CHARACTERIZATION AND REGULATION OF HUMAN *Kim-1* GENE PROMOTER: THE ROLE OF ACTIVATOR PROTEIN 1 (AP1) TRANSCRIPTION FACTOR

by

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ABSTRACT

CHARACTERIZATION AND REGULATION OF HUMAN *Kim-1* GENE PROMOTER: THE ROLE OF OF ACTIVATOR PROTEIN 1 (AP1) TRANSCRIPTION FACTOR

Kidney Injury Molecule-1 (KIM-1) is an immunoglobulin superfamily cell surface protein highly upregulated on the surface of dedifferentiated renal proximal tubule epithelial cells regenerating after toxic or ischemic injury. KIM-1 is known to be the most sensitive biomarker of kidney injury approved by FDA for preclinical safety studies in 2008. Therefore, characterization of the human *Kim-1* gene promoter and the associated transcription factors regulating its transcription under toxic injury is very crucial. For this purpose, a human-derived proximal tubule epithelial cell line HK2 was utilized. Ochratoxin A (OTA), Gentamicin (GM) and Cisplatin (CP), which are known to induce nephrotoxicity, were used in *in vitro* HK2 cell culture system as chemotoxic stress inducers. Significant changes were observed in KIM-1 protein and mRNA amounts under chemotoxic stress as shown by Western Blot Analysis and Quantitative Real Time PCR, respectively. The minimal promoter region of *Kim-1* gene was characterized by deletional mutation analysis of *Kim-1* upstream region. AP1 protein, a well-known stress response transcription factor, was demonstrated to bind to the promoter region of *Kim-1* gene by employing Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP) assay. However, luciferase reporter assays did not reveal any significant change between the wild type and the mutant AP1 binding site at -1010 position bearing plasmids in transient transfections. In addition, KIM-1 open reading frame (ORF) was cloned into bacterial and eukaryotic recombinant protein expression vector systems in order to produce KIM-1 antibody and reveal the functional role(s) of KIM-1 in human cells, respectively. Our results suggest that, the 700 bp upstream region of *Kim-1* gene contains the minimal promoter region and *Kim-1* gene might be regulated by the cooperative activity of AP1 with other *trans*-acting or *cis*-regulatory elements.

ÖZET

İNSAN *Kim-1* GEN PROMOTORUNUN KARAKTERİZASYONU VE REGÜLASYONU: AKTİVATÖR PROTEİN 1 (AP1) TRANSKRİPSİYON FAKTÖRÜNÜN ROLÜ

Böbrek Hasarı Molekülü-1 (KİM-1), immünoglobulin süper ailesi hücre yüzeyi proteini olup, toksik veya iskemik hasar sonrası dediferansiye olmuş renal proksimal tübül epitel hücrelerinin yüzeylerinde büyük ölçüde üretilmektedir. KİM-1, böbrek hasarının bilinen en hassas belirteçidir ve FDA tarafından preklinik çalışmalar için 2008 yılında onay almıştır. Bu nedenle, insan *Kim-1* geni promotorunun ve toksik hasar altında bu geni regüle eden transkripsiyon faktörlerinin karakterizasyonu büyük önem taşımaktadır. Bu amaçla, insan proksimal tübül epitel hücre hattı HK2 kullanılmıştır. Nefrotoksisiteye yol açtığı bilinen Okratoksin A (OTA), Gentamisin (GM) ve Sisplatin (CP), *in vitro* HK2 hücre kültürü sisteminde kemotoksik stres indükleyicileri olarak kullanılmıştır. Kemotoksik stress altında, anlamlı KİM-1 protein ve mRNA değişim miktarları, sırasıyla Western Blotlama analizi ve Kantitatif Gerçek Zamanlı Polimeraz Zincir Reaksiyonu yöntemleriyle gözlemlenmiştir. *Kim-1* geninin minimal promotör bölgesi, *Kim-1* geninin yukarı bölgesinin delesyonel mutasyon analiziyle karakterize edilmiştir. Strese cevap verdiği iyi bilinen AP1 proteininin *Kim-1* geninin promotor bölgesine bağlandığı, Elektroforetik Hareketlilik Kayma Analizi (EMSA) ve Kromatin Immün Çöktürme (ChIP) tahlilleri kullanılarak gösterilmiştir. Ancak, geçici transfeksiyonlarda, lusiferaz raportör tahlilleri sonucunda yabanıl ve mutant AP1 -1010 pozisyon bağlanma bölgesini içeren plazmitler arasında anlamlı bir değişim açığa çıkmamıştır. Ayrıca, KİM-1 açık okuma çerçevesi (ORF), bakteriyel ve ökaryotik rekombinant protein ekspresyon vektör sistemlerine, sırasıyla KİM-1 antikoru üretimi ve KİM-1'in insan hücrelerindeki fonksiyonel rolünün açığa çıkarılması için klonlanmışlardır. Sonuçlarımız, *Kim-1* geninin 700 bazlık yukarı bölgesinin minimal promotörü içerdiğini ve *Kim-1* geninin AP1 ve diğer *trans*-hareket eden ya da *cis*-düzenleyici elementlerin kooperatif aktivitesiyle regüle edilebileceğini önermektedir.

