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PhD THESIS

The Effect of the ref-1 on the Promoter Activity of the GFRα1 and JAG1

Graduate School of Chosun University

Department of Bio-Material Engineering

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Ref-1 에의한 GFRα1 및 JAG1 Promoter 조절연구.

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Advisor Ho Jin You, Professor & Chair, KDRRC

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ABBREVIATIONS

A, C, G, T - Nucleotides/bases

AS antisense

BAC bacterial artificial chromosome

bHLH basic helix-loop-helix

bp - Base pair

cDNA complementary DNA

CHIP Chromatin Immunoprecipitation

DNA - Deoxyribonucleic acid

DNA deoxyribonucleic acid

ECM extracellular matrix

EMSA Electrophoretic Mobility Shift Assay (Gel Shift Assay)

GDNF glial cell line-derived neurotrophic factor

GFL GDNF family ligand

GFP green fluorescent protein

GPI glycosylphosphatidyl inositol

mRNA - Messenger RNA

NCBI - National Center for Biotechnology Information

NF-κB BS#I NF-κB DNA Binding Site 1

NF-κB BS#II NF-κB DNA Binding Site 2

NF-κB BS#III NF-κB DNA Binding Site 3

PCR polymerase chain reaction

qRT-PCR quantitative real-time PCR

RNA - Ribonucleic acid

siRNA small interfering RNA

SMT Site directed mutagenesis

TF - Transcription factor

TFBS - Transcription factor binding site

TK tyrosine kinase

TSS - Transcription start site

WT Wilms' tumor

ABSTRACT

The Effect of the ref-1 on the Promoter Activity of the GFRα1 and JAG1.

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(I) Ref-1 Induced GFRα1 Promoter Activity in Human fibroblast cell line GM00637.

The overall goal of the research in this thesis was to gain the data that will eventually help in elucidating the Human apurinic/apyrimidinic (AP) endonuclease, APE 1/ref-1, induced Glial cell line derived neurotrophic factor family receptor alpha1; GFRα1 expression in Human fibroblast Cell Line GM00637. The mechanisms by which DNA binding of transcription factors NF-κB of GFRα1 promoter regulates the significant induction of functional GFRα1 in APE1 expressing GM00637 cell line. In addition to DNA repair activity, APE 1 is also capable of regulating the DNA binding activity of many transcription factors in vitro by a redox mechanism (ref-1). Here an active NF-κB binding site was identified in the GFRα1 putaive promoter region. By using EMSA super shift assays with NF-κB-p50 was identified as APE1

interacting protein followed by a nuclear translocation of p50 NF-κB subunits binding to NF-κB site of GFRα1 promoter. Further functional reporter assays and by means of in-vivo CHIP indicated that this NF-κB binding DNA elements associated with GFRα1 promoter. Further more, mutation of this site significantly reduced the promoter activity. Therefore APE1 induced human GFRα1 is controlled by NF-κB binding motifs, thereby providing a novel mechanism by which APE1 up-regulates GFRα1 functions via transcription factors of NF-κB binding sites of GFRα1 promoter. Taken together, this thesis demonstrates a novel Promoter analysis of GFRα1 in associated with APE1/ref-1. The studies also indicate that the precise mechanism of APE1/ref-1 mediated GFRα1 induction as well as GFRα1 promoter analysis.

(II) Ref-1 Induced JAG1 Promoter Activity in Human fibroblast cell line GM00637.

The Jagged 1 protein encoded by JAG1 is the human homolog of the Drosophila jagged protein. Human jagged 1 is the ligand for the receptor notch 1 which is a human homolog of the Drosophila jagged receptor notch. This work reports that, ref-1 induced JAG1 gene expression activity requires EGR-1 DNA binding site activation. The EGR-1 DNA binding sites of JAG1 promoter is capable of regulating the transcription of the ref-1 induced human JAG1 gene promoter. To assess the transcriptional regulation of JAG1 gene, the 5'-flanking region with [-98 to -1473] base pairs of the human JAG1 gene was cloned. By measuring the promoter activity using dozens of serial deleted luciferase constructs, the highest JAG1 transcriptional activity was found at the region from -850 to -382 upstream of ATG from JAG1 gene and the region is associated with three EGR-1 DNA binding sites and one is functional. In particular, it was demonstrated by chromatin immunoprecipitation and EMSA that EGR-1 assembles on the JAG1 promoter and, by site directed mutagenesis experiments, that it significantly reduces its transcriptional effects by acting through the 468 bp minimal promoter. Taken together, this data add to the body of evidence implying that EGR-1 binding site may fulfill a generic role at the promoters of JAG1 in response to ref-1 in GM00637 cells.

Introduction

1.0. Introduction

Gene regulation has been recognized as an important line of research due to its crucial biological significance. One of the essential regulatory regions of the gene is its promoter region. Recognition and annotation of promoter regions besides other regulatory regions in the genomes remains a fundamental task even today. This is because the genomic data continue to stay largely unannotated, particularly the regulatory regions. One reason that can be attributed to this problem is that promoter recognition and annotation is an extremely challenging problem in part due to the complexity of the data involved.

1.1. BIOLOGICAL BACKGROUND

An eukaryotic organism contains the complete genome in the nuclei of most of the cells. The genome is the complete set of genetic information inherited from the parents and comprises al the genes. The genome is physically present in the form of a polymer called DNA (deoxyribosenucleic acid). The basic unit of DNA is a nucleotide which comprises sugar-phosphate backbone and one of the four bases adenine (A), cytosine (C), guanine (G) and thymine (T). The genetic instructions encoded in genomic sequences are very less understood. The human genome, for example, is extraordinarily complex. The protein-coding bases of its 30,000 genes span only less than 2% of the entire 3 billion base pairs long genomic sequence (IHGSC). Of the rest noncoding segment of the genome, another small part contains regulatory regions controlling the expression of these genes.

1.2. Regulation of Gene expression and Promoter

Genes in DNA act as a blueprint for the production of RNA and proteins (another polymer) inside the cells. Proteins play an essential role in cellular functions. A vast majority of genes are known to produce proteins as their end products. The process of synthesizing proteins in cells is known as gene expression. Gene expression involves transfer of sequential genetic information from DNA to proteins and broadly involves following stages (Fig. 1.A.).

i) transcription, where a gene's DNA sequence is transcribed into a single stranded sequence of

primary transcript or pre-mRNA.

ii) capping, where primary transcript is capped on the 5' end, which stabilizes the transcript by protecting it from degradation enzymes.

iii) poly-adenylation, where a part of 3' end of the primary transcript is replaced by a poly-A tail for providing stability.

iv) splicing, where introns are removed from the primary transcript to form messenger RNA (mRNA).

v) mRNA is transported from nucleus to cytoplasm.

vi) translation, where a ribosome produces a protein by using the mRNA template.

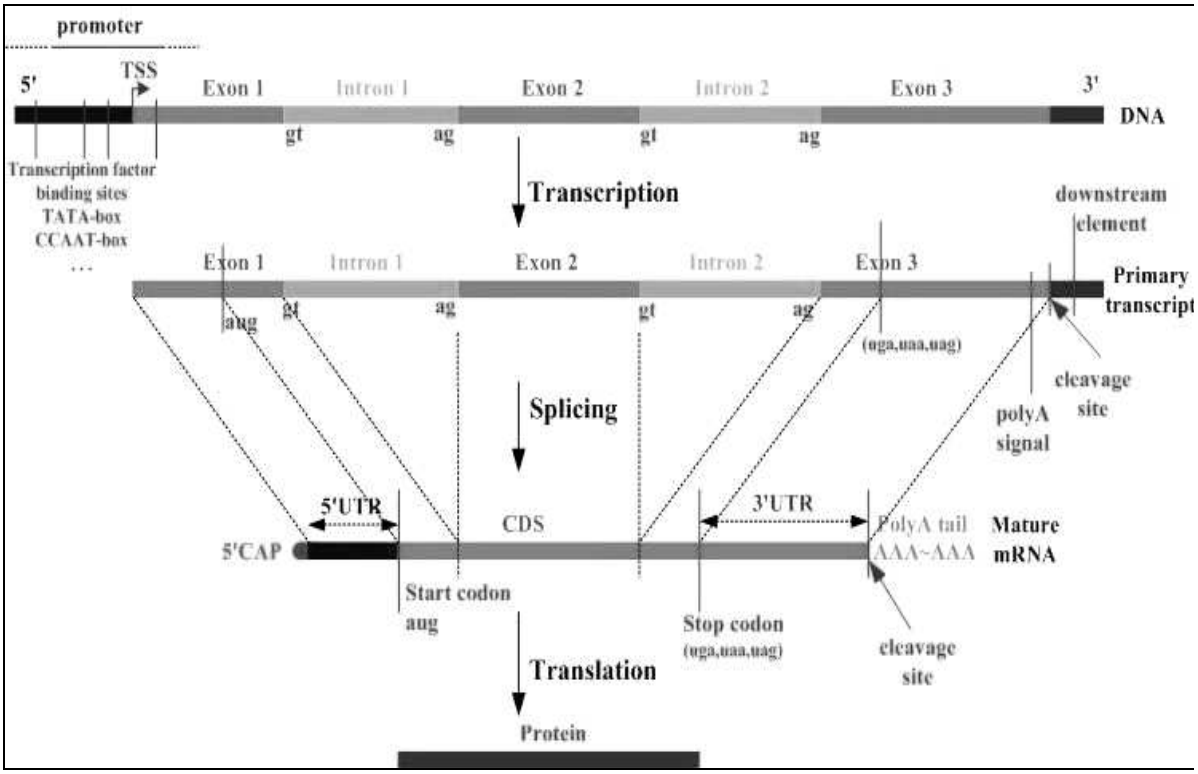


Fig. 1.A. Stages of gene expression in cell.

Gene expression is a strictly regulated process in cells. The regulation of gene expression is important as it determines where (cell-type), when (developmental stage), how, and in what quantities various proteins are produced in cells. This decides how cells develop, differentiate and respond to external stimuli. Gene regulation occurs at various stages of gene expression from transcription to translation (stages shown above), though transcription is generally

believed to be the most important stage. The transcription stage of gene expression involves regulatory DNA regions known as promoters. Every gene has at least one promoter that mediates and controls its transcription initiation. This control mechanism occurs through a complex interaction between various TFs that get attached to their specific TFBSs present in the gene's promoter region. A promoter is usually defined as a non-coding region of DNA that covers the TSS or the 5' end of the gene. Bulk of promoter region typically lies upstream of the TSS. The promoter region in Eukaryotes is usually difficult to characterize because of high variability. For example, promoters may vary from a few hundred bases in some genes to several kilo bases in the others. A promoter may be typically classified as,

i) Core promoter

-usually lies up to 30 bp upstream with respect to the TSS

- contains the TSS
- contains binding site for RNA polymerase
- contains general binding sites (i.e. binding sites commonly found in many promoter types)
- example of a binding site in this region is TATA-box

ii) Proximal promoter

-usually lies between 200 bp to 300 bp upstream with respect to the TSS

-contains specific binding sites that control temporal and spatial expression of a gene

- example of a binding site in this region is CAAT-box

iii) Distal promoter

-lies upstream of the proximal promoters, may be located thousands of bases away from the TSS

- contains specific binding sites that control temporal and spatial expression of a Gene.

Aside a promoter, there are some additional regulatory regions on the DNA that work cohesively with the promoter in regulating a gene at the transcription stage. These regions are usually located thousands of bases upstream or downstream of the TSS and regulate the rate of transcription of the associated gene. Alike promoters, the regulation here also occurs through specific regulatory TFBSs present in these regions. Examples of such regions include enhancers, silencers and boundary elements; enhancers increase the gene's transcription rate while silencers decrease it. Promoter regions are interspersed with characteristic short TFBSs

patterns (~6-20 bp in length) that provide functionality to these regions. These patterns are usually conserved across species and are degenerate in nature. As TFBS motifs are short they tend to occur frequently anywhere in the genome, however, only those that are present in the regulatory regions of the genome may be functionally active. TFBSs show large variations across promoters of a species; some promoters may have particular TFBSs that others do not have. Between promoters, TFBSs do not intrinsically have any bias towards a particular location or orientation (Werner 1999).

However for a particular class of promoters such a bias may be observed (Wasserman and Fickett 1998). Adding to the complexity, the nature of function of a TFBS may depend on its context/location within the promoter. For example, the factor AP1 suppresses gene transcription when it binds to its binding site in the distal promoter, while it supports the transcription when it binds to its binding site in the core promoter (Werner 1999). Such contextual behavior of a TFBS may be dictated by factors such as, tissue specificity, and cell-cycle & developmental stage. Overall, there are large variations in TFBS distributions across promoters and their associated functions. An existing paradigm is that within a promoter, TFBSs uniquely combine to form a module that imparts a specific functionality to the promoter. A typical functional module organization is shown in Fig. 1.1. The module is characterized by its features, such as specific order of TFBSs, their orientation, their location, and mutual distance between them. The module functions as a single cohesive unit and may not work if any of the module elements is absent or if any of its features gets disturbed. A module may be more specific on the DNA compared to a single TFBS. Due to this, modules are sometimes preferred over single TFBSs for modeling promoters.

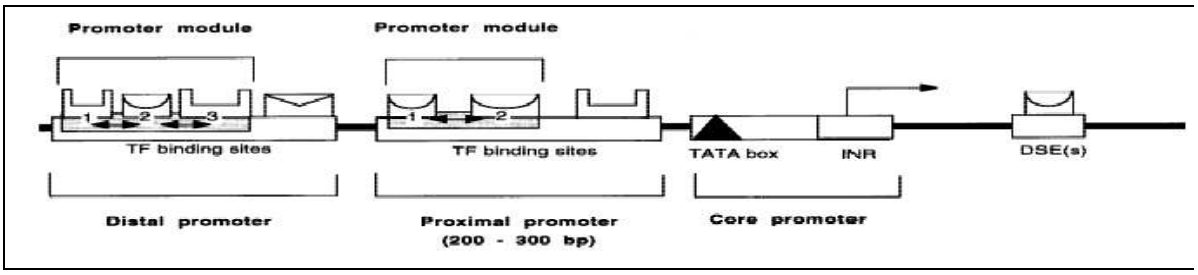


Fig. 1.1. A typical promoter structure showing modular organization of TFBSs. Taken from Werner 2003.

1.3.1. GFRα1

The GFR-alpha Nomenclature Committee (1997) proposed names and symbols for the GPI-linked receptors for the GDNF ligand family (1-5). GDNF and neurturin play key roles in the control of vertebrate neuron survival and differentiation (6). Both signal via a multicomponent receptor system formed by a glycosyl phosphatidylinositol (GPI)-linked ligand binding subunit (the 'alpha' component) and the receptor tyrosine kinase RET as a signaling (i.e., beta) subunit ((Jing *et al*1996; Nosrat *et al.*, 1997; Treanor *et al.*, 1996). The glial cell line-derived neurotrophic factor (GDNF) family ligands GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) are structurally related neurotrophic factors that signal through a multicomponent receptor composed of the transmembrane receptor tyrosine kinase RET and high affinity glycosylphosphatidylinositol (GPI)-anchored proteins, the GDNF family α receptors 1-4 (7).

Paratcha *et al.* (2001) showed that GFRα1 is released by neuronal cells, Schwann cells, and injured sciatic nerve. RET stimulation in trans by soluble or immobilized GFRα1 potentiates downstream signaling, neurite outgrowth, and neuronal survival, and elicits dramatic localized expansions of axons and growth cones. Soluble GFRα1 mediates robust recruitment of RET to lipid rafts via a mechanism requiring the RET tyrosine kinase. Activated RET associates with different adaptor proteins inside and outside lipid rafts.

Detail information about the difference between two variant with all the exon-intron information based on Ensemble database are summarized in here.

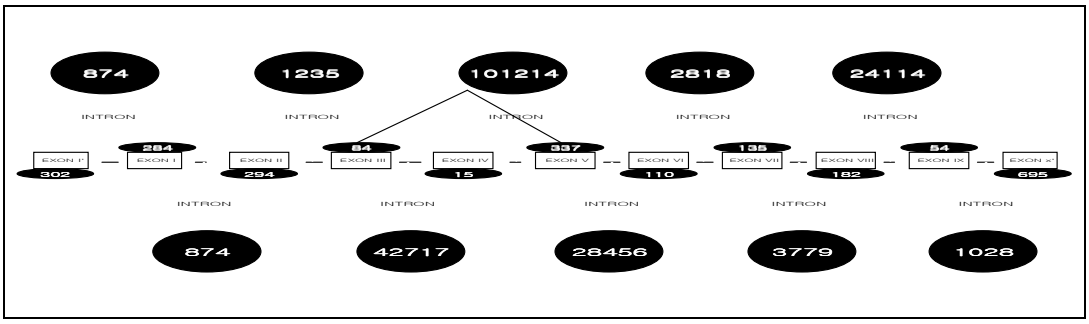


Fig. 1-3. Detail diagrammatic structure of the genomic configuration of exon-intron of the GFR α 1 gene.

1.3.2. Ref-1 Induced GFRα1 Promoter Activity in Human Fibroblast cell line GM00637.

Human GFRα1 expression is induced by ref-1 in human fibroblast cell line GM00637. To determine whether ref-1 mediated GFRα1 protein accumulation was associated with an increase in GFRα1 specific mRNA expression, total RNA from ref-1 expressing GM00637 cells were isolated and examined by RT-PCR (21-23) technique. As shown in fig. I-1A, GFRα1 mRNA was increased in ref-1 expressed GM00637 cells. Similarly to investigate whether ref-1 induced GFRα1 expression in human fibroblast cell line GM00637, the effect of ref-1 on GFRα1 protein level was determined by western blot using anti GFRα1 antibody. As shown in fig. I-1B, over expression of ref-1 in GM00637 significantly enhanced GFRα1 protein levels. These results confirmed that human GFRα1 expression is induced by ref-1 at a transcriptional level in GM00637 cells.

To further understand the control of ref-1 induced GFRα1 gene transcription, dozens of serial deleted reporter constructs of 5'-flanking region of the GFRα1 gene had cloned, mapped its transcriptional regulatory factor as NF-KB by EMSA and CHIP, characterized the regulatory activities in transcription of its different sub segments and demonstrated NF-κB as a critical transactivator interacting with the cognate *cis*-element in the promoter region of GFRα1 gene activation. To determine if ref-1 activates the human GFRα1 promoter, a GFRα1 promoter-driven luciferase reporter vector (-2291/-7) was transiently transfected into GM00637 cells with its ref-1 expresssing cells and cultured at least 48 hr after transfection. Ref-1 induced GFRα1 promoter activity in an over expressed cells was observed up to ~20-fold (Fig. I-4B) than that of control cell line, documenting a significant transcriptional response of the GFRα1 promoter. The precise mechanisms for their activation in human fibroblast cells were intended to be analyzed in this present study.

A computer based analysis [Genomatix-Matinspector] (28-30) of the GFRα1 promoter sequence (NCBI accession no. AC005872) revealed several putative binding sites for NF-κB transcription factors. These elements are located between -575 bp and -66 bp upstream of the translation start site in the GFRα1 gene. There are three NF-κB DNA binding domains [NF-κB

BS #I lies -349, NF-κB BS #II lies -300 and NF-κB BS #III lies -155 from ATG(+1) respectively] in reporter construct of ~0.59 kbp, but only NF-κB BS #I which is required for the DNA binding activity. In this work, EMSA supershift (17) and CHIP (18,19) as in-vitro and in vivo with site directed mutagenesis (20) showed GFRα1 gene promoter contains an active NF-κB binding site which play a important role to ref-1 induced GFRα1 gene activation.

The transcription factor NF-κB is implicated in the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses1 (8,9). NF-κB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family. There are five subunits of the NFκB family in mammals: p50, p65 (RelA), c-Rel, p52 and RelB (11,12). These proteins share a conserved 300 amino acid sequence in the N-terminal region, known as the Rel homology domain, that mediates DNA binding, protein dimerization and nuclear localization. This domain is also a target of the IκB inhibitors, which include IκBα, IκBβ, IκBγ, Bcl-3, p105 and p1004. Various dimer combinations of the NFκB subunits have distinct DNA binding specificities and may serve to activate specific sets of genes such as adhesion molecules, immunoreceptors and cytokines (13-16). The p50/p65 (NFκB1/RelA) heterodimers and the p50 homodimers are the most common dimers found in NFκB signaling pathway (11,12).

In order to determine which site(s) is responsible for this activation, we performed EMSA using double stranded oligonucleotide probes containing each NF- kB binding site from the –575/-66 region of the GFRα1 promoter. As shown in fig. I-6A, the intensity of DNA-protein complexes generated by the –300 and -155 probe, containing the NF-κB binding sites at the –300 and -155 position, respectively, did not change following ref-1 induced effect in comparison with non-treated parental nuclear extracts. However, the intensity of one band generated by the –349 probe containing the NF-κB binding site at the –349 position of the GFRα1 promoter was significantly higher in ref-1 expressed nuclear extracts as compared with parental nuclear extracts (Fig. I-6B).

