 밝히고자 한다.

또한 이러한 결과는 Protein chip의 probe를 제시해 줄수 있으므로 당뇨병과 관련된 유전자 발현 변화를 확인하기 위한 Protein chip 개발에 이용할 계획이다.

One hybrid assay 결과, 전사인자로 작용할 가능성이 있는 clones들의 단백질을 얻은후 기능에 대해 연구할 계획이다. 이러한 연구를 통해 지금까지 알려지지 않은 전사인자를 확보할수 있으리라 여겨진다.

제 6 장 참고문헌

1. Takeda, J., Kayano, T., Fukumoto, H., and Bell, G. I. (1993). Organization of the human GLUT2 (pancreatic beta-cell and hepatocyte) glucose transporter gene. *Diabetes* **42**, 773-777.

2. Permutt, M. A., Chiu, K. C., and Tanizawa, Y. (1992). Glucokinase and NIDDM. A candidate gene that paid off. *Diabetes* **41**, 1367-1372.

3. Steiner, D. F., Tager, H. S., Chan, S. J., Nanjo, K., Sanke, T., and Rubenstein, A.H. (1990). Lessons learned from molecular biology of insulin-gene mutations. *Diabetes Care* **13**, 600-609

4. Taylor, S. I., Cama, A., Accili, D., Barbetti, F., Quon, M. J., de la Luz Sierra, M., Suzuki, Y., Koller, E., Levy-Toledano, R., and Wertheimer, E. (1992). Mutations in the insulin receptor gene. *Endocr Rev* **13**, 566-595

5. Froguel, P., Zouali, H., Vionnet, N., Velho, G., Vaxillaire, M., Sun, F., Lessage, S., Stoffel, M., Takeda, J., and Pasa, P. (1993). Familial hyperglycemia due to mutations in glucokinase. Definition of a subtype of diabetes mellitus. *New Engl J Med* **328**, 697-702

6. Burant, C. F., Sivitz, W. I., Fukumoto, H., Kayano, T., Nagamatsu, S., Seino, S., Pessin, J. E., and Bell, G. I. (1991). Mammalian glucose transporters: structure and molecular regulation. *Recent Prog Horm Res* **47**, 349-387

7. Fukumoto, H., Seino, S., Imura, H., Seino, Y., Eddy, R. L., Fukushima, Y., Byers, M. G., Shows, T. B., and Bell, G. I. (1988). Sequence, tissue distribution, and chromosomal localization of mRNA encoding a human glucose transporter-like protein. *Proc Natl Acad Sci USA* **85**, 5434-5438

8. Gould, G. W., and Bell, G. I. (1990). Facilitative glucose transporters: an expanding family. *Trends Biochem Sci* **15**, 18-23

9. Johnson, J. H., Newgard, C. B., Milburn, J. L., Lodish, H. F., and Thorens, B. (1990).

The high Km glucose transporter of islets of Langerhans is functionally similar to the low affinity transporter of liver and has an identical primary sequence. *J Biol Chem* **265**, 6548-6551

10. Mueckler, M. (1994). Facilitative glucose transporters. *Eur J Biochem.* **219**, 713-725

11. Unger, R. H. (1991). Diabetic hyperglycemia: link to impaired glucose transport in pancreatic beta cells. *Science* **251**, 1200-1205

12. Johnson, J. H., Ogawa, A., Chen, L., Orci, L., Newgard, C. B., Alam, T., and Unger, R. H. (1985). Underexpression of cell high Km glucose transporters in noninsulin-dependent diabetes. *Science* **250**, 546-549

13. Orci, L., Ravazzola, M., Baetens, D., Imnan, L., Amhert, M., Peterson, R. G., Newgard, C. B., Johnson, J. H., and Unger, R. H. (1990). Evidence that down-regulation of beta-cell glucose transporters in non-insulin-dependent diabetes may be the cause of diabetic hyperglycemia. *Proc Natl Acad Sci USA* **87**, 9953-9957