To further characterize this increased DNA-protein complex from ref-1 expressed nuclear extracts, an unlabeled NF-κB consensus probe were used as competitors in EMSA (31-33). As

shown in fig. I-6 BI and BII (lane 4), unlabeled consensus probe completely competed the increased DNA-protein complex. In contrast, the -349 probe had significant effect on nucleoprotein complex formation fig. I-6 BI and BII (lane 3). These results indicate that the NF-κB binding site at the –349 position of the GFRα1 promoter confers the ref-1 mediated differential transcription factor binding. Supershift experiments with anti-p50 antibody resulted in a retarded mobility of the complex (indicated by red arrow), demonstrating the presence of p50, a subunit of NF-κB in the nucleoprotein complex [Fig. I-6 BI and BII (lane 5)]. But the incubation with the NF-κB family subunits such as p52 (Fig.I-6C II lane 6) and p65 ((Fig.I-6C II lane 4) had not any role to make supershift complex. Similarly it was not found any role to make nucleoprotein complex with antisera of c-Rel and RelB (Data are not shown). This data suggested that ref-1 stimulated the binding of p50 to the GFRα1 specific –349 position NF-κB probes while p52, Rel-B and c-Rel had not any effect to the NF-κB consensus sequences as well as GFRα1 specific probes..

Taken together these data indicate that the ref-1 increases binding of NF-κB to the –349 NF-κB binding site in the GFRα1 promoter. To further clarify the role of the –349 NF-κB binding site of the GFRα1 promoter, NF-κB DNA elements of the promoter-reporter construct (NF-κB SMT-575/-66) were mutagenized in each site separately (Fig. I-7). These constructs were transiently transfected into ref-1 expressed GM00637 cells and 48 hour after transfection luciferase activities were measured. Mutation in -349 NF-κB DNA binding element containing luciferase reporter construct NF-κB SMT-575/-66 significantly reduced response to ref-1 as well as basal GFRα1 promoter activity but not by other two -300 and -155 NF-κB DNA binding site confirming as previously shown that first NF-κB site at -349 is important for the ref-1 induced GFRα1 promoter activity.

To determine cellular NF-κB binding to the GFRα1 promoter region, the ChIP assay was carried out using an antibody against NF-κB P50 and P65 (66-70). As demonstrated, there were at least one functionally active NF-κB binding site located at the regions -349 in the GFRα1 promoter region -575/-66 that displayed the maximal promoter activity (Fig. I-5B). Thus, this active NF-κB BS #I was tested for their cellular protein binding by using specific primer pairs as tabulated in table I-4. Specificity of the experiment was evaluated by comparison to that of

Anti-Acetyl-Histone H3 binding to the GADPH promoter under the same conditions. According to the designed primer pairs, we anticipated that GFRα1 promoter fragments –575/-66 which contain active NF-κB BS#I, would result following immunoprecipitation with the NF-κB-p50 antibody and amplification by PCR. Indeed, gel analysis of PCR products confirmed our expectation. Approximately, ~150-200 a bp DNA bands were observed on gels as the PCR amplified using primer pairs that encompass NF-κB BS #I, (Fig. I-9A). In contrast, no signal was detected in control experiments with a nonspecific antibody . These results suggest that the assay conditions were appropriate and can be used to measure the relative levels of NF-κB BS #I binding to the GFRα1 gene in response to ref-1 stimuli. There was no significant difference in the yields of PCR products among groups using “input” (before immunoprecipitation) and DNA as a template (Fig.I-9).

1.4.1. JAGGED1

Jagged1 ligand for the Notch cell surface receptor, has an important role in blood vessel development (34). Signal activation leads to cleavage of the intracellular part of the Notch receptor from the membrane which translocates to the nucleus and activates transcription factors (35). JAGGED1 is a member of the Delta/Serrate/Lag-2 (DSL) family of proteins that are cell-bound ligands for Notch receptors. Initiation of Notch signaling occurs through a series of proteolytic events upon the binding of Notch to a DSL protein presented on neighboring cells (36). Whether DSL proteins themselves are capable of initiating an intrinsic signaling mechanism within the cell they are expressed is not known. Aberrant misexpression of JAGGED1 and DELTA1 has been documented in several human tumors; however, the mechanism by which misexpression of JAGGED1 contributes to oncogenesis has not been elucidated (37,38).

Notch proteins are a family of closely related transmembrane receptors demonstrated to be instrumental in cell fate decisions. Notch ligands 'Delta' and 'Jagged' (Lindsell *et al.*, 1995) were identified in Drosophila and rat, respectively. Oda *et al.* (1997) isolated the human homolog of the rat Jagged gene (symbolized JAGL1 by them) from a CpG island in a YAC clone covering the Alagille syndrome (ALGS; 118450) critical region on 20p12. This region

had been previously defined as flanked by SNAP (600322) distally and D20S186 proximally. The 5,942-bp cDNA possesses 3 alternative polyadenylation sites. Northern blot analysis of RNA from adult tissues indicated that JAGL1 is widely expressed in many tissues. The most abundant expression was observed in ovary, prostate, pancreas, placenta, and heart.

Bell *et al.* (2001) determined that JAG1 was one of several transcripts upregulated by umbilical vein endothelial cells during capillary morphogenesis in 3-dimensional collagen matrices.Li *et al*. (2006) found that JAG1 activated Notch signaling in rats and enhanced the differentiation of mesenchymal stem cells into cardiomyocytes.

1.4.2. Ref-1 Induced JAG1 Promoter Activity in Human fibroblast cell line GM00637

Human JAG1 expression is induced by ref-1 in human fibroblast cell line GM00637. To determine whether ref-1 mediated JAG1 protein accumulation was associated with an increase in JAG1 specific mRNA expression, total RNA from ref-1 over-expressed GM00637 cells were isolated and examined by RT-PCR (21-23) method. As shown in fig. II-1A, JAG1 mRNA was increased in ref-1 expressed GM00637 cells. Similarly to investigate whether ref-1 induces JAG1 expression in human fibroblast cell line GM00637, the effect of ref-1 on JAG1 protein level was determined. As shown in fig. II-1B, over expression of ref-1 in GM00637 significantly enhanced JAG1 protein levels. These results confirm that human JAG1 expression is induced by ref-1 at a transcriptional level in GM00637 cells.

In this study, we found that ref-1 activates the human JAG1 promoter. To determine if ref-1 activates the human JAG1 promoter, a JAG1 promoter-driven luciferase reporter vector (-850/-382) was transiently transfected into GM00637 and its ref-1 over expressed stable transfectant cultured at least 48 hr after transfection. Ref-1 induced JAG1 promoter activity in an over expressed cells was observed up to ~40-fold (Fig. II-4B), documenting a significant transcriptional response of the JAG1 promoter. The precise mechanisms for their activation in human fibroblast cell line was tried to be analyzed in this present study.

A computer based analysis Genomatix-Matinspector (28,29,30) of the JAG1 promoter sequence (NCBI accession no. AL035456) revealed three binding sites for EGR-1 transcription factors. These elements were located between -850 bp and -382 bp upstream of the transcription start site in the JAG1 gene. The results of the experiment indicate that the human JAG1 promoter is activated by EGR-1 DNA binding sites. There are three EGR-1 DNA binding sites [EGR-1 BS I -540-556, EGR-1BSII -622-638, EGR-1 BSII -778-794] of JAG1 promoter which was initially predicted by GENOMATIX Mat inspector software.

Egr-1 is involved in the regulation of cell proliferation in response to extracellular signal such as mitogens and growth factors, as well as oxidative stress (97,98). Studies of gene expression in human tumour cells and tissues support Egr-1's function as a tumour suppressor (99,100). The regulatory functions of the different APE1 activities could be implemented via three different mechanisms: (a) APE1's relocalization from the cytoplasm to the nucleus; (b) increase in APE1's level after transcriptional activation (86,90,96; and (c) APE1's post-translational modifications (such as acetylation and phosphorylation).

In order to determine which site(s) is responsible for this activation, we performed EMSA using double stranded oligonucleotide probes containing each EGR-1 specific DNA binding site from the -540/-556, -622/-638 and 778/-794 region of the JAG1 promoter. As shown in Fig. II-6A, the intensity of DNA-protein complexes generated by the -622/-638 probe, containing the EGR-1 DNA binding site #II of JAG1 promoter was significantly higher in ref-1 over-expressed cells than compared with nuclear extracts of parental cell line GM00637/GM00637-pcDNA3.1. To further characterize this increased DNA-protein complex from ref-1 over expressed nuclear extracts, an unlabeled double stranded probe [an EGR-1consensus probe (sequence: GGATCCAGCGGGGGCGAGCGGGGGCGA)] (39) were used as competitors in EMSA. As shown in fig. II-6B (lane 4), unlabeled probe completely competed the increased DNA-protein complex. In contrast, the EGR-1 Antibody had effect on nucleoprotein complex formation (Fig. II-6A).

These results indicate that the EGR-1 DNA binding site at the -622/-638 position of the JAG1 promoter confers the ref-1 mediated differential transcription factor binding. Supershift experiments with EGR-1 antibody resulted in a retarded mobility of the complex (indicated by

red arrow), demonstrating the presence of EGR-1 nucleoprotein complex (Fig. II-6C).

Taken together these data indicate that EGR-1 increases ref-1 induced JAG1 expression via EGR-1 binding -622/-638 site in the JAG1 promoter. To clarify the role of the -622/-638 EGR-1 binding site of the JAG1 promoter, a mutant JAG1 promoter-reporter construct -850-382 containing a mutation (ctt gga agg ggt tgg **aaa a**ag cgg gat gct cag ggc) in the EGR-1 BS #II was generated (Fig. II-7). This construct was transiently transfected into ref-1 expressing GM00637 cells, and the transfected cells were used for measurement of lucifearse activity. In addition, the mutation of EGR-1 BS#II binding site significantly reduced the ref-1 induced JAG1 promoter activity confirming as previously shown that at least one EGR-1 sites of -622 is important for the ref-1 induced JAG1 promoter activity.

To determine cellular EGR-1 binding to the JAG1 promoter region, the ChIP assay was carried out using an EGR-1 antibody against gel supershift and ChIP applications, sc-20689 X, Santa Cruz Biotechnology, Inc. (66-70). As demonstrated, there was at least one functionally active EGR-1 binding site located at the regions -622/-638 in the JAG1 promoter region -850/-382 that displayed the maximal promoter activity (Fig. II-5B). Thus, this active EGR-1 BS #II was tested for their cellular protein binding by using specific primer pairs as tabulated in table II-4. Specificity of the experiment was evaluated by comparison to that of Anti-Acetyl-Histone H3 binding to the GADPH promoter under the same conditions. According to the designed primer pairs, we anticipated that JAG1promoter fragments –850/-382 which contain active EGR-1 BS #II binding site, would result following immunoprecipitation with the EGR-1 antibody and amplification by PCR. Indeed, gel analysis of PCR products confirmed our expectation. Approximately, ~150-200 a bp DNA bands were observed on gels as the PCR amplified using primer pairs that encompass EGR-1 BS #II, (Fig. II-9A). In contrast, no signal was detected in control experiments with a nonspecific antibody. These results suggest that the assay conditions were appropriate and can be used to measure the relative levels of EGR-1 BS #II binding to the JAG1 gene in response to ref-1 mediated induction. There was no significant difference in the yields of PCR products among groups using “input” (before immunoprecipitation) and DNA as a template (Fig. II-9). These results indicate that the EGR-1 binding site at the -622/-638 position is critical for ref-1 mediated JAG1 promoter activation.

1.5. APE (ref-1)

Apurinic/Apyrimidinic (AP) endonuclease (APE) is a multifunctional protein involved in the maintenance of genomic integrity and in the regulation of gene expression (40). After the initial discovery in Escherichia coli, APE was purified from calf thymus DNA and extensively characterized as an endonuclease that cleaves the backbone of double-stranded DNA containing AP sites. APE homologues were subsequently identified and characterized in many organisms, including yeast as APN1 , mice as Apex , and humans as HAP1 (41). In addition to its major 5'-endonuclease activity, APE also expresses minor 3'-phosphodiesterase, 3'-phosphatase, and 3' →5'-exonuclease activities, the biological significance of which is controversial. Independent of its discovery as a DNA repair protein, APE was also characterized as ref-1, for redox factor-1, a redox activator of cellular transcription factors. Although the molecular detail of APE redox activity is still unclear, the discovery of APE as a regulator of transcriptional activity may underscore the importance of its involvement in cellular stress-response pathways (40,41).

The APE1/Ref-1 protein plays a central role in the highly regulated process of cellular response to oxidative stress (86-87). Its activation is part of a complex network of cellular events that determines the final outcome, namely cell growth arrest, death or survival of cells exposed to oxidative stress (86,88 ,91). In addition to well-characterized toxic effects, mild oxidative stress activates survival/proliferative signalling (92). Thus, tight temporal control and the extent of APE1 activation in response to oxidative stress could modulate cell growth and survival. APE1 is a ubiquitous multifunctional protein possessing both DNA repair and transcriptional regulatory activities. APE1 enhances DNA binding of a number of transcription factors including p53, Egr-1 and NF-κB (86,87, 93-96) by acting as a transcriptional co-activator.

The DNA base excision repair (BER) pathway is responsible for the repair of cellular alkylation and oxidative DNA damage. A crucial and the second step in the BER pathway involves the cleavage of baseless sites in DNA by an AP endonuclease. The major AP endonuclease in mammalian cells is Ape1/ref-1. Ape1/ref-1 is a multifunctional protein that is not only responsible for repair of AP sites, but also functions as a reduction-oxidation (redox)

factor maintaining transcription factors in an active reduced state. Ape1/ref-1 has been shown to stimulate the DNA binding activity of numerous transcription factors that are involved in cancer promotion and progression such as Fos, Jun, NF (B, PAX, HIF-1,HLF) and p53 (40). APE1/ref-1 has also been implicated in the activation of bioreductive drugs which require reduction in order to be active and has been shown to interact with a subunit of the Ku antigen to act as a negative regulator of the parathyroid hormone promoter, as well as part of the HREBP transcription factor complex. Ape1/ref-1 levels have been found to be elevated in a number of cancers such as ovarian, cervical, prostate, rhabdomyosarcomas and germ cell tumors and correlated with the radiosensitivity of cervical cancers. Hence, incorporation of the rapidly growing information on APE1/ref-1 may lead to a wide variety of functions and systems (41-43).

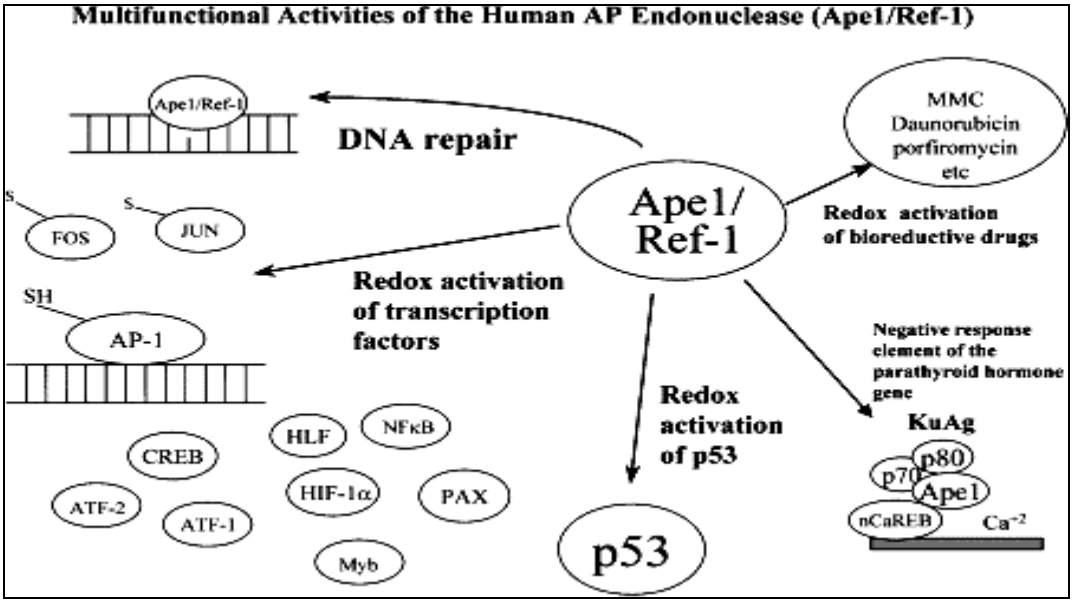


Fig.1.4. Multifunctional activities of the human AP endonuclease. Ape1/ref-1 is a multifunctional protein involved in BER, transcription factor regulation, and oxidative signaling. In DNA BER, it functions as an AP endonuclease. It is also involved in the activation of transcription factors such as p53, AP-1, HIF-1, and HLF. This activation can be through redox-dependent and/or redox-independent mechanisms.

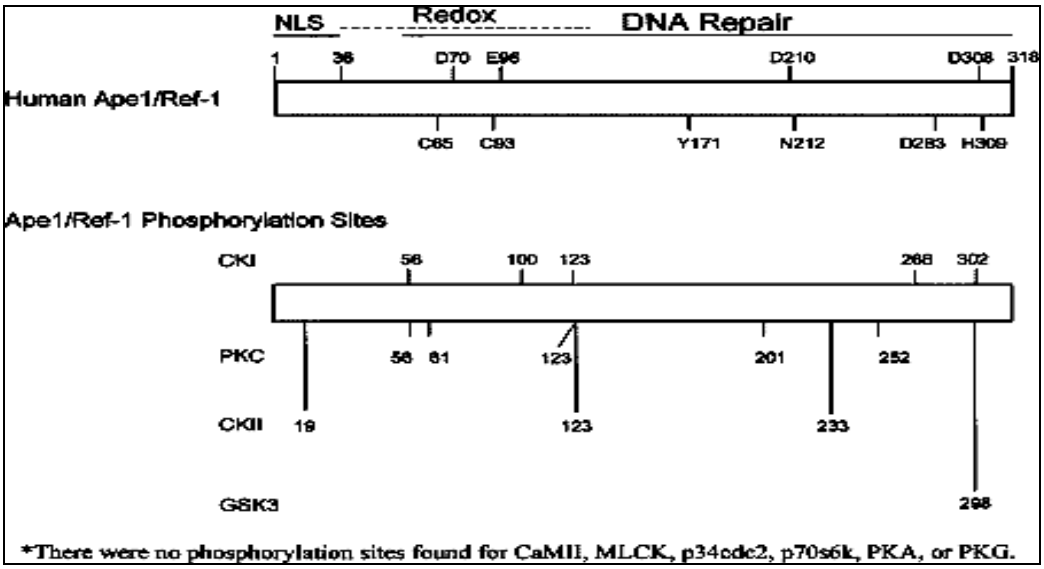


Fig. 1.5. Schematic diagram of important residues and putative phosphorylation sites in Ape1/ref-1. CKI, CKII, and PKC have been shown to phosphorylate Ape1/ref-1 in vitro, however, the other phosphorylation sites are putative. There were no phosphorylation sites found for CaMII, MLCK, p34cdc2, p70s6k, PKA, or PKG. NLS: nuclear localization signal, CKI: casein kinase I, PKC: protein kinase C, CKII: casein kinase II, GSK3: glycogen synthase kinase 3, CaMII: calcium/calmodulin-dependent protein kinase II, MLCK: myosin-light-chain kinase, PKA: protein kinase A and PKG: protein kinase G.

Table 1. APE-1/ref-1 enhances DNA binding of transcription factors.

TF	Name of TF
AP-1	Activator protein 1
CREB	Cyclic AMP response binding protein
ATF	Activating transcription factor
NF-κB	Nuclear factor-κB
Myb	Myb
Pax-5, Pax-8	Paired box containing family of genes
HIF-1α	Hypoxia-inducible factor 1α
HLF	HIF-like factor
PEBP2	Polyoma virus enhancer-binding protein 2
Egr-1	Early growth response-1
NF-Y	Nuclear factor-Y
p53	p53

APE1/ref-1 is a multifunctional protein that is critical to the survival of animals and, presumably, humans (44). It impacts on a wide variety of important cellular functions. As a major member of the BER pathway, APE1/ref-1 acts on AP sites in DNA, which, if left unrepaired, can result in a block to DNA replication, cytotoxicity, mutations, and genetic instability (45). APE1/ref-1 has also been found to stimulate the transcriptional activity of numerous transcription factors that have physiological functions as diverse as cell cycle control, apoptosis, angiogenesis, cellular growth, cellular differentiation, neuronal excitation, hematopoiesis and development. Consequently, APE1/ref-1 is a pivotal signaling factor involved in coordinating the cellular adaptation to a wide array of environmental stimuli. Furthermore, dysfunctional repair/redox activities of Ape1/ref-1 may underlie the pathology of neurodegenerative diseases, including aging. Thus, APE1/ref-1 appears to form a unique link between the DNA BER pathway, cancer, transcription factor regulation, oxidative signaling, and cell-cycle control.

APE1/ref-1 is regulated at both the transcriptional and post-translational level, and it may autoregulate by binding to its own promoter and inhibiting transcription (46). Ape1/ref-1 is ubiquitously expressed in cells, however, it exhibits a complex and heterogeneous staining pattern that differs among tissue types and even between neighboring cells, suggesting that localization of Ape1/ref-1 is highly regulated. Expression levels of Ape1/ref-1 are inversely related to apoptosis in many cell types (47-52). This characteristic may prove to be a valuable marker and predictor of cells undergoing the apoptotic process. Altered levels or cellular location of APE1/ref-1 have also been found in some cancers, including ovarian, cervical, prostate and germ cell tumors (53-56). Hence, APE1/ref-1 expression levels and/or patterns may serve as a diagnostic aid in cancer screening.

Although it is a great deal about this unique protein, many unanswered questions remain, including fundamental questions concerning the regulation of Ape1/ref-1 gene expression to questions concerning the role of APE1/ref-1 plays in cell growth, development and cancer.

The purpose of the present study was to investigate the relationship between APE1 to its GFRα1 and JAG1 expression. Here is observed direct correlation between the levels of APE1 with GFRα1 and JAG1 suggesting a functional link. In this study, APE1-mediated GFRα1 and

JAG1 activation appears to involve the transcription factor NF-kB and Egr-1 respectively. Collectively, this study has unravelled a novel, although indirect, regulatory function of APE1 on GFRα1/JAG1 activation, opening new perspectives in the comprehension of the many functions exerted by this multifunctional protein.

Site-directed mutagenesis of the human DNA repair enzyme APE1

Mutagenesis was performed using a PCR-based technique using site directed mutagenesis kit from Intron Biotech according to the manufacturer's protocol (20). All site specific mutant DNAs were authenticated by DNA sequencing. (NCBI-BC008145)

Primer sets of 65 cysteine to alanine mutations.

Primer F C65A

5'- aaacctgccacactcaagatc**gcc**tcttggaatgtggatgggc -3'

Primer R C65A

5'-gcccatccacattccaaga**ggc**gatcttgagtgtggcaggttt -3'

Primer sets of 93 cysteine to alanine mutations.

Primer F C93A

5'- ggaagaagccccagatatactg**gcc**cttcaagagaccaaatgttcag -3'

Primer R C93A

5'- ctgaacatttggtctcttgaag**ggc**cagtatatctggggcttcttcc -3'

Primer sets of 296 cysteine to alanine mutations.

Primer F C296A

5'- cactctctgttacctgcattg**gct**gacagcaagatccgttccaagg -3'

Primer R C296A

5'- ccttggaacggatcttgctgtc**agc**caatgcaggtaacagagagtg -3'

& AIMS OF THE STUDY

Transcriptional regulation in some of the important eukaryotic genes have been a subject of intensive research for decades but only recently here is started to understand how different signaling cascades regulate activation or repression of transcription factors, and how various pathways cross-talk with each other in the guidance of promoter analysis. The experiments in the present study have elucidated the function and regulatory relationships of two important receptors GFRα1 and JAGGED1 in regards to APE (ref-1) in human fibroblast cell line GM00637.

The overall goal of the research in this thesis was to gain the data that will eventually help in elucidating the trasncriptonal regulatory mechanism of APE 1 or ref-1 induced GFR α 1/JAG1 expression in Human fibroblast Cell Line GM00637.

- Examine the transcriptional activity of the GFRα1 and JAG1 luciferase reporter construct and to function regulation of GFRα1 and JAG1 by APE (ref-1) in human fibroblast GM00637 cells.

- For further identification, focusing to find out the possible regulatory DNA elements which might have role to enhance DNA repair APE in human fibroblast cell GM00637 line with the help of EMSA and CHIP.

- Besides that, also carry out the primer extension and RACE for finding out the transcription initiation site of both of the human GFRα1 and JAGGED1 to findout the typical eukaryotic promoter.

MATERIALS AND METHODS

(1) Reagents

NF-κB p65 (RelA), RelB, c-Rel, p50 and p52 with rabbit IgG and Anti-acetyl Histone H3 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). GFRα1 and JAG1 as well as EGR-1 antibodies were also purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Luciferase assay system with Renilla and b-Galactosidase Enzyme Assay System with reporter Lysis Buffer were bought from the Promega Corporation, Madison, WI, USA. All the EMSA related stuffs were purchased from Pierce Biotechnology. Site Mutagenesis kit was used from the INTRON Biotech Korea. Upstate Biotechnology's Chromatin Immunoprecipitation (ChIP) Assay Kit was used to obesrve in-vivo DNA configuration. All the oligos were synthesized from the Bioneer and Sigma-Aldrich, Korea. Sequencing authenticity was approved from the bioneer and cosmogentech-Seoul, Korea.

(2) Maintenance of Cell Lines

Human Fibroblast GM00637 and it's stable transfected derivatives GM00637-pcDNA3.1 and GM00637-APE cells were grown in a humidified atmosphere containing 5% CO_2 at 37^0C in EMEM supplemented with 50 units/ml penicillin, 50 mg/ml streptomycin and 10% (vol/vol) FBS.

(3) Genomic DNA Extraction

High quality RNA-free genomic DNA was prepared from APE over expressed human fibroblast GM00637 by using DNAZOL (Invitrogen life technologies). In brief, Cells grown in monolayer were lysed directly in a culture dish by adding 0.75 - 1.0 ml of DNAzol per 10 cm^2 culture plate area followed by inversion or repeated pipetting. Following centrifugation, the resulting viscous supernatant was transferred to a fresh tube. DNA was precipitated from the lysate/homogenate by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzol used for the isolation .Then DNA precipitate was washed by twice with 0.8 - 1.0 ml of 75% ethanol. Finally DNA was dissolved (without drying) in 8 mM NaOH by slowly passing the pellet through a pipette.

(4) Cloning and DNA sequence Analysis

Genomic DNA of APE expressing GM00637 cells were amplified by PCR using the GFRα1 and JAG1 oligo Primer using following sets (Table I-1 and II-1) of upstream and downstream sites of different regions.

PCR was carried out under the following conditions: initial denaturation for 3 min at 94^0C followed by 32 cycles of denaturation at 94^0C for 30 s, annealing at 58^0C for 30s, and extension at 72^0C for 1 min.; followed by a single final cycle extension at 72^0C for 10 min. The resulting PCR products were then purified by Gel Extraction kit of iNtRON Bio-Tech, Korea. Then PCR amplified fragments were cloned into a T-Easy vector (3,015 bp, pGEM-T Easy vector) from Promega Corp. which has blue/white selection, also T overhangs for easy of cloning PCR fragments. Transformation of recombinant plasmid into competent cell Of *E. coli* and plating of cell on agar medium with 1000x Ampicillin plus x-gal and IPTG. White colonies growth on ampicilin agar plate inoculated into liquid LB Broth medium with Ampicilin were grown over night at 37^0 C and miniprep DNA prepared from each of these cultures. After DNA extraction, sequence is confirmed by analyzing for comparison to mother sequences at the GenBank database with the NCBI Blast Search analysis.

Then Miniprep DNA digested with either Xho1 and HindIII (NEB lab) or Kpn1 and Xho1 endonuclease since there is a Kpn1, Xho1 and HindIII site on each side of the cloning site so that a fragment of the required size was obtained. This recombinant plasmid having correct size inserts were ligated into PGL3 basic Vector (Promega Incorp.USA).

In the same manner a part of the coding region, the redox and full length size of Homo sapiens APEX nuclease (multifunctional repair enzyme) was amplified by PCR with primers of redox – sense - ggt acc atg ccg aag cgt ggg aaa aa and antisense ctc gag tga aca ttt ggt ctc ttg aag gca c and full length - sense ggt acc atg ccg aag cgt ggg aaa aa and antisense ctc gag tca cag tgc tag gta tag ggt gat agg . Similarly as above, in this case also PCR product were first ligated into T-easy Vector (Promega,USA) and this correct size inserts were ligated into pcDNA3.1Vector (neo) (Promega Incorp.USA).

(5) Site-Directed Mutagenesis

Mutations to NF-κB bidning sites of GFRα1 promoter and EGR-1 DNA binding sites of JAG1 promoter were introduced using the in vitro overlap extension method of PCR mutagenesis (20,81) using kit provided by INTRON biotech, Korea. PCR primers with mutations (TableI-3 and II-3) were synthesized by the Bioneer and Sigma –Aldrich, Korea. PCR amplification was performed using an eppendorf- mastercycler with reaction mixture recommended by user's guide. The mutated clones were initially screened for the presence of an introduced restriction site, and the entire fragment was sequenced to confirm the integrity of each mutant (Bioneer, Korea.).

(6) Transfection

Transient transfections were performed using the FuGENE 6 transfection reagent (Roche, Indianapolis, Ind.) as specified by the manufacturer (57). In brief, FuGENE6 was diluted with serum-free medium (without antibiotics and fungicides) in 3:1 ratio of media and FuGENE6 reagent. Then followed by manufacturer's protocol, DNA was added to dilute FuGENE6 reagent and incubated the mixed complex for a minimum of 15 minutes of room temperature. The complex containing reporter plasmid DNA was added to cells and returned the cells to the incubator until the assay for gene expression was performed. In some cases the Lipofectamine 2000™ transfection reagent from INVITROGEN was also used as specified by the manufacturer (24).

(7) Luciferase Activity Measurement

Human fibroblast Cell Line GM00637 and its stable APE over expressed cells were transiently transfected with the indicated constructs (500ng total) using FuGENE 6 (Roche Applied Science).

35 ng of pRL-CMV (Promega, Madison, WI), which encodes a Renilla luciferase gene was systematically added to the transfection mixture to assess transfection efficiency. When required, controls were carried out by replacing constructs with corresponding empty vectors. After 48 hr treatment, transfected cells were washed once with the phosphate-buffered saline (PBS) and lysed in a 1x lysis buffer (5x PLBR, Promega) with gentle shaking at room temperature for 20 min.The 2 ul of cell lysate of each wells were transferred to a fresh

sterilized e-tube, and the dual luciferase activity in cell extracts was determined according to the manufacturer's protocol (Promega). Briefly, each well mixture contained 5 ul of the cell lysates and 10 µl of a firefly luciferase-measuring buffer (LAR ll R, Promega). The firefly luciferase activity was measured by a luminometer (the luminometer was programmed to perform a 2-s premeasurement delay, followed by a 4-s measurement period for each reporter). After measuring the firefly luciferase activity (Stop & GloR, Promega), a Renilla luciferase-measuring buffer was added, and the Renilla luciferase activity were then measured (25). Each transfection was performed in triplicate, and all were repeated at least three times. .Promoter activities were expressed as relative light units normalized for the protein content in each extract. The luciferase activity of each well was compared with that of the promoter less pGL3 basic vector (Promega).

The observed luciferase activity reported in each figure is expressed as the percentage of the activity obtained with the most active construct used in each figure. The DNAs and cell lines transfected are as indicated in individual experiments.

(8) Total RNA Isolation

RNA was isolated from GM00637 and GM00637-APE cell lines using the TRIzol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's protocol. Briefly, 1 ml TRIzol was added for each 100mm plate cells and cells were lysed 10-15 minutes at room temperature, until the solution was homogenized. Lysate was extracted with chloroform and precipitated with isopropanol. RNA was pelleted, washed in ethanol, and resuspended in DEPC-treated water. The concentration and purity of RNA was determined based on $O.D._{260/280}$ measurements.

(9) Transient Transfection of APE

Transient transfections were performed using the Lipofectamine 2000™ transfection reagent from INVITROGEN as specified by the manufacturer (24). In brief, Lipofectamine 2000™ was diluted with serum-free medium (without antibiotics and fungicides) and was used in 1:3 ratios of plasmid DNA and Lipofectamine 2000™ reagent. Then followed by manufacturer's protocol, APE full containing pcDNA3.1 plasmid construct in dose dependent manner as well as site mutagenesis constructs were added to diluted Lipofectamine 2000™

reagent and incubated the mixed complex for a minimum of 20 minutes of room temperature. The complex containing expression plasmid DNA was added to above mentioned cells and media was substituted after 3 hrs. The cells are then incubated to the incubator until the 48 hour of incubation..

The samples of transfection and optimization for transfection control while using Lipofectamine was developed as cell type specific in our laboratory. The lipofectamine complex was removed after 3-4 hrs of transfection as mentioned above. RNA was extracted after 48 hrs of transfection.

(10) Reverse Transcription (RT)-PCR

Total RNA was extracted from cells after transfection of APE using RNeasy Mini Kit from QUIAGEN according to the manufacturer's instructions. The first-strand cDNAs were synthesized using oligo (dT) by M-MLV reverse transcriptase (Invitrogen). Equal aliquots of the cDNA products were subjected to 18-24 cycles of PCR with GAPDH primer sets. PCRs were run with Top Tag DNA Polymerase of NOVAGEN with 10 pmol of each primer, and 0.2 mM each dNTP in a final volume of 20 µl. The PCR products were then electrophoresed on 1.3% agarose gel.

(11) Western Blotting

The cells were washed with phosphate-buffered saline (PBS) and lysed on ice for 10 minutes in the M-PER mammalian protein Extraction Reagent (PIERCE) added with protease Inhibitor Cocktail tablet (Roch). After incubation, extracts were vortexed for 5min and centrifuged at 13000rpm for 15min. The supernatant was diluted with 5X SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 20ug of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (58-60) (Amersham Biosciences, Piscataway, NJ).

After electro blotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20(TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes were rinsed with TBST and then

incubated with appropriate primary antibodies in TBST at 4℃ overnight. All antibodies used in this study are polyclonal antibody against human GFRα1 and JAG1 was obtained from Santa Cruz Biotech. We followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:4,000) in a blocking buffer for 2 hours at room temperature, and washed again. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

(12) Primer Extension

To find out GFRα1 and JAG1 Promoter transcriptional start sites by primer extension analysis (26), total RNA was purified from APE over expressed GM00637cell lines. Primer extension was performed using the AMV reverse transcriptase primer extension system (Promega, Madison, WI; protocol supplied by manufacturer) using 20-50 μg total RNA from APE over expressed Stable GM00637cell line with antisense primers specific for sequences at the 5' end of exon 1 of human GFRα1.

Oligonucleotide probes were end-labeled with γ -32P-ATP (3000 Ci/mmol) before primer extension using T4 polynucleotide kinase. These probes were used in separate RT reactions and the 32P-labeled extended products were separated on 8% denaturing polyacrylamide gels and visualized by autoradiography.

(13) Nuclear Protein Extraction

Nuclear protein extracts were prepared from Confluent cells (5 ml) of human fibroblast cell line GM00637 which were harvested, pelleted, and washed once in phosphate-buffered saline solution. All nuclear extraction procedures were performed on ice with chilled reagents using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the instructions of the manufacturer. The final supernatant derived from the nuclear pellet was placed in a clean, prechilled tube and stored at – 80°C until electro mobility shift assay (EMSA) was done on the nuclear protein extracts (27).

5′-Biotin-labeled DNA oligos containing the NAFkB of GFR α 1 or EGR-1 of JAG1 were synthesized and the two complementary oligos were annealed to obtain the double-stranded probe (76-80) .

Before doing the EMSA and super shift EMSA, were estimated labeling efficiency by Dot Blot with hand spotting using Light Shift Chemiluminescent EMSA Kit (Pierce) (71-75). The labeled oligos with respect to the control oligos were then transferred to positively charged Nylon membrane (Nitrane) and was Semi-Dried in air and cross-linked in UV cross linker at auto cross-link level for 1 min. The Biotin-labeled DNA probe was performed strictly following the manufacturer's protocol.

(14) EMSA-Binding Reaction

EMSA was used to detect specific binding of the transcription factor GFRα1 and JAG1 (Oligo was designed from NCBI-AC005872 for GFRα1 and, AL035456 for JAG1) to its specific DNA consensus sequence. Nuclear extraction was prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce) as mentioned above. Protein content of nuclear extracts was determined with a protein assay with Bradford solution (Bio-Rad).

Nuclear protein-DNA binding reactions were conducted for 20 min at room temperature in a 20-µl volume containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 1 µg/µl polyIC, 1% Nonidet P-40, (all the reagents are included in the LightShift Chemiluminescent EMSA kit; Pierce), 20 fM biotin-labeled probe of the double-stranded NF-κB of GFRα1 and EGR-1 of JAG1 consensus sequence containing a binding site and 5 µg of nuclear protein. Binding reactions were analyzed using 5% native PAGE. After blotting to a nylon membrane, labeled oligonucleotides were detected with the LightShift Chemiluminescent EMSA kit following the instructions of the manufacturer (Pierce).

The binding specificity was confirmed by adding to the nuclear protein-DNA binding reaction either an excess of unlabeled NF-κB or EGR-1 consensus oligonucleotides respectively.

(15) Detection

Labeling efficiency of standards and different test oligonucleotides complex were detected by using a Light Shift chemiluminescent EMSA kit (Pierce) (61-64). Briefly, serial dilution of

standards and different test oligos were blotted to a nylon membrane. After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin-HRP conjugate for 30 min at room temperature. The membrane was incubated with chemiluminescent substrate for 5 min, and allowed to expose radiographic film. Band intensity of serial dilution of standards and different test oligonucleotide complex was digitized by image using an image scanner (Hp Director).

(16) Chromatin immunoprecipitation assays

ChIP assays were performed as described in manufacturer's protocols with some necessary modifications. Human fibroblast cells GM00627 and its stably over-expressed GM00637-pcDNA3.1 with GM00637-APE were incubated with 1% formaldehyde at 37^0C for 10 min, washed twice in ice cold PBS with 125mM glycine, 1mM EDTA, 1mM PMSF and collected in 1ml of ice-cold PBS. Cell pellets were re-suspended in lysis buffer (50mM Tris–HCl pH 8.0, 1% SDS, 10mM EDTA, 1_ protease inhibitor cocktail) and sonicated to produce DNA fragments of 200–500 bp. Lysates were diluted 10-fold in 20mM Tris– HCl, pH 8.0, 1% Triton X-100, 2mM EDTA, 150mM NaCl, 1_ protease inhibitor cocktail and incubated with antibodies over night at 4^0C. Immune complexes were incubated with sheared salmon sperm DNA for 1 h at 48^0C before the addition of protein G Sepharose beads, pre-blocked with BSA and further incubation for 1 h. Immunoprecipitates were washed with TSE I (20mM Tris–HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl); TSE II (20mM Tris–HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl); Buffer III (10mM Tris–HCl pH 8.0, 1% NP-40, 1mM EDTA, 1% deoxycholate, 0.25M LiCl) and twice with TE buffer. DNA–protein complexes were eluted twice with 1% SDS in 0.1M NaHCO3. Eluates were pooled and cross-links reversed at 658C for 6 h. After proteinase K digestion for 1 h at 458C, DNA fragments were purified with a PCR purification kit (iNtRON, Korea). Primers for the human GFAR1 and JAG1 are tabulated in I-4 and II-4 respectively.

(17) Statistical analysis

Data were expressed as mean ± SD of at least three independent experiments. Statistical differences between means were determined using two-tailed t test when appropriate. A p value < 0.05 was considered significant.