14. Orci, L., Unger, R. H., Ravazzola, M., Ogawak, A., Komiya, I., Baetens, D., Lodish, H. F., and Thorens, B. (1990). Reduced beta-cell glucose transporter in new onset diabetic BB rats. *J Clin Invest* **86**, 1615-1622

15. Thorens, B., Wu, Y. J., Leahy, J. L., and Weir, G. C. (1992). The loss of GLUT2 expression by glucose-unresponsive beta cells of db/db mice is reversible and is induced by the diabetic environment. *J Clin Invest* **90**, 77-85

16. Matsubara, A., Tanizawa, Y., Matsutani, A., Kaneko, T., and Kaku, K. (1995). Sequence variations of the pancreatic islet/liver glucose transporter (GLUT2) gene in Japanese subjects with noninsulin dependent diabetes mellitus. *J Clin Endocrin Metab* **80**, 3131-3135

17. Patel, P., Bell, G. I., Cook, J. T. E., Turner, R. C., and Wainscoat, J. S. (1991). Multiple restriction fragment length polymorphisms at the GLUT2 locus: GLUT2 haplotypes for genetic analysis of type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* **34**, 817-821

18. Elbein, S. C., Hoffman, M. D., Matsutani, A., and Permut, M. A. (1992). Linkage analysis of GLUT1 (HepG2) and GLUT2 (liver/islet) genes in familial NIDDM. *Diabetes* **41**, 1660-1667

19. Mueckler, M., Kruse, M., Strube, M., Riggs, A. C., Chiu, K. C., and Permutt, M. A. (1994). A mutation in the Glut2 glucose transporter gene of a diabetic patient abolishes transport activity. *J Biol Chem* **269**, 17765-17767

20. Moller, A. M., Jensen, N. M., Pildal, J., Drivsholm, T., Borch-Johsen, K., Urhammer, S. A., Hansen, T., and Pedersen, O. (2001). Studies of genetic variability of the glucose transporter 2 promoter in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* **86**, 2181-2186

21. Diabetes Mellitus: report of a WHO study Group (1985). *WHO Tech Rep Ser* **727**, Geneva pp1-113

22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Strategies for cloning in plasmid vectors. In: Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory, New York, vol 1:53-73

23. Sanger, F., Nicklen, S., Coulson, and A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Biotechnology* **74**,5463-5467

24. Gorman, D. M., Moffat, L. F., and Howard, B. H. (1982). *Mol Cell Biol* **2**, 1044-1051

25. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-264

26. Cha, J. Y., Kim, H. I., Kim, K. S., Hur, M. W., and Ahn, Y. H. (2000). Identification of transacting factors responsible for the tissue-specific expression of human glucose transporter type 2 isoform gene. Cooperative role of hepatocyte nuclear factors 1alpha and 3beta. *J Biol Chem* **275**, 18358-18365

27. Shimada, F., Makino, H., Iwaoka, H., Miyamoto, S., Hashimoto, N., Kanatsuka, A., Bell, G. I., and Yoshida, S. (1995). Identification of two novel amino acid polymorphisms in beta-cell/liver (GLUT2) glucose transporter in Japanese subjects. *Diabetologia* **38**, 211-215

28. Milburn, J. L., Ohneda, M., Johnson, J. H., and Unger, R. H. (1993). Beta-cell GLUT-2 loss and non-insulin-dependent diabetes mellitus: current status of the hypothesis. *Diabetes Metab Rev* **9**, 231-236

29. Kim , H. I., Kim, J. W., Kim, S. H., Cha, J. Y., Kim, K. S., and Ahn, Y. H. (2000) Identification and functional characterization of the peroxisomal proliferator response element in rat GLUT2 promoter. *Diabetes* **49**, 1517-1524

30. Kim, J. W., and Ahn, Y. H. (1998). CCAAT/enhancer binding protein regulates the promoter activity of the rat GLUT2 glucose transporter gene in liver cells. *Biochem J* **336**, 83-90

31. Doria, A., and Plengvidhya, N. (2000) Recent advances in the genetics of maturity-onset diabetes of the young and other forms of autosomal dominant diabetes. *Curr Opin Endocrinol Diabetes* **7**, 203-210