Table I-1, I-2, I-3, I-4 & II-1, II-2, II-3, II-4 Sequence of the oligonucleotides during the study

Table I-1. GFRα1Primer sets for Cloning			
Genes	**Forward primer**	**Reverse primer**	**Accession No.**
GFRα1	GFRα1XhoPF78176	GFRα1HindIII80399	AC005872
	ctc gag att caa act cgg tct ccc cga	aag ctt gga gct cag cat gca gcg at	
GFRα1	GFRα1PF78421	GFRα1HindIIIPR78780	AC005872
	ctcgagtaatttactctcgaaacagccaaaat gga	aag ctt tac acg ggc aca agg gct ctt t	
GFRα1	GFRα1XhoPF78761	GFRα1PRHindIII79140	AC005872
	ctc gag agc cct tgt gcc cgt gta tgt	aag ctt cag gac gat ttc cga gca gag	
GFRα1	GFRα1XhoPF79121	GFRα1HindIIIPR79339	AC005872
	ctc gag tct gct cgg aaa tcg tcc tg	aag ctt acc aac ctg gac tca acc g	
GFRα1	GFRα1PF3Kpn	GFRα1HindIIIPR78780	AC005872
	ggt acc tcg agc tct cga aga tta ccg ca	aag ctt tac acg ggc aca agg gct ctt t	
GFRα1	GFRα1PF5Kpn	GFRα1PRHindIII79140	AC005872
	ggt acc aac cca aca gac acc ccc t	aag ctt cag gac gat ttc cga gca gag	
GFRα1	GFRα1PF78421	GFRα1HindIIIPR79339	AC005872
	ctcgagtaatttactctcgaaacagccaaaat gga	aag ctt acc aac ctg gac tca acc g	

GFRα1	GFRα1XhoPF7876	GFRα1PRXhoPF3	AC005872
	ctc gag agc cct tgt gcc cgt gta tgt	ctc gag ggg ccg agt tgg gcc agg a	
GFRα1	GFRα1XhoPF79121	GFRA1RH 79162	AC005872
	ctc gag tct gct cgg aaa tcg tcc tg	aag ctt agc tcg aag ggc cga gtt	
GFRα1	GFRA1FX 78751	GFRA1RH 79109	AC005872
	ctc gag aag aag aaa gag ccc ttg tgc	aag ctt gga tgc tcg tta ggg atg tt	
GFRα1	GFRA1FX 78882	GFRA1RH 79056	AC005872
	ctc gag aga aca ctg cgt gtc ctg tg	aag ctt gtt ccg agg cca gat ttc tt	
GFRα1	GFRA1FX 78971	GFRA1RH 78992	AC005872
	ctc gag agc tta gga ggg agc tgg ag	aag ctt agc tcc agc tcc ctc cta ag	
GFRα1	GFRA1FKpnI 80065	GFRα1HindIIIPR79339	AC005872
	ggt acc gga aaa cac gca cat gca c	aag ctt acc aac ctg gac tca acc g	
GFRα1	GFRA1FKpnI 80147	GFRα1PRXhoPF3	AC005872
	ggt acc gag ggg aga ggg ttc tgt g	ctc gag ggg ccg agt tgg gcc agg a	

Table I-2. Oligos for EMSA

Genes	**Forward primer**	**Reverse primer**	**Accession No.**
GFRα1	GFR-NF-κB 1F(5’Biotin)	GFR-NF-κB 1R (5’Biotin)	AC005872
	cct cac ccc ggt gtt gga aat tcc	tcc cgc ctt tgg gga att tcc aac acc	

	cca aag gcg gga	ggg gtg agg	
GFRα1	GFR-NF-κB 2F(5'Biotin)	GFR-NF-κB 2R (5'Biotin)	AC005872
	gag ggt tct gtg ggg gga gtc tcc ggc gct ctc cgc	gcg gag agc gcc gga gac tcc ccc cac aga acc ctc	
GFRα1	GFR-NF-κB 3F (5'Biotin)	GFR-NF-κB 3R (5'Biotin)	AC005872
	ctg ctg cca gac ccg gag ttt cct ctt tca ctg gat	atc cag tga aag agg aaa ctc cgg gtc tgg cag cag	

Table I-3. Oligos for Site Directed Mutagenesis

Genes	Forward primer	Reverse primer	Accession No.
GFRα1	Smut 80118 NF-KBF	Smut 80118 NF-KBR	AC005872
	ccc ggt gtt gga aat cgc cca aag gcg gga ac	gtt ccc gcc ttt ggg cga ttt cca aca ccg gg	
GFRα1	SMut 80167 NF-KB F	SMut 80167 NF-KB R	AC005872
	gag agg gtt ctg tgg gta gag tct ccg gcg ctc t	aga gcg ccg gag act cta ccc aca gaa ccc tct c	
GFRα1	SMut 80312 NF-KB F	SMut 80312 NF-KB R	AC005872
	gcc aga ccc gga gtt ggc tct ttc act gga tg	cat cca gtg aaa gag cca act ccg ggt ctg gc	
GFRα1	SM80118aaaaF	SM80118aaaaR	AC005872
	ccc ggt gtt gga aaa aaa cca aag gcg gga ac	gtt ccc gcc ttt ggt ttt ttt cca aca ccg gg	

Table I-4. GFRα1 Primer sets for Chromatin Immunoprecipitation			
Genes	**Forward primer**	**Reverse primer**	**Accession No.**
GFRα1	GFRA1F355	GFRA1R512	AC005872
	ttc ccc ata tga acc agt	tgt act tct tgc aaa tgt cg	
GFRα1	GFRα1PF5Kpn	GFRA1R 79162	AC005872
	ggt acc aac cca aca gac acc ccc t	agctcgaagggccgagtt	
GFRα1	GFRA1F 78751	GFRA1R 79109	AC005872
	aagaagaaagagcccttgtgc	ggatgctcgttagggatgtt	
GFRα1	GFRA1F 78882	GFRA1R 79056	AC005872
	agaacactgcgtgtcctgtg	gttccgaggccagatttctt	
GFRα1	GFRA1F 78971	GFRA1R 78992	AC005872
	agcttaggagggagctggag	agctccagctccctcctaag	
GFRα1	CHIP 3NF-κB F1	CHIP 3NF-κB R1	AC005872
	CAAAGGCGGGAACGGG	ACTCAGCTCCGGGATGGC	
GFRα1	CHIP 3NF-κB F2	CHIP 3NF-κB R2	AC005872
	GGGAGAGGGTTCTGTGGG	CTGGCAGCAGCCACCG	
GFRα1	CHIP 4NF-κB F1	CHIP 4NF-κB R1	AC005872
	AAAGCGGAACCGCCTCCC	AGCTCAGCATGCAGCGATCC	
GFRα1	CHIP 4NF-κB F2	CHIP 4NF-κB R2	AC005872
	TTGGGTCGGACCTGAAC	ACAGCTGTGCTGCTCTGG	

GFRα1	CHIP 80066F12	CHIP 80134R12	AC005872
	gga aaa cac gca cat gca c	ttg ggg aat ttc caa cac c	
GFRα1	CHIP 80057F12	CHIP 80156R12	AC005872
	gcg gcc cct gga aaa c	ctc ccc tcc ccc gtt c	

Table II-1. JAG1Primer sets for Cloning

Genes	**Forward primer**	**Reverse primer**	**Accession No.**
JAG1	XhoJAGPF1	HindIIIJAGPR1	AL035456
	ctc gag aag ttt ttc aaa gtt ccc agc ag	aagctt ccc gcg ccg cgc gcc gcg gg	
JAG1	XhoJAGPF4	HindIIIJAGPR3	AL035456
	ctc gag ccg cgg cgc cta cac ctg	aag ctt cta ata tac tcc gcc gat tgg a	
JAG1	JAGKpnPF	HindIIIJAGPR4	AL035456
	ggt acc tca tga ata tta ata agc gcg cat	aag ctt aaa acc agc cta gct cgc gg	
JAG1	JAGKpnPF4	JAGXhoPR	AL035456
	ggt acc cgc ggc gcc tac acc tg	ctc gag tct aat ata ctc cgc cga ttg ga	
JAG1	JAG1FX 65408	AG1RH 65187	AL035456
	ctcgaggcgcataatcataataataaagaag g	aag ctt gcg tcc cgg ctc taa tat ac	

JAG1	JAG1FX 65600	JAG65471HindIII (PR4)	AL035456
	ctc gag gcg tgc tgg gta gag gtg	aag ctt aaa acc agc cta gct cgc gg	
JAG1	JAG1FX 65761	JAG65579RHindIII	AL035456
	ctc gag ggg gcc ctg gtt ctt cta c	aag ctt gcc acc tct acc cag cac	
JAG1	JAG1 65719FXho1	JAG1PR 65495 HindIII	AL035456
	ctc gag ccc aac ccc tcc aag ttc	aag ctt gcc gca ggt aac aca atg ac	
JAG1	JAG1 65761F Xho1	JAG1P 65939R HindIII	AL035456
	ctc gag ggg gcc ctg gtt ctt cta c	aag ctt cag gtg tag gcg ccg cg	
JAG1	JAG1 65812FXho1		AL035456
	ctc gag gca ctc tcc ctt ccc tcc t	Foorward Primer	
JAG1	JAG1 65889FXho1	JAG65939FXhoI (PF4)	AL035456
	ctc gag cgg ggc gcc cga ggg ggc g	ctc gag ccg cgg cgc cta cac ctg	
JAG1	JAG65684FXhoI	AG1P 66562F	AL035456
	ctc gag ctc ccc agc aac gtg aag	ctt gcc tca agg tgg aaa ac	
JAG1	JAG65839FXhoI	JAG1P 66562 Xho1F	AL035456
	ctc gag gca acg atc cct tcc aag ta	ctc gag ctt gcc tca agg tgg aaa ac	

Table II-2. JAG1 Oligos for EMSA

Genes	Forward primer	Reverse primer	Accession No.

JAG1	EGR-1 Consensus F 5’Biotin	EGR-1 Consensus R 5’Biotin	AL035456
	GGATCCAGCGGGGGCGAG CGCGGGGGCGA	TCGCCCCCGCGCTCGCCCCC GCTGGATCC	
JAG1	EGR-1 BS**I** F 5’Biotin	EGR-1 BS**I** R 5’Biotin	AL035456
	ccc agg gtg agc acg ccc tct cat gaa tat taa t	att aat att cat gag agg gcg tgc tca ccc tgg g	
JAG1	EGR-1 BS**II** F 5’Biotin	EGR-1 BS**II** R 5’Biotin	AL035456
	gag cat ccc gct gcc ccc aac ccc ttc caa gtt c	gaa ctt gga agg ggt tgg ggg cag cgg gat gct c	
JAG1	EGR-1 BS**III** F 5’Biotin	EGR-1 BS**III** R 5’Biotin	AL035456
	gcc cgg ggc gcc cga ggg ggc ggt ccc cgc tgg g	ccc agc ggg gac cgc ccc ctc ggg cgc ccc ggg c	
JAG1	c-RelF(5'Biotin EMSA)	c-RelR(5'Biotin EMSA)	AL035456
	cag gct ttg gga agg gcc ggc gga gat gct	agc atc tcc gcc ggc cct tcc caa agc ctg	
JAG1	JP NF-KB F[5'Biotin]	JP NF-KB R[5'Biotin]	AL035456
	gcc ccc agc ggg gac cgc ccc ctc ggg cgc	gcg ccc gag ggg gcg gtc ccc gct ggg ggc	

Table II-3. Oligos for Site Directed Mutagenesis

Genes	**Forward primer**	**Reverse primer**	**Accession no.**
JAG1	EGR-1 BSI SMTF	EGR-1 BSI SMTR	AL035456
	gcc cag ggt gag cac ttc ctc tca	aat att cat gag agg aag tgc tca ccc	

	tga ata tt	tgg gc	
JAG1	EGR-1 BSII SMTF	EGR-1 BSII SMTR	AL035456
	cct gag cat ccc gct ttc ccc aac ccc ttc ca	tgg aag ggg ttg ggg aaa gcg gga tgc tca gg	
JAG1	EGR-1 BSIII SMTF	EGR-1 BSIII SMTR	AL035456
	ggg gcg ccc gag ggg ttg gtc ccc gct ggg gg	ccc cca gcg ggg acc aac ccc tcg ggc gcc cc	
JAG1	EGR-1 BSII SMTF2	EGR-1 BSII SMTR2	AL035456
	tga gca tcc cgc ttt ccc caa ccc ctt c	gaa ggg gtt ggg gaa agc ggg atg ctca	
JAG1	EGR-1 BSII SMTF3	EGR-1 BSII SMTR3	AL035456
	gcc ctg agc atc ccg ctt tcc cca acc cct tcc aag	ctt gga agg ggt tgg gga aag cgg gat gct cag ggc	
JAG1	JP NF-KB SMTF	JP NF-KB SMTR	AL035456
	gcc cga ggg ggc ggt ttt cgc tgg ggg cct cca	tgg agg ccc cca gcg aaa acc gcc ccc tcg ggc	

Table II-4. JAG1 Primer sets for Chromatin Immunoprecipitation

Genes	**Forward primer**	**Reverse primer**	**Accession no.**
JAG1	BS1 -619F	BSI -406R	AL035456
	cca agt tcc tcc tcg cac ta	gcc gca ggt aac aca atg ac	
JAG1	BSI-604F	BSI-490R	AL035456
	act acc ccc tcc cca gca	gcc acc tct acc cag cac	

JAG1	BSII-750F	BSII-600R	AL035456
	gca acg atc cct tcc aag ta	tag tgc gag gag gaa ctt gg	
JAG1	BSII-669F	BSII-572R	AL035456
	gcc ctg gtt ctt cta cgc	cct ccc ctt cac gtt gct	
JAG1	BSIII-872F	BSIII-731R	AL035456
	gct ccg acc gct cag tca	tac ttg gaa ggg atc gtt gc	
JAG1	BSIII-894F	BSIII-646R	AL035456
	ttc gga agg cgg aag ctg	agg gcg gcg tag aag aac	

GAPDH-175bp	TTCCAGGAGCGAGAT CCC	CACCCATGACGA ACATGGG	BC83511.1

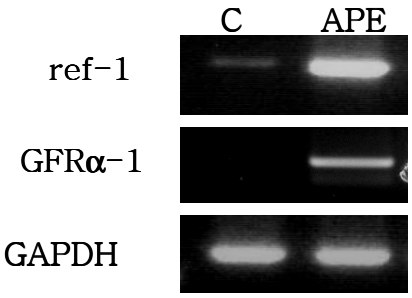
RESULTS

I. Ref-1 Induced GFRα1 Promoter Activity in Human fibroblast cell line GM00637.

I-1) GFRα1 transcript levels were increased in ref-1expressed GM00637 cells.

As shown in following figure, GFRα1 transcript levels were increased significantly in ref-1 expressed GM00637 cells. GFRα1 mRNA levels increased only in ref-1 expressed conditions (Fig.I-1A). We performed western blot analysis to investigate whether the rise of GFRα1 mRNA in ref-1 expressed cells was accompanied by a similar increase in GFRα1 protein. Using a GFRα1 antibody, could be detected in membrane preparations of ref-1 expressed GM00637 cells (Fig. I-1B). Equal protein transfer was assessed by re-probing of the stripped membrane with anti-β-actin antibody.

A]



B]

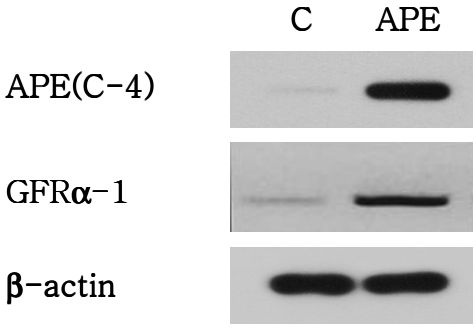


Fig. I-1. Relative transcript levels of the GFRα1 receptor in GM00637 cells. A.Messenger RNA levels were determined by a RT-PCR method and normalized to GAPDH transcripts. **B.** Relative GFRα1 protein content in ref-1 expressed cell lines were identified by western blot using Anti GFRα1 antibody. Note, that GFRα1 expression was stimulated significantly in ref-1 expressed cells in comparison to the C-control cells.

I-2) Isolation of the 5' flanking region of the human GFRα1 gene.

A genomic DNA fragment containing -2291/-7 base pairs compassing the 5' flanking region of the human GFRα1 gene upstream of the translation start codon ATG was isolated by PCR from ref-1 expressing GM00637 cells and inserted into the pGL3-Basic vector. This DNA fragment was shown below to drive the expression of the luciferase reporter gene indicating its promoter property. Sequence analysis revealed that it contains putative binding sites for the following transcription factors which used for EMSA are; NF-κB, HES-1, SP1, FOXO, CREB, NRSE, ZF5, E2F etc. These analyses suggest that this putative promoter region might play a fundamental role in the regulation of ref-1 induced GFRα1 transcription in vivo.

(NCBI AC005872)

-2466 tcctcctctt cttctccccg ccctgacacc cgggcctcaa acttcaacca aagcccgtgc

-2406 ccttttcaat ttacccccct cgatcaaaat gagccattct tgtctgtcct ccgcggcggc

-2346 ccattgtctg gcgtgatagg tttgcagatt tgacagctgg gcgcacgcag atttgattca

HES-1A

-2286 aactcggtct ccccgagaga tgaacttgga catcagcaaa gatcccgagc actgccggct

-2226 ggctcctaga ccggtctccc gacccagtgt agacttcggt gccccgggcg ccccccggcg

-2166 tgcgggaagg ggagcgtgtg tcaggggtgg ggggcggggg gtgagcagca cgactgggaa

-2106 ccagcggtcc caggggttgg ggcgaagggc tgtgtacatg ttaggctttt tttgttgttg

FOXO

-2046 ttaatttact ctcgaaacag ccaaaatgga ggtcagctta taaattttct aaagccaggt

-1986 ctggccgggt ttaaatgcag tgggctgggg gctccgcttt tgtctccttg tcggagctcc

-1926 aggagagagg tagagactgc gtgtgcatgt gtgcgtgtga gtgtgagtgt gcacgcgctg

-1866 gcgtggacga acgggtgggc gcatcccagg atcgccgctc cagcgtcctg ggtctgagtg

-1806 tgtcttgaaa gcaccaagtc ttgaatcaca gccactagtg acgtggggga aaactttgtg

CREB

-1746 ttccgaagaa gggtctggct cgattgccct aagaagaaag agcccttgtg cccgtgtatg

-1686 tgtgtgtgtg tgtctgtgtg tgtgtgtgag agagagagag agagagagac agagagagag

-1626 agagagagag tgtgtgtctg gcagcacccg cggccggctg cagaacactg cgtgtcctgt

-1566 gcgcgcgtac gggtccggcc gcgggtccca ccgcaagcgg gcgtgtgcag ctgcggctgc

HES-1B

-1506 ctggcgggcg agcttaggag ggagctggag ctgcggagcg gcgggaacag gagcaggccg

-1446 agggtcctct ggccagaaga aatctggcct cggaacacgc cattctccgc gccgcttcca

Exon I (79031-79333) NRSE

-1386 ataaccacta acatccctaa cgagcatccg agccgagggc tctgctcgga aatcgtcctg

-1326 gcccaactcg gcccttcgag ctctcgaaga ttaccgcatc tattttttt ttctttttt

-1266 tcttttccta gcgcagataa agtgagcccg gaaagggaag gagggggcgg ggacaccatt

EGR-1

-1206 gccctgaaag aataaataag taaataaaca aactggctcc tcgccgcagc tggacgcggt

-1146 cggttgagtc caggttggtg gcatttggag tttttttcgt tctgtttggg aacccattgc

-1086 cgcttcccgt ccctcttccc cactccttct tccccccctcc tccggccacc cctttctttt

-1026 tgctggcgtc tcccaacttt agggcccggg gggcgctcgt ggcgccgggc tgcacctgcc

HES-1C

-966 ggggcacgcc ggactgcgg gccggcgcgc tcgccctggg ctggggtgcg gaagcgcccg

ZF5

-906 agacctgggc accgggcgag agtgtgagag gagcggacgc ccaggaggga gccgaggacg

-846 cgcggcgggt agggggtagg ggcgcaggaa ccgggtttca gccccagtcg ggacatcggt

-786 tccccctggc ttggcggcct ctggagggag acgagtcccg gggagaagga gggcggcgac

-726 tggggctagg gattggcgat cggagtgggg agggccggcg gctgagcgag ctcgctggct

-666 gacgcgagga gggggaggcc aacttcccgg gagaaggggc tctttcggcg ccagggctgg

E2F

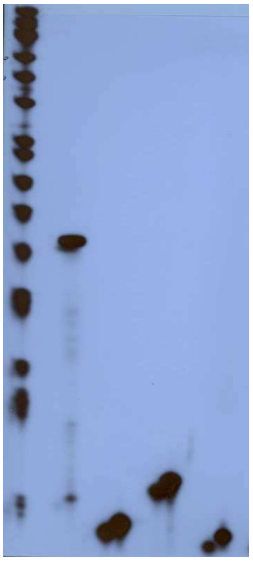
```
-606  ctagggagct gccgccgccg ccaccgaggg ctcggactcc aggcagctgc aggctccgct

-546  cgcgacgctc ggaaagtacc gtttatttat ttatttgctt gcgttcagcg tctcgggttg

-486  aacccaacag acaccccctt ggactttagg aaccctcttc agacctctcc agccctcacc

-426  tccatccccg tgtcagcggc ccctggaaaa cacgcacatg caccccgacc tgggtggggg

-366  tgggtcctca ccccggtgtt ggaaattccc caaaggcggg aacgggggag gggagagggt
                (NF-κB (p65)Binding site I)
-306  tctgtgggg gagtctccgg cgctctccgc tctcatctca aagcgcgccc tccctttcag
      (NF-κB Binding site II)
-246  gttgggtcgg acctgaaccc ctaaaagcgg aaccgcctcc cgccctcgcc atcccggagc
                        GFRα1 PE 80254R
-186  tgagtcgccg gcggcggtgg ctgctgccag acccggagtt tcctctttca ctggatggag
                                (NF-κB (p65)Binding site III)
-186  tgagtcgccg gcggcggtgg ctgctgccag acccggagtt tcctctttca ctggatggag
                                                GFRα1 PE 80340R
-126  ctgaacttttg ggcggccaga gcagcacagc tgtccgggga tcgctgcatg ctgagctccc
            GFRα1 PE 80370R                     GFRα1 PE 80401R
-66   tcggcaagac ccagcggcgg ctcgggattt ttttgggggg gcggggacca gccccgcgcc

-6    ggcaccatgt tcctggcgac cctgtacttc gcgctgccgc tcttgggtaa gtcgaggccc
        [TSS +1]          ExonII(80221-80506)
```

Fig. I-2. Nucleotide sequence of the 5′flanking region of the GFRα1 gene. Nucleotides are numbered corresponding to the translational initiation site at + 1 at the start. On the based of Ensemble data, first and second exon as shown highlighted. Binding sites for the transcription factors NF-κB and others are boxed. Primers which were used for primer extension analysis are boxed in blue letters.

I-3) The transcription start sites of the human GFRα1 gene.

To identify start sites of the GFRα1 transcription, we first carried out a primer extension assay. Total cellular RNA isolated from ref-1 expressing GM00637 cells were incubated with the [γ-32P]-labeled GFRα1 antisense primer in the presence of reverse transcriptase. Following analysis of the reaction product (Fig I-3 lanes 3, 4 and 5) on the sequencing gel, the transcription start sites could not be identified by the sequencing ladders (lane 1) directly parallel to the run-off reverse transcripts. As shown (lane 2), there was a conspicuous extension product of the control RNA with control primer supplied by Promega.

From the promoter activity of GFRα1 luciferase reporter constructs, the promoter construct having DNA elements of upstream of ExonII showed the highest promoter activity. Moreover, deletion construct of those DNA elements showed gradual loss in promoter activity. So, we focussed to those DNA sequences which might direct basal level of transcription. Taking consideration of these data, just we designed to find out the possible transcription initiation site by Primer extension system, but it was failed to find it in this research work. For reference, the DNA sequences and reverse primers are shown in fig I-2.



Where Lane 1st-Hinf DNA Marker
Lane 2nd-Cotrol Primer
Lane 3rd-GFRA1 Primer Extension R79090
Lane 4th-GFRA1 Primer Extension R79107
Lane 5th-GFRA1 Primer Extension R79140

Fig. I-3. Primer extension analysis for GFRα1 transcription start sites. Reverse oligonucleotide as indicated above was end-labeled with [γ-32P] ATP and used in a primer extension experiment with 20 μg of total cellular RNA isolated from ref-1 expressing GM00637 cells. Lanes C, D and E showed no extension products. But lane B showed extended product while using control primer and control RNA as supplied by Promega PE kit.

I-4) Deletion analysis of the GFRα1 promoter.

To characterize the 5' regulatory region of the GFRα1 gene, a set of luciferase reporter gene constructs containing successive 5' deletions of the GFRα1 promoter were prepared (Fig. 4A). After transient transfection into ref-1 expressing cells, the transcription activities derived from these constructs were tested. The background luciferase expression levels were evaluated by transfection of equal amounts of the pGL3-Basic reporter vector without the GFRα1 promoter insert. After having a series of systematic luciferase assays, an important reporter constructs was screened out as shown in fig. 4B. Highest level of the GFRα1 promoter activity was found in the construct Prom-575/-66 reaching higher than of the full-length control (Prom-2291/-7), when the sequence length extended from -2291/to -1327, no significant alterations of luciferase activities were observed. Thus, critical control elements that regulate GFRα1 gene expression may not be present in the region from -2291 to -1327 and it appears that the major role of the fragment between -575 and -66 is transactivation of the ref-1 induced GFRα1 gene.

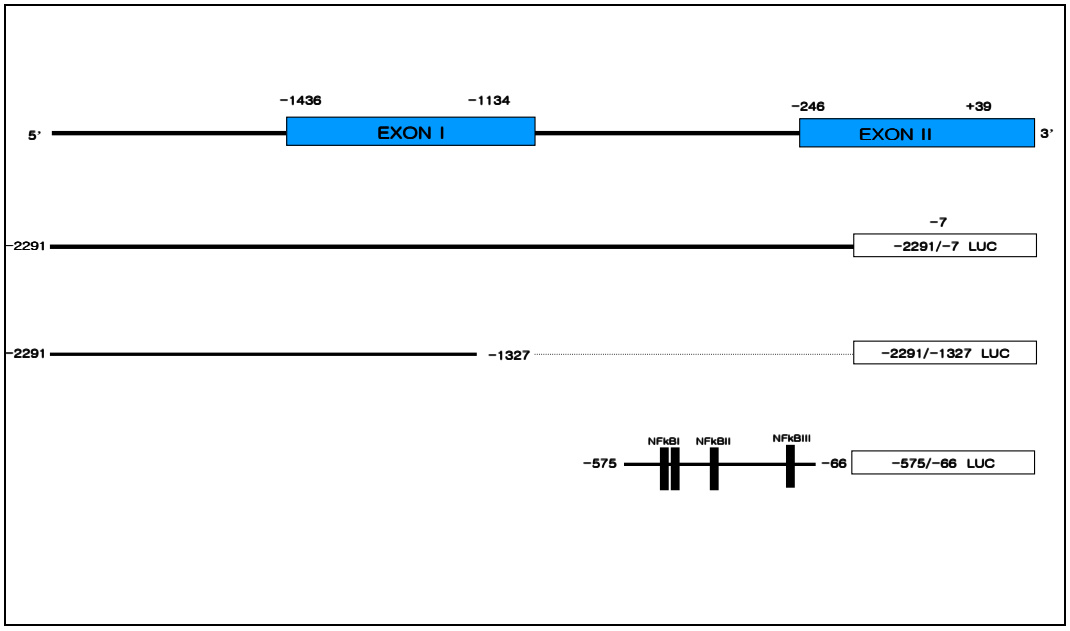


Fig. I-4.A Schematic representation of GFRα1 promoter-reporter chimeras with putative NF-κB binding sites. Scheme of the 5'-upstream region of the human GFRα1 promoter showing major luciferase constructs which were used to identify the promoter activity and distribution of NF-κB binding sites.

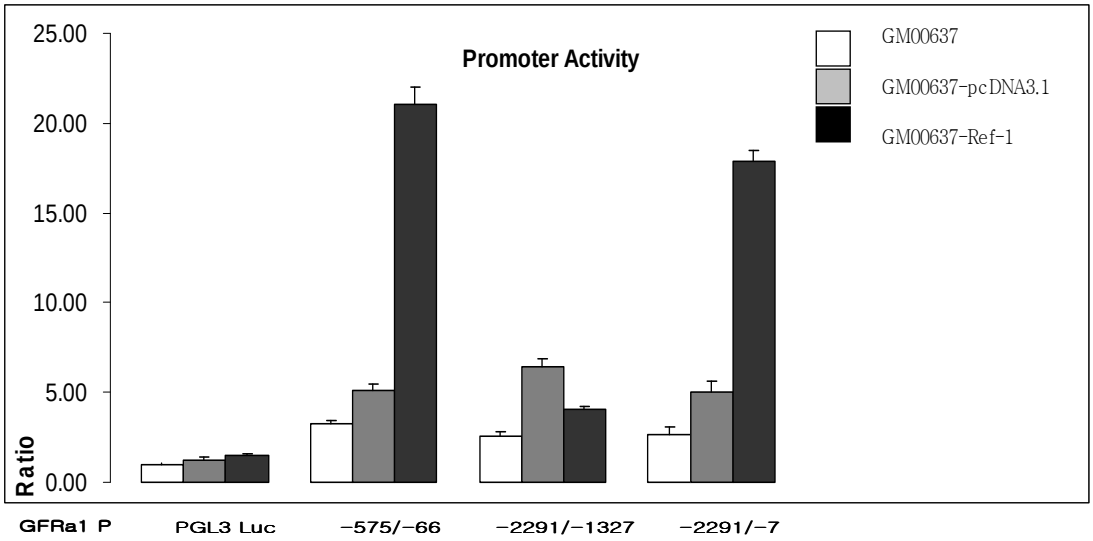


Fig. I-4.B. Relative luciferase activities of GFRα1 promoter-deletion constructs. GFRα1 promoter-deletion constructs were transiently transfected into ref-1 expressing GM00637 cells. Forty eight hour after transfection, cells were harvested for assaying luciferase activities. The plasmid pSV-β-Gal was co-transfected with GFRα1 deletion constructs for data normalization. All values represent the mean ± SD of three experiments, each determined with triplicate dishes. The lengths of the reporter constructs relative to the translation initiation site are indicated as in above schematic diagram Fig.I-4A.

I-5) Roles of the Putative NF-κB DNA Binding sites in the GFRα1 promoter activation.

A computer based analysis (Genomatix-Matinspector) of the GFRα1 promoter sequence (NCBI accession no. AC005872 revealed several putative binding sites for NF-κB transcription factors. These elements were located between -575 bp and -66 bp upstream of the putative transcription start site in the GFRα1 gene. More 5'-deletions of nucleotides from the Prom-575/-66 induced serially reduction of luciferase activities based on the NF-κB DNA binding sites such that the Prom -366/-66, Prom-320/-66 and Prom-287/-66 decreased GFRα1 promoter activity (Fig.I-5A).

Furthermore, deletions of the 3'proximal regions upstream of ATG from the Prom-575/-66 also decreased luciferase activities as shown by those in constructs Prom -575/-66, Prom-575/-300 and Prom-575/-349 (Fig.I-5C). It should be noted that the construct Prom-575/-66 significantly induced the highest expression of the luciferase gene to that of the pGL3-Basic control while the construct Prom—575/-349 or -387/-66 displayed only basal level of luciferase activity in comparison to the pGL3-Basic. Thus, the GFRα1 promoter contains functionally active NF-κB sequences which act as independent basal promoter modules for the ref-1 induced GFRα1 gene transcription.

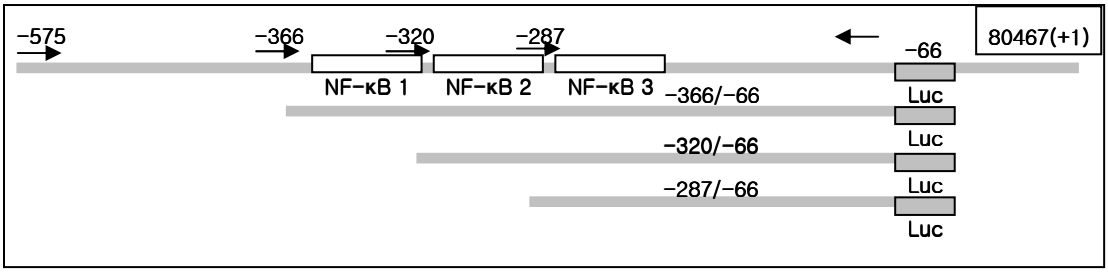


Fig. I-5.A. General overview of 5'deleted luciferase reporter constructs in human GFRα1 gene focusing to purposed NF-κB regulatory region.

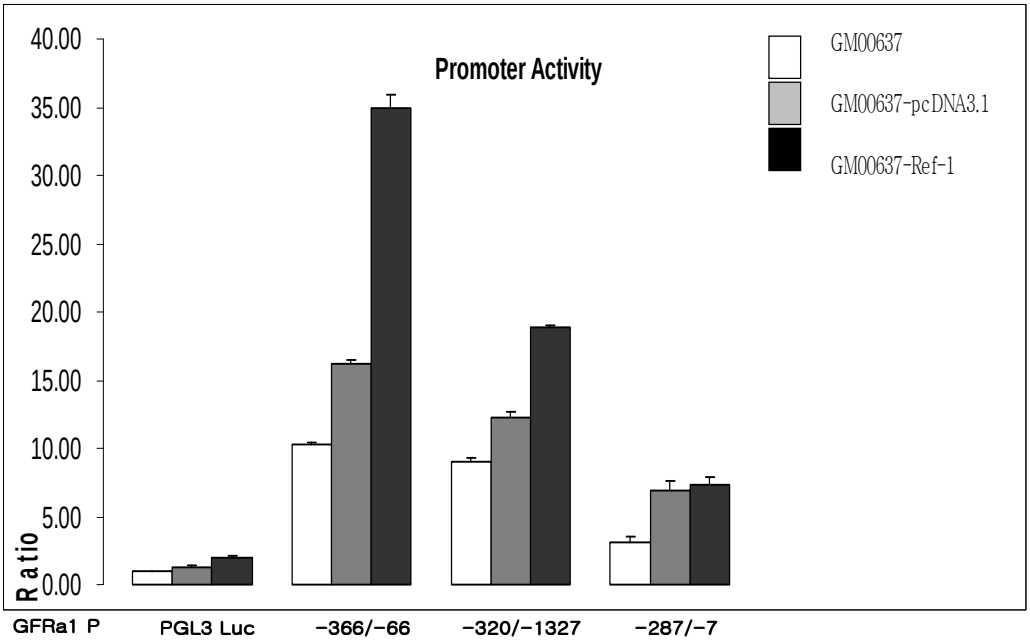


Fig.I-5.B. Relative luciferase activities of NF-κB oriented GFRα1 promoter-reporter constructs. The GFRα1 promoter-reporter constructs each as shown in Fig.I-5.A and the PGL3 luciferase reporter vector, an internal control, were transiently co-transfected into ref-1 expressed GM00637 cells. Forty eight hour after transfection, cells were harvested for assaying the reporter gene expression. Firefly luciferase activities elicited by the GFRα1 promoter with NF-κB binding sites were normalized to Renilla luciferase activities derived from the PGL3 basic vector. Data shown are the mean ± SD of three experiments, each determined with triplicate dishes.

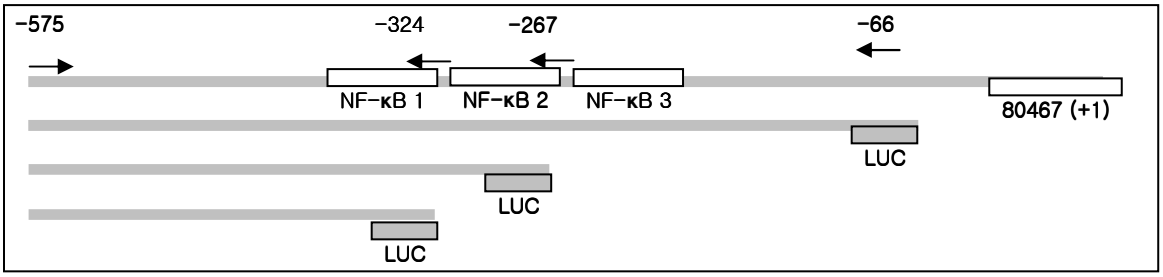


Fig. I-5.C. General overview of 3'deleted luciferase reporter constructs in human GFRα1 gene focusing to purposed NF-κB regulatory region.

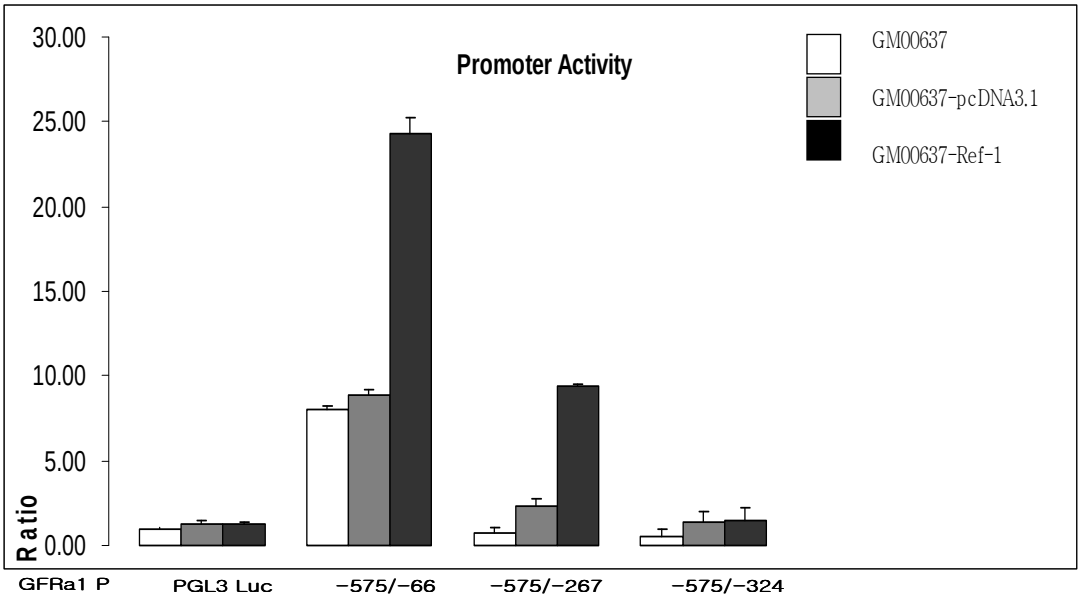


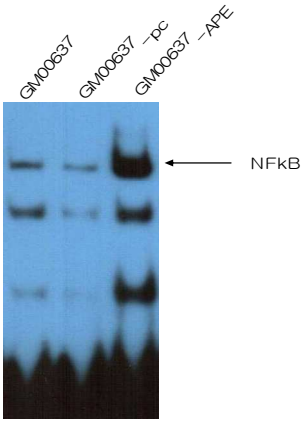
Fig. I-5.D. Relative luciferase activities of 3'deleted NF-κB oriented GFRα1 promoter-reporter constructs. The GFRα1 promoter-reporter constructs each as shown in Fig.I-5.A and the PGL3 luciferase reporter vector, an internal control, were transiently co-transfected into ref-1 expressing GM00637 cells. Forty eight hour after transfection, cells were harvested for assaying the reporter gene expression. Firefly luciferase activities elicited by the GFRα1 promoter with NF-κB binding sites were normalized to Renilla luciferase activities derived from the PGL3 basic vector. Data shown are the mean ± SD of three experiments, each determined with triplicate dishes.

I-6) Identification of NF-κB BS #I binding to the sub-region -575/-66 of the GFRα1 promoter.

To identify transcription factors that bind to the putative *cis*-element -575/-66 as described above, we conducted electrophoretic mobility shift and supershift assays (EMSA) with antibodies against subunits of the NFκB family in mammals: p50, p65 (RelA), c-Rel, p52 and RelB. As shown (Fig. I-6B,C), only the NF-κB-p50 antibody, rather than other antibodies tested, yielded a supershifted band, indicating that the putative *cis*-element in this sub-region is a NF-κB binding site.

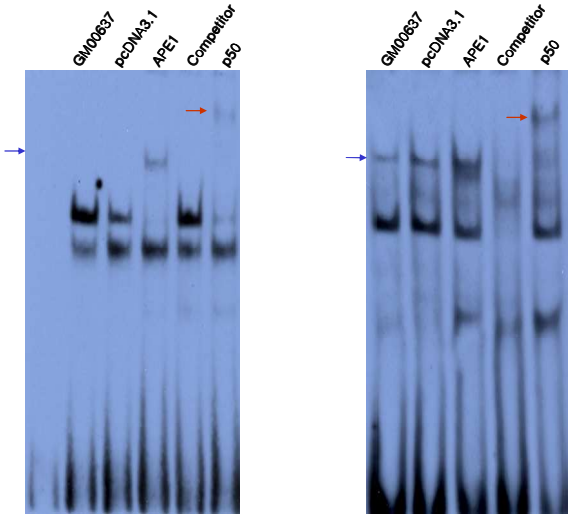
The consensus NF-κB oligonucleotides were used as competitors as in competion assays (Fig. I-6B II lane 4) in the presence of the ref-1 expressing GM00637 cell nuclear extract using 5' Biotin labeled NF-κB DNA binding site-I (NF-κB BS #I) as a probe. As expected (Fig. I-6B lane 4), approximate 100-fold molar excess of cold NF-κB binding site-consensus (NF-κB-BS-Cons) oligonucleotides competed successfully with labeled probes to bind to the NF-κB protein. From this competition as well as supershift assays result strongly support the hypothesis that the NF-κB binding site exists in the -575/-66 fragment of the GFRα1 promoter and the transcription factor, NF-κB, specifically binds to this *cis*-element.

[A]



[B]

(I) **(II)**



NFkB Consensus Probe GFRa1Putative NFkB-1 Probe

[C]

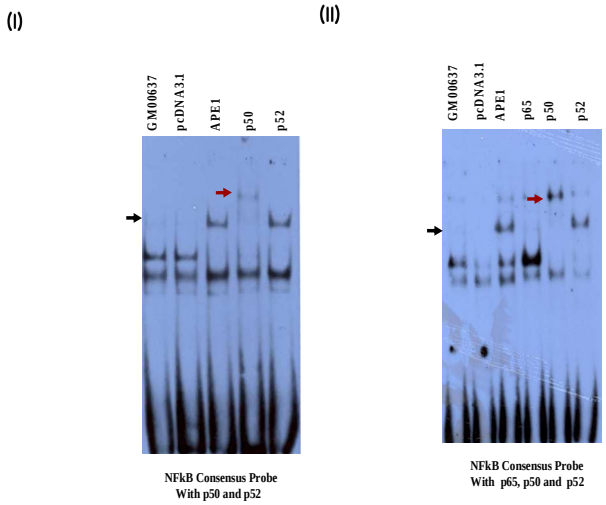


Fig. I-6. Identification of the NF-κB binding site in the sub-region -575/-66 of the GFRα1 promoter. A. NF-κB binding to the sub region -348/-332 of the GFRα1 promoter determined by EMSA assays. 5'-Biotin labeled synthetic GFRα1 promoter oligonucleotide probe -349/-337 containing NF-κB BS #I and 5 μg nuclear extracts (NE) prepared from ref-1 expressing GM00637 cells were incubated in the reaction mixture. After reaction, samples were analyzed on the native 5% polyacrylamide gel followed by autoradiography. **B. NF-κB BS #I binding to the sub region -348/-332 of the GFRα1 promoter determined by EMSA competition and supershift assays**. Experiments were performed as described in **A**, consensus competitors were added into the reaction mixture before addition of the probe (Lane 4) and antibody against NF-κB subunit p50 with NF-κB binding consensus oligo (Fig.I-6B-I) as well as putative NF-κB binding motif (Fig.I-6B-II Lane 6) **C. Electrophoretic mobility supershift assay of oligonucleotide -348/-332 transcription factor complexes in the presence of different antisera**. Fig. I-6C-I Lane 5 and Fig. I-6C-II Lane 4 and 6 show no binding reactions and no supershift with the end-labeled **-349/-337** probe with ref-1 expressing GM00637 nuclear extract (NE) in the presence of antibodies against p52 (C-I lane 5), p65 (C-II lane 4), or p52 (C-II lane 6). Reaction products were analyzed on the native 6% polyacrylamide gel followed by autoradiography.

I-7) Evaluations of transcriptional activities of all putative NF-κB binding sites in the GFRα1 promoter region -575/-66.

Computational analysis showed that there are 3 putative NF-κB binding sites located at -349/-337, -300/-288 and -155/-143 in the GFRα1 promoter region from -576to -66 which yielded the maximal reporter gene expression (Fig. 4B). To evaluate roles of the NF-κB in the GFRα1 gene activation, we introduced site-directed mutations at the NF-κB DNA binding site in the GFRα1 promoter-reporter gene construct spanning from -575 to -66 (Fig. I-7). GFRα1 promoter activities were tested by transfection assays in ref-1 expressing cells.

Mutations were introduced of the NF-κB DNA binding sites #I SM 80118 NF-KB 5'ccc ggt gtt gga aat **cg**c cca aag gcg gga ac 3', NF-κB DNA binding sites #II SM 80167 NF-KB 5' gag agg gtt ctg tgg g**ta** gag tct ccg gcg ctc t 3' and NF-κB DNA binding sites #III SM 80312 NF-KB 5'gcc aga ccc gga gtt **gg**c tct ttc act gga tg 3'(mutations are bold and labeled with underlines). As shown in fig. I-7, mutations in NF-κB DNA binding site #I significantly reduced the luciferase gene expression to the wild type control, respectively, whereas NF-κB DNA binding site #II and #III mutations didn't affect the promoter activity to that of wild type control. Thus, the GFRα1 promoter contains functionally an active NF-κB sequences which acts as independent basal promoter modules for the ref-1 induced GFRα1 gene transcription. From the mutation analysis, the NF-κB binding site (-341/-337) containing GFRα1 promoter fragment (-575/-66) was demonstrated to enhance the SV40 promoter activity (Fig. 7A).

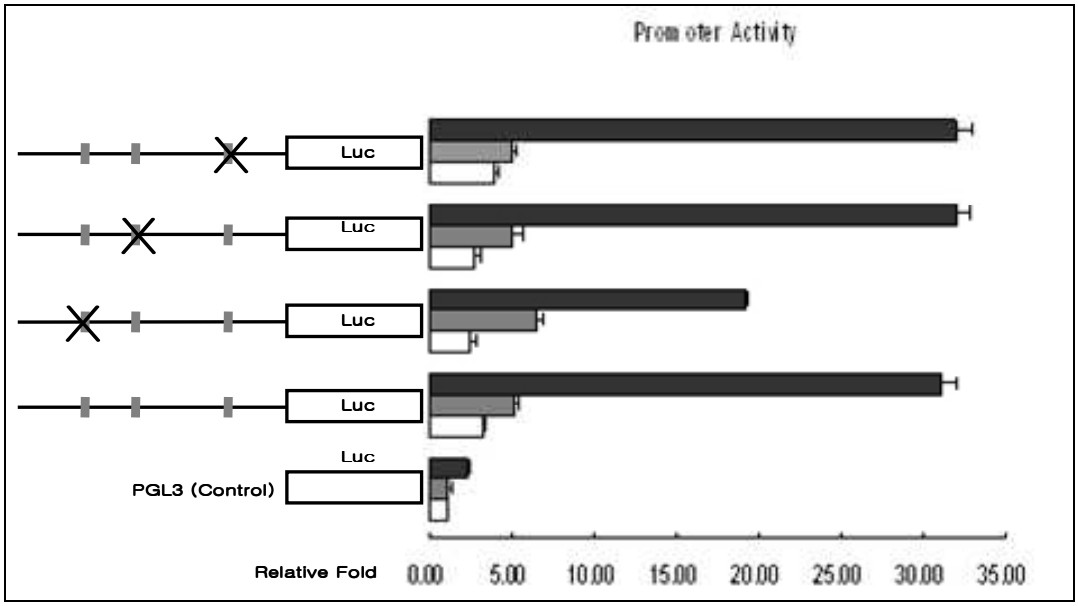


Fig. I-7. Effects of NF-κB-BS-WT and its mutants on the SV40 promoter-directed reporter gene expression. The NF-κB-BS-WT and various mutants as shown in schematic drawing in the left were inserted upstream of the SV40 promoter in the pGL-3 basic reporter vector. Resulting chimeric constructs were co-tranfected with the plasmid pSV-β-Gal into ref-1 expressing GM00637 cells. After a 48 hour post-transfection, luciferase and β-Gal activities in cell lysates were determined. NF-κB-BS-WT or mutant modified SV40 promoter-directed luciferase activities were normalized to the transfection efficiency. All values represent the mean ± SD of three experiments, each determined with triplicate dishes.

I-8) Determination of functional NF-κB-BS #I-binding sites in the GFRα1 promoter region –575/-66. To examine whether other putative NF-κB binding sites are also capable of incorporation with NF-κB BS #I and regulation of the GFRα1 gene expression, we carried out electrophoretic mobility shift and supershift assays with synthetic oligonucleotides spanning GFRα1 promoter sequences oligos GFR-NF-κB BS #1F 5'Biotin cct cac ccc ggt gtt gga aat tcc cca aag gcg gga, GFR-NF-κB BSIIF 5'Biotin gag ggt tct gtg ggg gga gtc tcc ggc gct ctc cgc and GFR-NF-κB BSIIIF 5'Biotin ctg ctg cca gac ccg gag ttt cct ctt tca ctg gat as shown in fig. I-8. After incubation with nuclear extracts from ref-1 expressing GM00637 cells, labeled oligonucleotides induced mobility shift bands in the absence of and supershift bands in the presence of the antibody against NF-κB BS #I in gel electrophoresis (Fig. I-8) but not in the BS #II and BS #III oligo. Thus radio-labeled synthetic GFRα1 promoter fragments containing putative NF-κB BS #II of -300/-288 and NF-κB BS #III of -155/-143 had not any role to make DNA –protein complex as shown in following figure. These results indicated that the only NF-κB BS #I is functional.

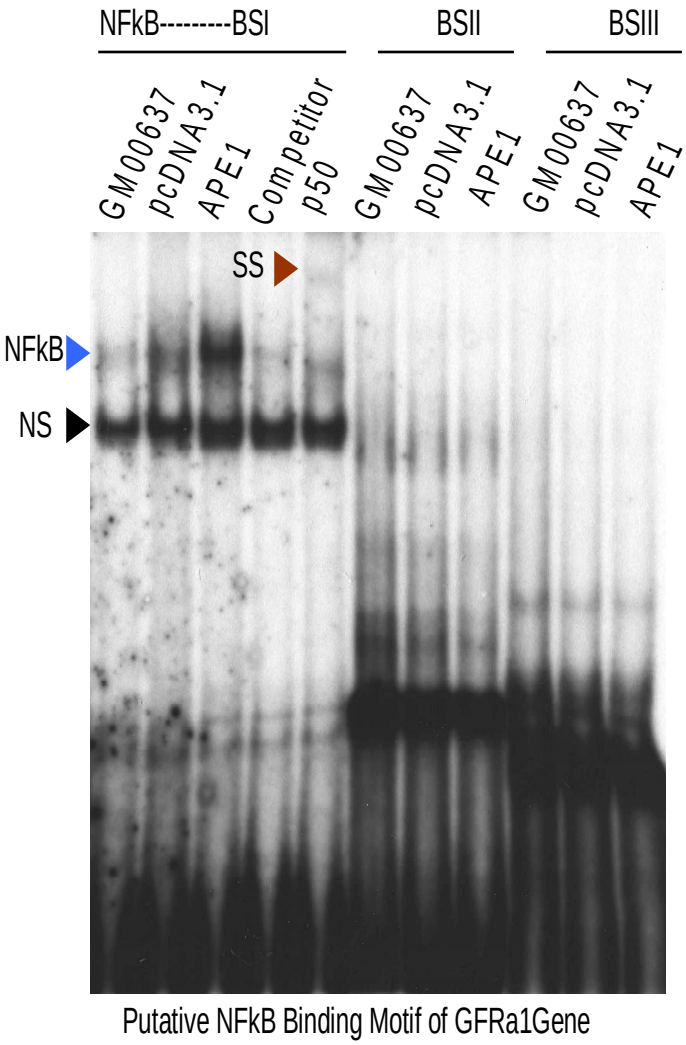


Fig. I-8. Determination of functional NF-κB-binding sites in the GFRα1 promoter region -575/-66. [32P]-labeled synthetic GFRα1 promoter fragments containing putative NF-κB BS#I binding sites as shown in fig. I-8 and 5 μg nuclear extracts (NE) prepared from ref-1 expressed GM00637 cells or control cells, an internal control, were incubated in the reaction mixture in the absence or presence of the specific antibody against NF-κB. After reaction, samples were analyzed on the native 6% polyacrylamide gel followed by autoradiography. Reaction products are shown in lanes 1-5 with the probe NF-κB-BS #1, lanes 6-8 with the probe NF-κB-BS #2 and lanes 9-11 with the probe NF-κB-BS #3 .

I-9) Cellular NF-κB binding to the cognate *cis*-element in the GFRα1 promoter.

To determine cellular NF-κB binding to the GFRα1 promoter region, the ChIP assay was carried out using an antibody against NF-κB-p50 and NF-κB p-65. As demonstrated, there were at least one functionally active NF-κB binding site among three NF-κB located at the regions in the GFRα1 promoter region -575/-66 that displayed the maximal promoter activity (Fig. I-4B). Thus, this active NF-κB-binding site was tested for their cellular protein binding by using specific primer pairs of table I-4. According to the designed primer pairs, we anticipated that GFRα1 promoter fragments -575/-66 which contain active NF-κB-binding sites #1, would result following immunoprecipitation with the NF-κB-p50 antibody and amplification by PCR. Indeed, gel analysis of PCR products confirmed our expectation. Approximately, a ~100 bp and a 150 bp DNA bands were observed on gels as the PCR amplified using primer pairs that encompass NF-κB binding site #1 (Fig. I-9B). In contrast, no signal was detected in control experiments with a nonspecific antibody (Fig. I-9B). These results suggest that the assay conditions were appropriate and can be used to measure the relative levels of NF-κB binding to the GFRα1 gene. As noted, the same cell number (2 x 106) for each group was used for DNA extraction, and there was no significant difference in the yields of PCR products among groups using “input” (before immunoprecipitation) DNA as a template (Fig.I-9A). These results reflect the status of cellular NF-κB binding to the GFRα1 gene which is highly sensitive to ref-1 stimulated endogenous expression.

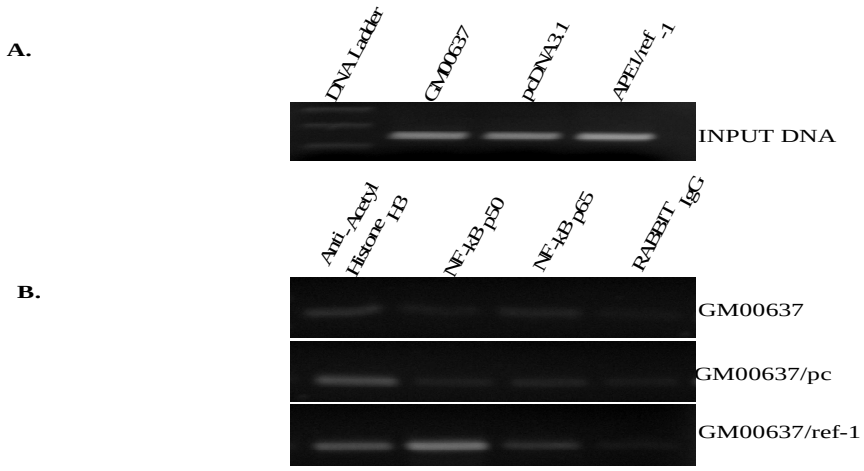


Fig.I-9. Determination of cellular NF-κB binding to the GFRα1 gene promoter by the ChIP assay. GM00637,GM00637-pcDNA3.1 and GM00637-ref-1 cells were incubated from 2 x 106 cells for each group, and immunoprecipitated with an antibody against all NF-κB-p50 and NF-κB-p65, Anti acetyla Histone H3, a positive control or nonspecific rat IgG, a negative control, Using unimmunoprecipitated (input) DNA as a template, the PCR with primer pairs leading to NF-κB BS #I as shown in table I-4 amplified GFRα1 gene fragments -349/-322 region of -575/-66 putative promter constructs. PCR with "input" DNA was performed to monitor amplification efficiency of individual primer pair along the GFRα1 gene. PCR products were analyzed on 2.2% agarose gels.

I-10] Precise mechanism of the ref-1 regulation to the functionalities of the GFRα1 promoter.

To evaluate the functional importance of the important amino acids of ref-1 to the transcriptional activity of GFRα1, we made the various APE1 mutants as described above into the mammalian expression TOPO- pcDNA3.1 (neo) vector. Transient co-transfections were carried out by using this various ref-1 mutants with the NF-κB binding site containing luciferase reporter constructs -575/-66 in GM00637 cells. After 48 hr of transfection, cells were harvested and measured the trasncriptional activity by using b-galactosidase assay syatem. As shown (Fig. I-10), cells transfected with the wild-type ref-1 expression plasmid (TOPO-pcDNA3.1 APE Full Length) and with GFRα1 promoter fragments -575/-66 which contain active NF-κB-binding sites as well as redox or c-terminus part of the ref-1 expression plasmid (TOPO-pcDNA3.1-APEF1/R300) exhibited higher levels of luciferase activities of the pGL3-Promoter control. However, ref-1 repair part or N-terminal region containing expression vector (TOPO-pcDNA3.1-APE Repair) showed significantly reduced transcriptional activity.

A point mutation was introduced in the first half of the redox part containing conserved c-terminus site which is considered as a residue thought to be important or redox activity [138] in all mouse, rat, and human. Therefore an active cysteine of position 65 of redox part was changed to alanine (a cysteine t position 65) [147] which results significant decrease of

luciferase activities in comparison to the empty vector as a control while a point mutation in both halves and others show not a significant reduction. Thus, the NF-κB interaction with its binding site in the GFRα1 promoter region is a critical molecular target for ref-1 induction to its redox part.

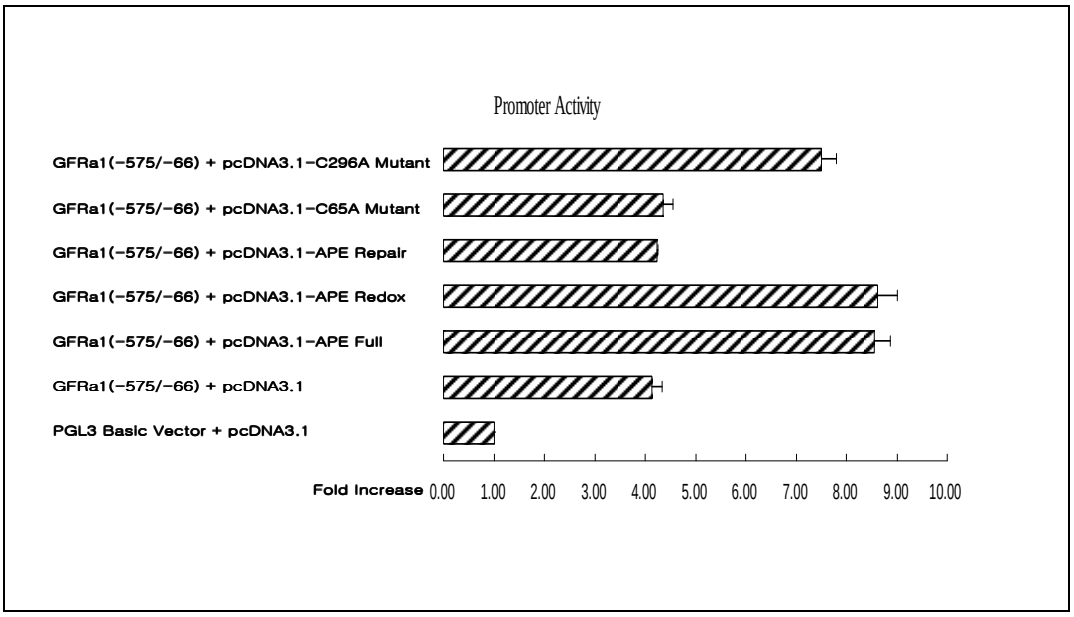


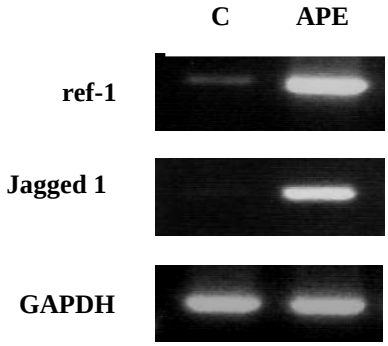
Fig. I-10. Ref-1 regulation to the functionalities of the GFRα1 promoter. Ref-1regulated GFRα1 transcriptional activity was determined by using various ref-1 expression constructs with site directed mutations and NF-κB GFRα1 Promoter-reporter constructs (-575/-66) were transiently co-transfected in human fibroblast GM00637 cells and 48 hr after transfection, promoter activities were measured by using b-Galactosidase assay system. Various constructs with its mutations are named in the left.

II. Ref-1 Induced JAG1 Promoter Activity in Human fibroblast cell line GM00637.

II-1) Ref-1 Induces JAG1 Expression in human fibroblast GM00637 Cells.

To determine whether ref-1 mediated JAG1 expression was associated with an increase in JAG1-specific mRNA expression, total RNA from ref-1 expressing GM00637 cells were isolated and examined by RT-PCR. As shown in Fig. II-1A, JAG1 mRNA was increased significantly in ref-1 expressing GM00637 cells. To investigate whether ref-1 induces JAG1 protein accumulation in GM00637, JAG1 protein level was determined by using ref-1 expressing GM00637 cells. As shown in Fig. II-1B, ref-1 expressed cells significantly enhanced JAG1 protein levels. However parental cells had no effect on JAG1 expression in response to ref-1(Fig. II-1B). Equal protein transfer was assessed by re-probing of the stripped membrane with anti-β-actin antibody (Fig. II-1B). These results confirm that human JAG1 expression is induced by ref-1 at a transcriptional level in GM00637-ref-1 cells.

A)



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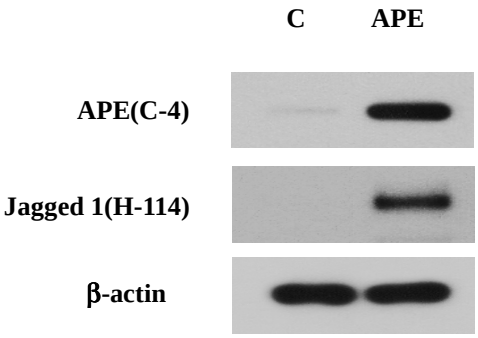


Fig. II-1. Relative transcript levels of the JAG1 receptor in GM00637 cells. A. Messenger RNA levels were determined by a RT-PCR method and normalized to GAPDH transcripts. **B.** Relative JAG1 protein content in ref-1 expressed cell lines were identified by western blot using anti JAG1 antibody. Note, that JAG1 expression was stimulated significantly in ref-1 expressing cells.

II-2) Isolation of the 5' flanking region of the human JAG1 gene.

A genomic DNA fragment containing -1473/-98 base pairs compassing the 5’ flanking region of the human JAG1gene upstream of the translation start codon ATG was isolated by PCR from ref-1 expressing GM00637 cells and inserted into the pGL3-Basic vector. This DNA fragment was shown below to drive the expression of the luciferase reporter gene indicating its promoter property. Sequence analysis revealed that it contains putative binding sites for the following transcription factors which used for EMSA are, EGR-1,EGR-2,3,NF-κB, HES-1, SP1, FOXO, CREB, NRSE, ZF5, E2F etc. These analyses were helpful to find out the regulatory region which might play a fundamental role in the regulation of JAG1 transcription in vivo.

+49 ggaggcttag ggggcgcccg gaccggccgc gcgtccgtgg ggaacgcatc gctgcgccgc

Translation Initiation Site +1

-12 gcgccgcggg cactcgggac gccgccgctg ctgttcgcgc tggtgctgcc gccggtgctg

-72 ccgtcgccgc tgcccctgcg gccgccgcgt cccggctcta atatactccg ccgattggag

-132 catgcacgac tggaaaacaa caccactttt caaaagccct ttcaagagcg gcccgttcca

-192 gaaggcaaag agcccggcct ccttttatta ttctgatcgc ttctttgaga cgctccccct

-252 cctcttccac ctcccggctt tctttccttc tctcgcgctc cccttctttt attattatga

-312 ttatgcgcag ccttttattc ccttttagat cagctgcatg gaaaaagggg ggagggaggg

-372 gagaaaaaaa aaaaccagcc tagctcgcgg gccggccgca ggtaacacaa tgacgcgtgc

c-Krox-1 JAG PE65468R JAG PE65499R

-432 ccgcccggct ctcggagaag gacccggaga gcccgtctgg cagcagcggc cggggctggc

HES-1

-492 cacctctacc cagcacgccg ggcagggcgc atgcgcgctt attaatattc atgagagggc

(EGR-1 BSI)

-552 gtgctcaccc tgggcacgcc cctccccttc acgttgctgg ggagggggta gtgcgaggag

-612 gaacttggaa ggggttgggg gcagcgggat gctcagggcg gcgtagaaga accagggccc

(EGR-1 BSII)

-672 cacaccaacc cccgccctcg ggagcttcgg gccaggaggg aagggagagt gcggggaggt

-732 acttggaagg gatcgttgct cagggacgcc tggaggcccc cagcgggggac cgcccccctcg

(EGR-1 BSIII)

-792 ggcgccccgg gccgtgcgcc tttgcccgcg cgtcggggcc ccaggtgtag gcgccgcgg

-852 gctgactgag cggtcggagc ggggcagctt ccgccttccg aaccgcctgc gtcagctgcg

```
-912  gctttcgccc cgggaggagg gcctgcctgc ccgccgggag ttcggcccgc ttcccgcgag

-972  cgagccgccc agagcgctct gctggcggca gaggcggcgg cgaggctggc gcgcttgccg

-1032 ccgtctgctc gccccgcgga ggcgacctgg gcagacgctg ctgggaactt tgaaaaactt

-1092 tcctggagcc aggcttgccg cagattcgag gggaagcctc ggccgcgtcc cacccccctcc
-1152 caaatccgag tctgcggagc ctgggagggc tcccagcttc ctatccaaac cgcgccgggg

-1212 cagaggcgcg gggaaaccgg gggtagaagg cggcgggcac gccggggccc tgttcaggct

-1272 ttgggaaggg ccggcggaga tgctggtggg cttggacgcc ctccccggcc cgacgccccg
       c-Rel Binding site
-1332 gccgcagtgc cgggattgca cctgtaggcg gcctccgagc agctctctgg gtggcaagag

-1392 atgggcctgg gagggaccgt gtccccagca ccgccagccg cggaacttgc cttctctggg

-1452 gtggcagtgg aaaccgatac tgttttccac cttgaggcaa gcctcgcaat taacagctac
```

Fig. II-2. Nucleotide sequence of the 5′flanking region of the JAG1 gene. Nucleotides are numbered corresponding to the translational initiation site at + 1 at the start. Binding sites for the transcription factors EGR-1 and others are boxed. Primers which were used for primer extension analysis are boxed .

I-3) The transcription start sites of the JAG1 gene.

Though it was failed to find out the start sites of the JGA1 transcription, a primer extension assay was carried out. Total cellular RNA isolated from GM00637-ref-1 over expressed cells was incubated with the [γ-32P]-labeled JAG1 antisense primer in the presence of reverse transcriptase. Following analysis of the reaction product (lanes C, D and E) on the sequencing gel, the transcription start sites could not be identified by the sequencing ladders (lane 1) directly parallel to the run-off reverse transcripts. As shown (lane 2), there was a conspicuous extension product of the control RNA with control primer supplied by Promega.

Fig. II.3. Primer extension analysis for JAG1transcription start sites.

We were unable to find out the TSS of the JAG1 gene. Oligonucleotide Reverse as indicated above was end-labeled with [γ-32P] ATP and used in a primer extension experiment with 20 μg of total cellular RNA isolated from GM00637-ref-1cells. Lanes F and G showed no extension products. But lane B showed extended product while using control primer and control RNA as supplied by Promega PE kit.

II-4) Deletion analysis of the JAG1 promoter.

To characterize the 5' regulatory region of the JAG1 gene, we further prepared a set of luciferase reporter gene constructs containing successive 5' deletions of the JAG1 promoter (Fig.II-4A). After transient transfection into ref-1 expressing GM00637cells, the transcription activities derived from these constructs were tested. The background luciferase expression levels were evaluated by transfection of equal amounts of the pGL3-Basic reporter vector without the JAG1 promoter insert. A series of systematic luciferase assays as shown in Fig.II-4B indicated that a high level of the JAG1 promoter activity was found in the construct Prom (-850/-382) than the full-length control (Prom—1473/-98). When the sequence length extended from -850 to -1473, no significant alterations of luciferase activities were observed. Thus, critical control elements that regulate JAG1 gene expression may not be present in the region from -1473 to -850. More 5'-deletions of nucleotides from the Prom-850/-98 induced reduction of luciferase activities such that the Prom-672, -98, -546/-98, -511/-98, and -319/-98 decreased JAG1 promoter activities in comparison with the full-length control, respectively. Note that there was one exception, i.e., the Prom-850/-382, which showed highest promoter activity relative to that of the other promoter constructs.

Thus, it appears that the major role of the fragment between -850 and -382 is transactivation of the GFRα1 gene. Taken together, these results suggest that there should be some regulatory region in between sequence from -850 to -382 which are essential for ref-1 inducedd JAG1 promoter functions.

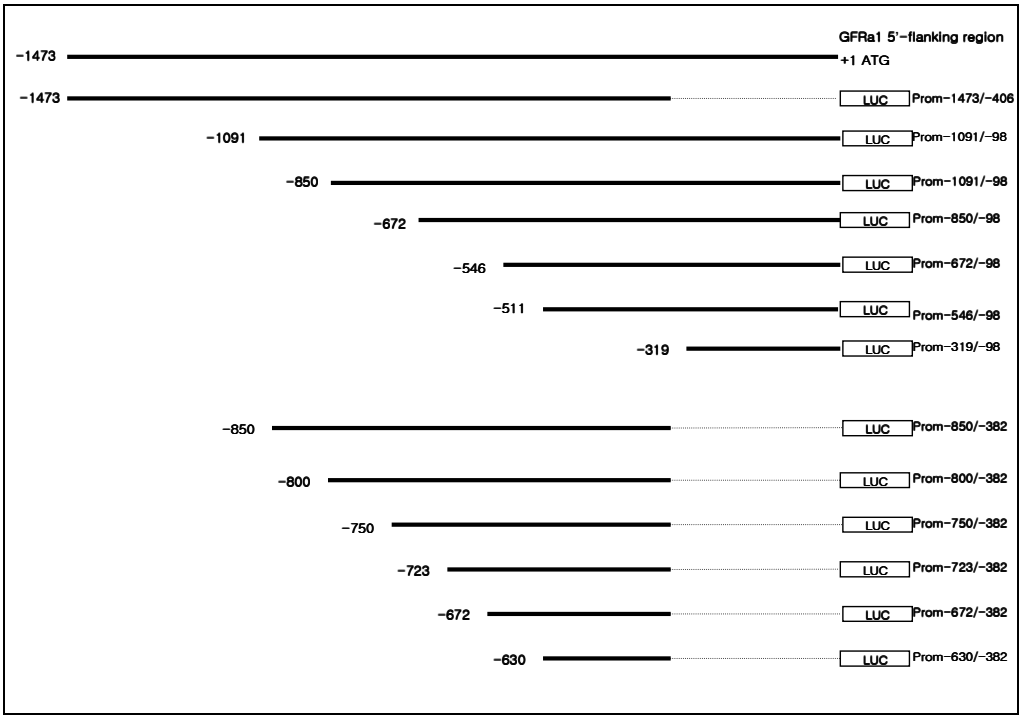


Fig. II-4.A. Schematic representation of JAG1 promoter-deletion constructs.

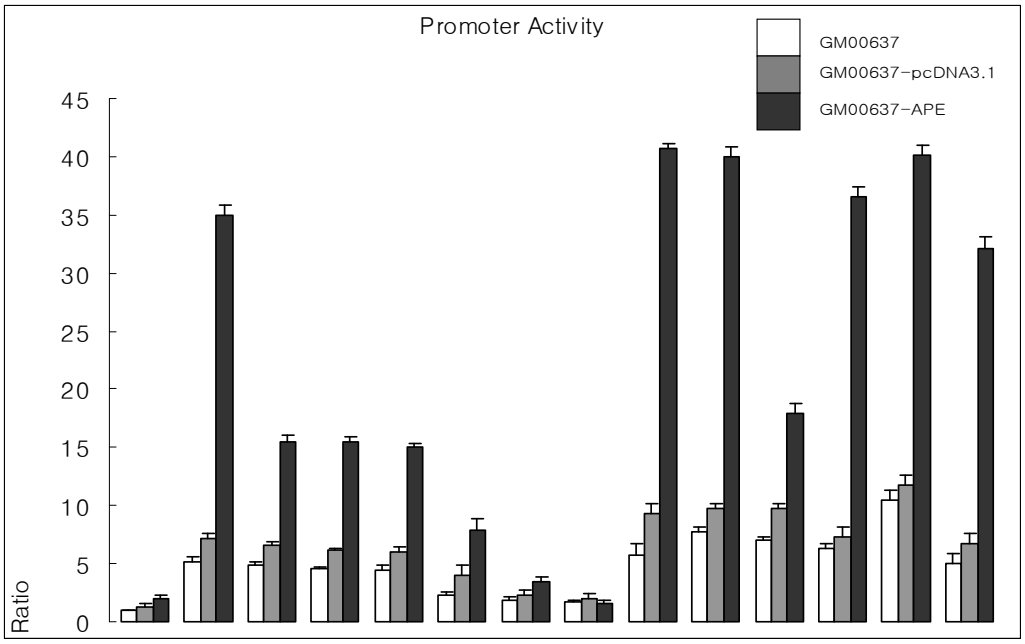


Fig. II-4.B. Luciferase activities in ref-1 expressing GM00637 cells transfected with JAG1 promoter-deletion constructs. JAG1 promoter-deletion constructs were transiently transfected into ref-1 expressed GM00637 cells. 48 h after transfection, cells were harvested for assaying luciferase activities. The plasmid pSV-β-Gal was co-transfected with JAG1 deletion constructs for data normalization. All values represent the mean ± SD of three experiments, each determined with triplicate dishes.

II-5) Roles of the putative EGR-1 in the JAG1 promoter activation.

A computer based analysis (Genomatix-Matinspector) of the JAG1 promoter sequence (NCBI accession no. AL035456) revealed several putative binding sites for EGR-1 transcription factors. These elements were located between -850 bp and -382 bp upstream of the putative translation start site in the JAG1 gene. Interestingly, deletions of the 3'proximal regions (-490/-382) DNA element upstream of ATG from the Prom -850/-490 also totally decreased luciferase activities as shown by Prom -850/-382. (Fig.II-5B). It should be noted that the construct Prom-850/-382 significantly induced the highest expression of the luciferase gene to that of the pGL3-Basic control while the construct Prom 490/-382 or -850/-490 displayed only basal level of luciferase activity in comparison to the pGL3-Basic. Thus, it appears that the major role of the fragment between -850 and -382 is transactivation of the ref-1 induced JAG1 gene.

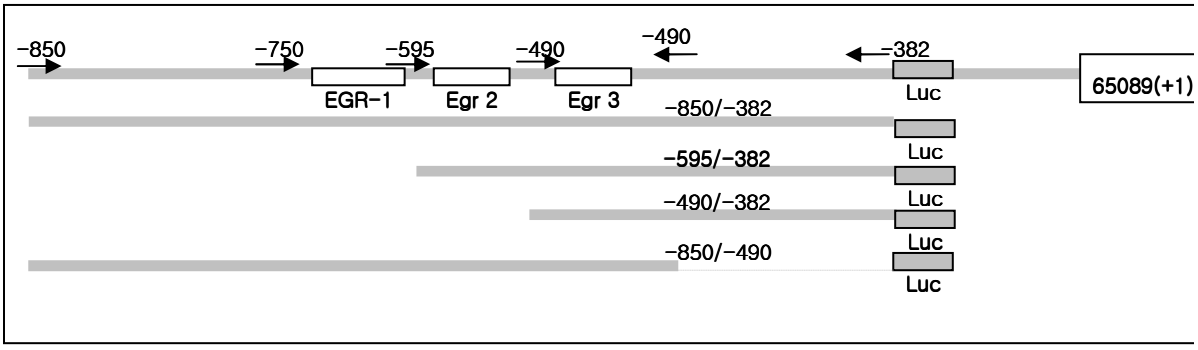


Fig. II-5.A. General overview of 5'deleted luciferase reporter constructs in human JAG1 gene focusing to purposed EGR-1 regulatory region.

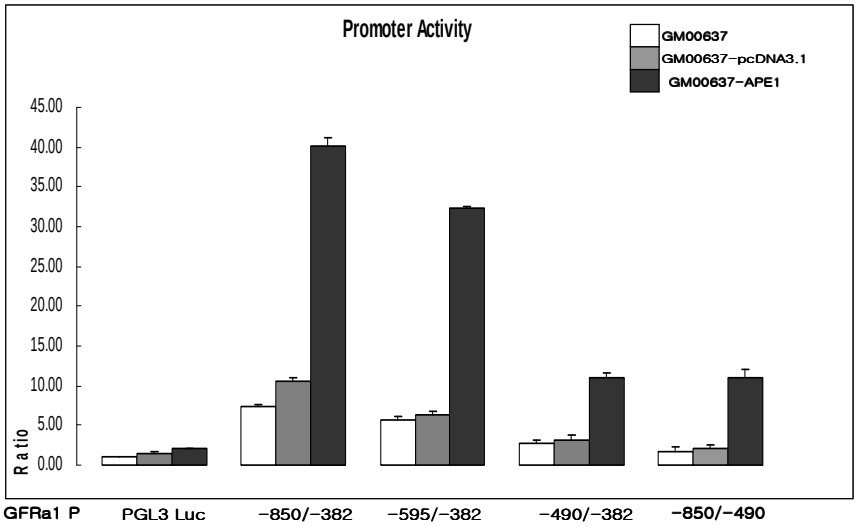


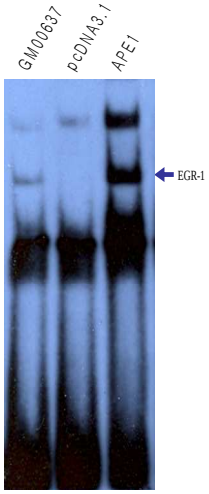
Fig. II-5.B. Relative luciferase activities of EGR-1 oriented JAG1 promoter-reporter constructs. The JAG1 promoter-reporter constructs each as shown in Fig.I-5.A and the PGL3 luciferase reporter vector, an internal control, were transiently co-transfected into ref-1 expressing GM00637 cells. Forty eight hour after transfection, cells were harvested for assaying the reporter gene expression. Firefly luciferase activities elicited by the JAG1 promoter with EGR-1 binding sites were normalized to b-Galactosidase activities derived from the PGL3 basic vector. Data shown are the mean ± SD of three experiments, each determined with triplicate dishes.

II-6) Identification of EGR-1 binding to the sub-region -850/-383 of the JAG1 promoter.

To identify transcription factors that bind to the putative *cis*-element -638/-622 as described above, we conducted electrophoretic mobility shift and supershift assays (EMSA) with antibodies against EGR-1 as shown (Fig. II-6A & 6B), yielded a supershifted band, indicating that the putative *cis*-element in this sub-region is an EGR-1 binding site. The consensus EGR-1 oligonucleotides were used as competitors as in supershift assays (Fig. II-6B) in the presence of the GM00637-ref-1 nuclear extract using 5' Biotin Labeled EGR-1 DNA binding site-II (EGR-

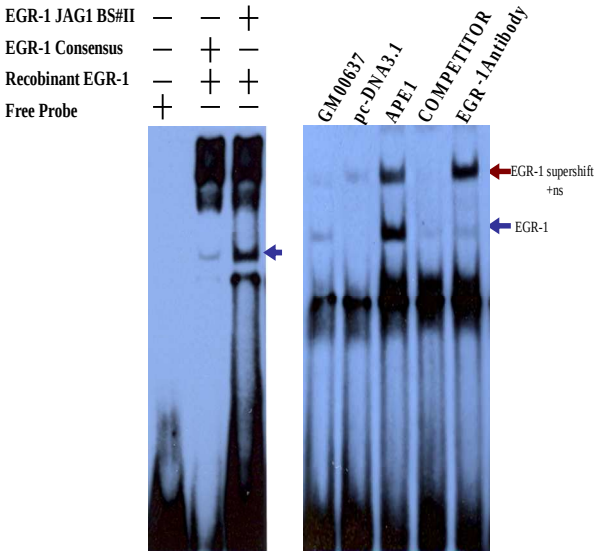
1-BS-II) as a probe. As expected (Fig. II-6B), approximate 100-fold molar excess of cold EGR-1 binding site-consensus (EGR-1-BS-Cons) (lanes 4) oligonucleotides competed successfully with labeled probes to bind to the EGR-1 protein. From this supershift assays result strongly support the hypothesis that the EGR-1 binding site exists in the -850/-382 fragment of the JAG1 promoter and the transcription factor, EGR-1, specifically binds to this *cis*-element.

[A]



Putative EGR-1 Binding Motif

[B]



EGR-1 Binding Activity Putative EGR-1 Binding Motif

Fig. II-6.A. Identification of the EGR-1 binding site in the sub-region -850/-382 of the JAG1 promoter. A. EGR-1 binding to the subregion -638/-622 of the JAG1 promoter determined by EMSA assays. 5'-Biotin labeled synthetic JAG1 promoter oligonucleotide probe –638/-622 containing EGR-1 BS #II and 5 μg nuclear extracts (NE) prepared from ref-1 expressed cells were incubated in the reaction mixture. After reaction, samples were analyzed on the native 5% Polyacrylamide gel followed by autoradiography**.** **B.EGR-1 BS #II binding to the sub region -638/-622 of the JAG1 promoter determined by EMSA supershift competition assays**. Experiments were performed as described in **A**, consensus competitors were added into the reaction mixture before addition of the putative EGR-1 binding oligo (Fig.II-6B lane 4) as well as antibody against EGR-1 was incubated with putative EGR-1 binding motif (Fig. II-6B Lane 5). Left panel shows the EGR-1 specific band was confirmed by using EGR-1 recombinant protein incubated with EGR-1 binding consensus oligo and putative EGR-1 binding motif respectively.

II-7) Evaluations of transcriptional activities of putative EGR-1 BS #II in JAG1 promoter region -850/382.

To evaluate roles of the EGR-1 in the JAG1 gene activation, site-directed mutation was intoduced at the EGR-1 BS #II in the JAG1 promoter-reporter gene construct spanning from -850/382 (Fig. II-7). JAG1 core promoter activities were tested by transfection assays in ref-1 expressing cells GM00637. As shown in Fig. II-7, mutations of the EGR-1-BS #II {EGR-1 BSII SMTF: cct gag cat ccc gct **tt**c ccc aac ccc ttc ca} (mutations are labeled with Bold Letters) reduced the luciferase gene expression than that of the wild type control. Thus, the JAG1 promoter contains functionally active EGR-1 sequences which are considered to be an important for ref-1 induced JAG1 promoter activation.

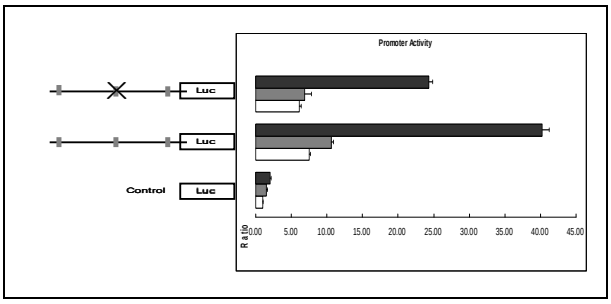


Fig. II-7. Effects of EGR-1-BS-WT and its mutants on the SV40 promoter-directed reporter gene expression. The EGR-1-BS-WT and EGR-1 BS #II mutant as shown in schematic drawing in the left were inserted upstream of the SV40 promoter in the pGL-3 promoter reporter vector. Resulting chimeric constructs were co-tranfected with the plasmid pSV-β-Gal into ref-1 expressing GM00637 cells. After a 48 h posttransfection, luciferase and β-Gal activities in cell lysates were determined. EGR-1-BS-WT or mutant modified SV40 promoter-directed luciferase activities were normalized to the transfection efficiency. All values represent the mean ± SD of three experiments, each determined with triplicate dishes. Luciferase activity was measured by using b-Galactosidase assay system of Site Directed EGR-1 JAG1-II Promoter-reporter constructs in human fibroblast GM00637 and its DNA repair protein APE over-expressed cell line.

II-8) Determination of functional EGR-1 BS #II in the JAG1 promoter region.

Computational analysis showed that there are 3 putative EGR-1 binding sites located at -540/-556, -622/-638 and -778/-794 in the JAG1 promoter region from -850 to -382 which yielded the maximal reporter gene expression (Fig. II-4B). From the mutation analysis, The EGR-1 BS#II binding site (-622/-638) containing JAG1 promoter fragment (-850/-382) was demonstrated to enhance the SV40 promoter activity (Fig. II-7). To examine whether other putative EGR-1 binding sites are also capable of incorporation with EGR-1 BS #II and regulation of the JAG1 gene expression, we carried out electrophoretic mobility shift with synthetic oligonucleotides spanning JAG1 promoter sequences oligos EGR-1 BSI F 5’Biotin ccc agg gtg agc acg ccc tct cat gaa tat taa t, EGR-1 BSII F 5’Biotin gag cat ccc gct gcc ccc aac ccc ttc caa gtt c and EGR-1 BSIII F 5’Biotin gcc cgg ggc gcc cga ggg ggc ggt ccc cgc tgg g as shown in Fig. 8A. After incubation with nuclear extracts from ref-1 expressed GM00637 cells, labeled oligonucleotides induced mobility shift bands in the presence of EGR-1 BS #II (Fig. II-8) but not in the BS#I and BS#III oligo. These results indicated that the only EGR-1 BS #II is functional.

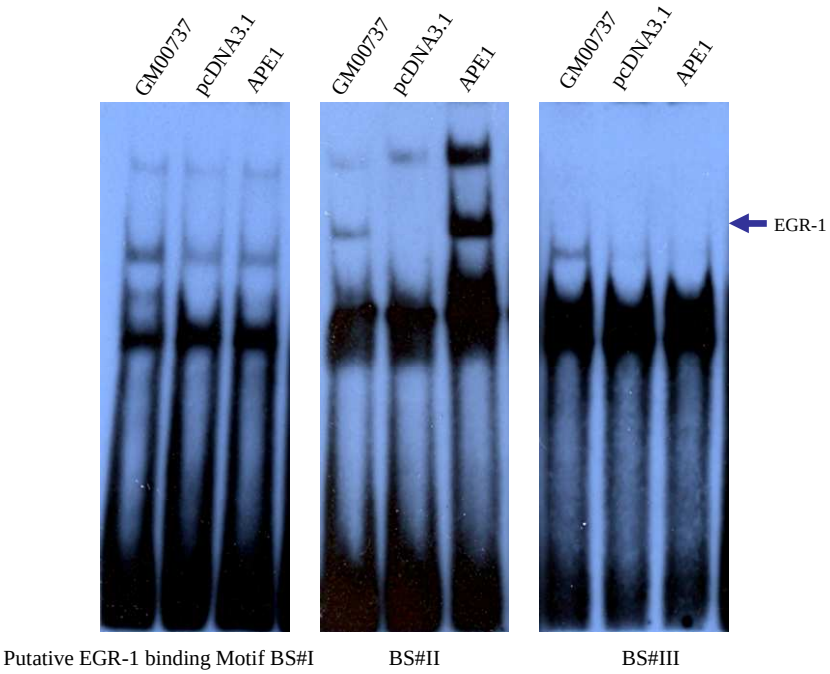
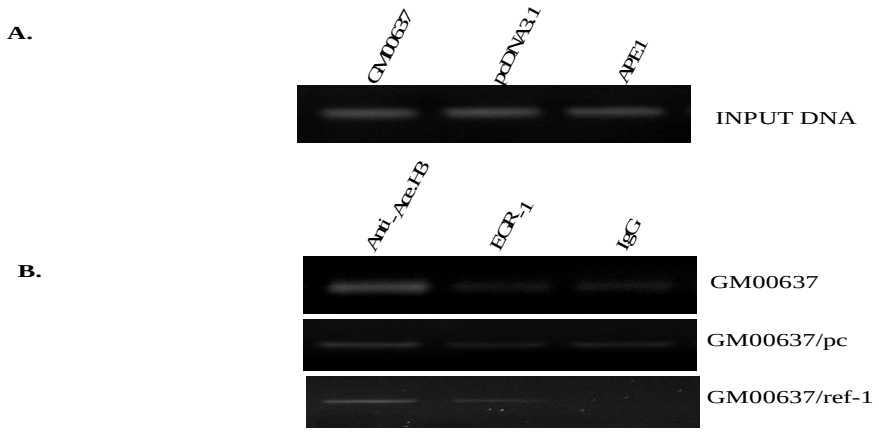


Fig. I-8. Determination of functional EGR-1-binding sites in the JAG1 promoter region – 850/382. 5'- biotin labeled synthetic JAG1 promoter fragments containing putative EGR-1 binding sites as shown in Fig. II-8 and 5 μg nuclear extracts (NE) prepared from ref-1 expressed GM00637 cells were incubated in the reaction mixture in the absence or presence of the specific antibody against EGR-1. After reaction, samples were analyzed on the native 6% polyacrylamide gel followed by autoradiography. Reaction products are shown in lanes 1-3 with the probe EGR-1-BS#1, lanes 4-6 with the probe EGR-1-BS #2 and lanes 7-9 with the probe EGR-1- BS#3 .

II-9) Cellular EGR-1 binding to the cognate *cis*-element in the JAG1 promoter.

To determine cellular EGR-1 binding to the JAG1 promoter region, the ChIP assay was carried out using an antibody against EGR-1 antibody. As demonstrated, there were at least one functionally active EGR-1 binding sites among three EGR-1 located at the regions in the EGR-1 promoter region -850/-382 that displayed the maximal promoter activity (Fig. II-4B). Thus, this active EGR-1 BS #II binding site was tested for their cellular protein binding by using specific primer pairs tabulated in table II-4. According to the designed primer pairs, we anticipated that JAG1 promoter fragments -850/-382/ which contain active EGR-1-binding sites #II , would result following immunoprecipitation with the EGR-1 antibody and amplification by PCR. Indeed, gel analysis of PCR products confirmed our expectation. Approximately, a ~100 bp and a 150 bp DNA bands were observed on gels as the PCR amplified using primer pairs that encompass EGR-1 binding sites #II (Fig. II-9). In contrast, no signal was detected in control experiments with a nonspecific antibody. These results suggest that the assay conditions were appropriate and can be used to measure the relative levels of EGR-1 binding to the JAG1 gene. As noted, the same cell number (2 x 106) for each group was used for DNA extraction, and there was no significant difference in the yields of PCR products among groups using “input” (before immunoprecipitation) DNA as a template (Fig II-9). These results reflect the status of cellular EGR-1 binding to the JAG1 gene which is highly sensitive to ref-1 stimulated endogenous expression.



F

Fig. II-9. Determination of cellular EGR-1 binding to the JAG1 gene promoter by the ChIP assay. GM00637, GM00637-pcDNA3.1 and GM00637-APE1 cells were incubated from 2 x 106 cells for each group, and immunoprecipitated with an antibody against all EGR-1, Anti acetyla Histone H3, a positive control or nonspecific rabbit IgG, a negative control (Fig.B), Using unimmunoprecipitated (input) DNA as a template, the PCR with primer pairs leading to EGR-1 BS #II. PCR with "input" DNA was performed to monitor amplification efficiency of individual primer pair along the JAG1 gene (Fig.A). PCR products were analyzed on 2.2% agarose gels.

II-10] Precise mechanism of the ref-1 regulation to the functionalities of the JAG1 promoter.

To verify the precise role of the ref-1 in the JAG1 expression, the various APE1 mutants were made as described above into the TOPO- pcDNA3.1 (neo) vector. Transient co-transfection was carried out by using various ref-1 mutants with the EGR-1 binding site containing luciferase reporter constructs -850/-382 in GM00637 cells. After 48 hour of transfection, cells were harvested and measured the trasncriptional activity by using b-galactosidase assay syatem. As shown (Fig. I-10), cells ransfected with the wild-type ref-1 expression plasmid (TOPO-pcDNA3.1 APE Full Length) and with JAG1 promoter fragments -850/-382 which contain an active EGR-1 binding site as well as redox or c-terminus part of the ref-1 expression plasmid (TOPO-pcDNA3.1-APEF1/R300) exhibited higher levels of luciferase activities of the pGL3-Promoter control. However, ref-1 repair part or N-terminal region containing expression vector (TOPO-pcDNA3.1-APE Repair) showed significantly reduced transcriptional activity.

A point mutation was introduced in the first half of the redox part containing conserved c-terminus site which is considered as a residue thought to be important or redox activity [138] in all mouse, rat, and human. An active cysteine of position 65 of redox part was changed to alanine (a cysteine t position 65) [147] which results significant decrease of luciferase activities in comparison to the empty vector as a control while a point mutation in both halves

and others show not a significant reduction. Thus, the EGR-1 interaction with its binding site in the JAG1 promoter region is a critical molecular target for ref-1 induction to its redox part.

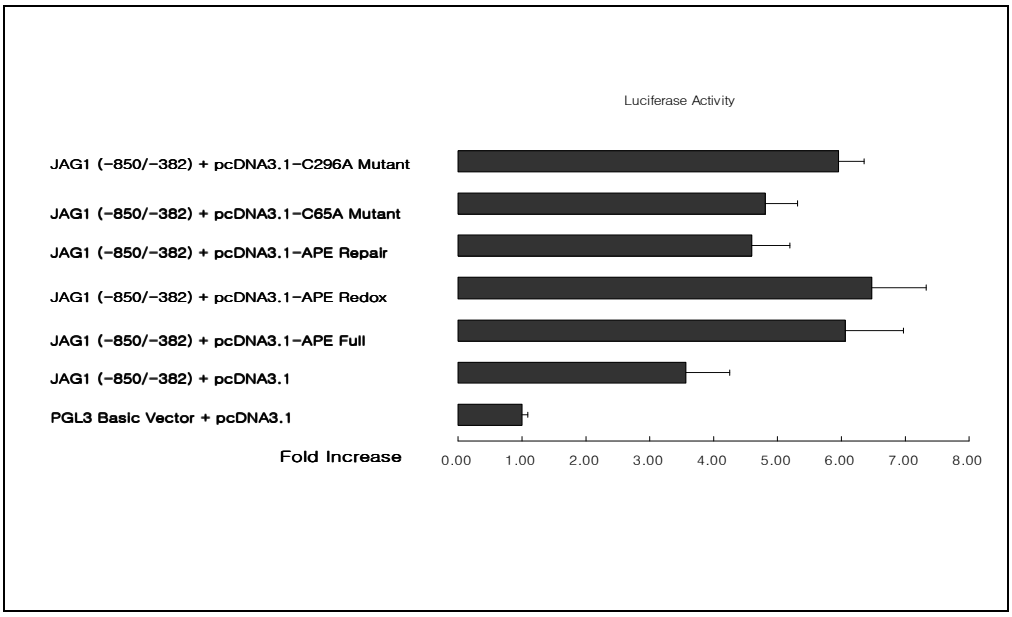


Fig. II-10. Ref-1 regulation to the functionalities of the JAG1 promoter. Ref-1regulated JAG1 transcriptional activity was determined by using various ref-1 expression constructs with site directed mutations and EGR-1 JAG1 Promoter-reporter constructs (-850/-382) were transiently co-transfected in human fibroblast GM00637 cells and 48 hr after transfection, promoter activities were measured by using b-Galactosidase assay system. Various constructs with its mutations are named in the left.

Discussion

I. Ref-1 Induced GFRα1 Promoter Activity in Human fibroblast cell line GM00637.

Regulation of GFRα1 transcription in response to ref-1 is a critical control point for the GFRα1 gene expression involved in human fibroblast cell line GM00637. This report presents the data for the GFRα1 gene promoter by addressing three issues. First, it identified the ref-1 induced GFRα1 expression in human fibroblast cell line GM00637. Second, it characterized functional NF-κB core elements and other active sub-regions of the GFRα1 gene promoter. And third, it demonstrated the NF-κB binding site as an important *cis*-element for transactivation of the GFRα1 gene.

Hence the GFRα1 gene promoter had cloned and characterized in an effort to understand mechanisms for the GFRα1 transcriptional regulation. The 5'-flanking region of the GFRα1 gene appears to contain no typical TATA or CCAAT box in experimental analysis, as it was failed to find out the TSS. The TATA box as the first core promoter element identified in RNA polymerase II-transcribed genes is generally present 25-30 bp upstream of the transcription start site (65). From the Primer extension assays we were not able to find transcription start sites in GFRα1 promoter region.

To determine whether the 5'-flanking region of the GFRα1 gene is functionally active for the regulation of transcription, an approximately -2.3 kbp genomic fragment spanning nucleotides from -2291 to -7 was inserted in front of the luciferase gene in the reporter gene vector (Prom -3,979). The promoter activity was assessed by transient transfection of the Prom –2291/-7 construct into ref-1 expressed GM00637 cells. As shown in Fig. I-4B, the Prom -2291/-7 induced around ~18-fold increase over the pGL3 basal level in reporter gene expression indicating its GFRα1 promoter property. A series of 5'-deletions of the Prom -2291/-7 indicated that the construct Prom -575/66 which retained promoter activity amounting to at least ~20-fold of the pGL3-Basic control. The maximal luciferase activity was found in the -575/-66 base pair region immediately upstream of ATG as shown in the construct Prom –575/-66.

Progressive 5'-deletion assays indicated the sequence from -1 to -287 as a basic region and the sequence from -575/-66 as a regulatory region, both are essential for the maximal expression of the ref-1 induced GFRα1 promoter activity. Screening of the -575/-66 region using gel shift and supershift, and competition assays demonstrated a NF-κB binding site #I, i.e., 5'Biotin cct cac ccc ggt gtt gga aat tcc cca aag gcg gga -3', at the region of 349- /-322 sequence of double stranded 5'biotin labeled DNA were used (Fig. I-6). Mutating in nucleotide in the GFRα1 promoter-NF-κB BS #I binding site at the region of -349- /-322 successfully inhibited luciferase activities in chimeric construct-transfected ref-1 expressed cells (Fig. I-7).

These results strongly support the hypothesis that NF-κB BS #I is one of the transcriptional activator that binds to the *cis*-element located at -349/-322, facilitating ref-1 induced GFRα1 transcription. The GFRα1 promoter region -575/-66 that yielded the maximal activity contains at least 3 putative NF-κB binding sites at regions of -349/-322, -300/-288, and -155 /-143. To gain insight into biological activities of all putative NF-κB-binding sites in the GFRα1 promoter we performed electrophoretic mobility shift and supershift assays. Results showed that except the oligonucleotides containing the sequence of -349/-337, two oligonucleotides with the sequences -300/-288 and -155/-143 had not any role to make nucleoprotein complex with the GFRα1 specific NF-κB binding motif (Fig.I-8).

Furthermore, site-directed mutations of the NF-κB BS #I at the sequences ccc ggt gtt gga aat **cg**c cca aag gcg gga ac strongly inhibited GFRα1 promoter activities. These results are consistent with the enhancement of SV40 promoter activity by the GFRα1 NF-κB binding site -349/-337 in chimeric construct transfected cells as shown above (Fig.I-4B) and further support the conclusion that the NF-κB binding site acts as an enhancer *cis*-element transactivating the GFRα1 gene.

So all of above promoter analysis data also showed that NF-κB is required for ref-1 induced GFRα1 activation. Thus, it was speculated that in vitro binding data could not completely explain the real situation in vivo. To address this possibility, ChIP assays were performed with an anti-p50 and anti-p65 antibody in ref-1 expressing GM00637 cells. The ChIP results are consistent with the promoter assay results. In the ref-1 expressing GM00637 cells, there is a

persistent interaction between the NF-κB -349 site and the residual p50, while very low or absence of interaction can be seen at NF-κB site and p65 residue (Fig. I-9). However, such a protein-DNA interaction can not be seen at NF-κB-300 and NF-κB-155 site .

The ref-1 regulation of gene expression is sensitive to redox part since its DNA binding domain contains a cysteine (C65) residue susceptible for GFRα1 induction. This possibility was tasted by using mutation in redox part of ref-1 (C65) (45,48,49) and showed that the luciferase reporter gene expression in GM00637 cells transfected with GFRα1 NF-κB binding site (-575/-66)-SV40 promoter chimeric constructs was significantly inhibited (Fig.I-10). Luciferase activities in NF-κB binding site-SV40 promoter chimeric construct-transfected cells treated with other ref-1 expression truncated or mutated constructs were expressed relative to controls obtained from cells transfected with NF-κB element free pGL3-Promoter vector under the same conditions to ensure the inhibition mediated by the redox part of the ref-1 and NF-κB *cis*-element interaction. Redox part of the ref-1 regulation to the GFRα1 promoter activity was further confirmed by as similar to above co-transfection assay of different mutated ref-1 expression constructs with the GFRα1 promoter-reporter vector (Fig. I-10). These results indicate the high sensitivity of NF-κB interaction with its cognate *cis*-element of the GFRα1 gene in response to ref-1 stimulation. Ongoing experiments should be focussed to determine the transcription Initiation site by Primer extension analysis and RACE to predict a typical eukaryotic promoter.

In sum, this report describes the cloning and characterization of the GFRα1 gene promoter in response to ref-1 in human fibroblasts. The maximal promoter activity was displayed within an 575/-66 bp 5’ flanking region upstream of the +1 as a putative translation initiation site including exon-1 composed of a basic domain from -287 to -1 and a regulatory domain from -575 to -66. One NF-κB binding site at the sequences -349/-337 was determined which acted as an enhancer element elevating the GFRα1 promoter activity in response to ref-1. NF-κB transactivation of the GFRα1 gene was strongly modulated in response to ref-1 in human fibroblast GM00637 cells. By pursuing these findings, it may elucidate mechanisms for controlling transcription of GFRα1, a key neuronal receptor, in response to ref-1.

II. Ref-1 Induced JAG1 Promoter Activity in Human fibroblast cell line GM00637.

In the current study, it was sought to identify factor(s) that could potentially upregulate JAG1 expression in the context of ref-1 in human fibroblast cell line GM00637. It was demonstrated that ref-1 upregulated JAG1 protein and mRNA expression. Furthermore, an EGR-1 binding DNA element was identified which was critical for basal and ref-1 induced activation of the JAG1 promoter. More importantly, EGR-1 transactivated the JAG1 promoter via this EGR-1 element in ref-1 expressing GM00637 cells.

More 5'-deletions of nucleotides from the Prom -850/-382 induced serially reduction of luciferase activities based on the EGR-1 DNA binding sites such that the Prom -850/-382, Prom-595/-382 and Prom -490/-382 decreased JAG1 promoter activity (Fig.II-5A & 5B). Interestingly, deletions of the 3'proximal regions (-490/-382) DNA element upstream of ATG from the Prom -850/-490 also totally decreased luciferase activities as shown by Prom -850/-382. (Fig.II-5B). It should be noted that the construct Prom-850/-382 significantly induced the highest expression of the luciferase gene to that of the pGL3-Basic control while the construct Prom 490/-382 or -850/-490 displayed only basal level of luciferase activity in comparison to the pGL3-Basic. Thus, it appears that the major role of the fragment between -850 and -382 is transactivation of the ref-1 induced JAG1 gene.

By 5' promoter deletion experiments, a region at bp -850 to -382 was identified which was important for ref-1induction of the JAG1 promoter (Fig. II-5B). Indeed, internal mutation of this region in the context of the larger promoter construct abolished ref-1 induction (Fig. II-7). Surprisingly, it also reduced the basal promoter activity by >50% (Fig. II-7). These complementary experiments demonstrated an essential role of the EGR-1 motif in conferring JAG1 basal and ref-1 inducible promoter activity in GM00637 cells.

EGR (Early Growth Response) proteins represent a family of transcription factors involved in cell cycle regulation (82). They contain three, nearly identical DNA - binding zinc finger regions, each possessing a unique flanking region. EGR-1, -2, -3, and -4, bind the EGR consensus sequence GCG T/GGG GCG, however the individual proteins show differing

binding affinities for related aaaaaa sequences (83,84). The EGR binding sites are present in promoters of several tissue specific genes regulating cytokines and growth factors as well as genes regulating the cell cycle.

These results strongly support the hypothesis that EGR-1 BS #II is one of the transcriptional activator that binds to the *cis*-element located at -622 /-638, facilitating ref-1 induced JAG1 transcription. The JAG1 promoter region -850/-382 that yielded the maximal promoter activity contains at least 3 putative EGR-1 binding sites at regions of -540/-556, -622/-638, and -778 /-794. To gain insight into biological activities of all putative EGR-1 binding sites in the JAG1 promoter, an electrophoretic mobility shift and supershift assays were performed (Fig. II-6, II-8). Results showed that except the oligonucleotides containing the sequence of -622/-638, two oligonucleotides with the sequences -540/-556 and -778/-794 had not any role to make nucleoproteincomplex with EGR-1 specific antibody. Screening of the -850/-382 region using gel shift and supershift, and competition assays demonstrated an EGR-1 BS #II at the region of 622- /-638 is important to regulate the ref-1 induced JAG1 promoter activity in GM00637 cells. Ongoing experiments should be focussed to determine the transcription Initiation site by primer extension analysis and RACE to predict a typical eukaryotic promoter.

In conclusion, an EGR-1 DNA element was identified in the JAG1 promoter that was critical for ref-1 induced JAG1 promoter activity. In the present study, it was elucidated the mechanism by which ref-1 induces JAG1 expression in human fibroblast cell GM00637. Ref-1 induces JAG1 expression at the transcriptional level (Fig.II-1). Recent reports established that the JAG1 promoter has three EGR-1 binding sites and only one EGR-1 binding site at the –622/-638 position of the JAG1 promoter is responsible for ref-1-mediated JAG1 promoter activation as well as DNA element of the region -850/-490 displayed only basal level of luciferase activity (Fig.II-7).

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<국문초록>

Ref-1 에의한 GFRα1 및 JAG1 Promoter 조절연구

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본 연구의 목적은 인간 섬유아세포 GM00637에서 GFRα1과 JAG1 의 발현을 유도하는 APE1/Ref-1의 역할을 밝히는 것이다. GFRα1 과 JAG1 promoter에 전사인자 NFk-B의 결합은 APE1을 발현하는 GM00637 세포에서 기능적인 GFRα1의 유도를 뚜렷하게 조절한다. 또한, DNA 복구 활성에서 APE1은 redox 기작에 의해서 많은 전사인자들의 DNA 결합 활성을 조절할 수 있다. 우리는 GFRα1 promoter와 JAG1 promoter 에서 3개의 잘 보존되고 기능적인 NFk-B와 EGR-1 결합부분을 찾았다. EMSA super shift assay를 이용하여 NFk-B p50 과 EGR-1 APE1 interacting 단백질로 확인되었고, nuclear translocation에 의해 GFRα1 promoter이 NFk-B 와 JAG1 promoter이 EGR-1 결합부분에 결합하였다. 또한, in vivo ChIP를 이용하여 NFk-B 와 EGR-1 결합 DNA 요소들이 GFRα1 promoter 와 JAG1 promoter와 결합하는것을 밝혔다. 더 나아가, 이러한 부분의 돌연변이는 promoter 활성을 뚜렷하게 감소시켰다. 그러므로, APE1 유도된 GFRα1은 NFk-B 와 JAG1은 EGR-1 결합 motifs에 의해 조절되고, 이러한 결과에 의해서 APE1이 GFRα1 과 JAG1 promoter의 NFk-B 와 EGR-1 결합부분의 전사인자들을 통해서 GFRα1과 JAG1 기능을 조절하는 새로운 기작을 뒷받침 한다. 결론적으로, 이 논문은 APE1과 관련된 GFRα1 과 JAG1의 새로운 promoter 분석을 증명하였다. 또한, APE1이 GFRα1과 JAG1 promoter 분석과 GFRα1 과 JAG1 유도를 매개하는 정확한 기작을 나타내었다.

저작물 이용 허락서

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	영문: The Effect of the ref-1 on the Promoter Activity of the GFRα1 and JAG1.				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(0) 반대( )

2008년 8 월 일

저작자: 사무드라아차르야 (서명 또는 인)

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