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LIST OF SYMBOLS

bp	Base Pairs
g	Gravity
gr	Gram
kb	Kilobase
kDa	Kilodalton
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
ng	Nanogram
^{o}C	Centigrade degree
s	second
S	Svedberg
V	Volt
µg	Microgram
µl	Microliter
α	Alpha
β	Beta
κ	Kappa

LIST OF ACRONYMS / ABBREVIATIONS

aa	Amino Acid
AKI	Acute Kidney Injury
AP1	Activator Protein 1
ARF	Acute Renal Failure
ATF	Activating Transcription Factor
BrdUrd	Bromodeoxyuridine
bZIP	Basic Region-Leucine Zipper
cDNA	Complementary DNA
C-terminus	Carboxyl Terminus
ChIP	Chromatin Immunoprecipitation
CO_2	Carbon Dioxide
CP	Cisplatin
CRE	cAMP-responsive element
DEPC	Diethylpyrocarbonate
DMEM	Dubecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleosidetriphosphate
EDTA	Ethylenediaminetetraacetate
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Signal Regulated Kinase
FDA	Food and Drug Administration
gDNA	Genomic DNA
GM	Gentamicin
HAVCR1	Hepatitis A Virus Cellular Receptor-1
HK2	Human Kidney Cell Line 2
HPV-16	Human Papilloma Virus 16

IgV	Immunoglobulin Variable Region
JNK	c-Jun N-Terminal Kinase
KIM-1	Kidney Injury Molecule-1
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
NGF	Neuronal Growth Factor
NLS	Nuclear Localization Signal
OCT2	Octamer Transcription Factor 2
ORF	Open Reading Frame
OTA	Ochratoxin A
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PS	Phosphatidylserine
PTEC	Proximal Tubular Epithelial Cell
PVDF	Polyvinylidene Fluoride
RACE	Rapid Amplification of cDNA Ends
RCC	Renal Cell Carcinoma
RDA	Representational Difference Analysis
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolution per minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
SDM	Site-Directed Mutagenesis
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
TEMED	Tetramethylethylenediamine
TF	Transcription Factor
TIM	T-Cell Immunoglobulin Mucin Protein
Thr/Ser/Pro	Threonine/Serine/Proline
TNF-α	Tumor Necrosis Factor-α
TPA	12-*O*-tetradecanoyl-phorbyl-13-acetate

TRE	TPA response element
tRNA	Transfer RNA
UTR	Untranslated Region
TAE	Tris acetic acid
TBS	Tris-buffered saline
TBST	Tris Buffered Saline with Tween
TEMED	N,N',N',N'-Tetramethylethylenediamine
Tris	Tris (hydroxylmethyl) aminomethane
Tween	Polysorbate
UV	Ultra Violet

1. INTRODUCTION

1.1. Acute Kindey Injury (AKI)

Acute kidney injury (AKI) also known as acute renal failure (ARF) is a rapid complication of critical illness which shows high mortality risk due to prolonged decrease in renal function (Bellomo *et al*., 2004). The effects of AKI can be reversed by the remarkable capacity of kidney tubules to repair and regenerate (Bonventre, 2010). The major cause of AKI is acute tubular necrosis induced by ischemic or nephrotoxic injury to the kidney (Lameire *et al*., 2005). Ischemic injuries can be due to decreased renal perfusion, tubulointerstitial inflammation and edema or reduction in the filtering capacity of the glomerulus. Nephrotoxic injury is mainly caused by the accumulation of toxic substances such as metabolites of antibiotics like Gentamicin (GM) or chemotherapeutic agents such as Cisplatin (CP) (Thadhani *et al*., 1996).

Nitrogenous waste products such as urea or creatinine and other metabolic waste products that are normally excreted by the kidney accumulate in kidney upon AKI (Lameire *et al*., 2005). Although the pathophysiology of AKI has been studied and understood widely, there are no effective therapies for the AKI patients other than supportive treatment methods (Schrier, 2010). Mostly, serum creatinine or blood urea nitrogen levels are checked in order to understand the presence and the level of injury rated from slight to severe in patients. However, these markers are usually nonspecific, insensitive and may change drastically only after significant kidney injury occurs with a delay, preventing early diagnosis and reliability (Vaidya *et al*., 2006). However, it is crucial to identify renal tubular injury at an early stage for AKI patients. Therefore, to establish effective treatment techniques for AKI, sensitive and reliable biomarkers should be identified. This is especially important for confirming the presence of kidney injury when the morphological changes are still at the minimum levels in the patient. Ideally, a number of biomarkers can be useful not only to understand the presence of injury but also understand at which part of the kidney tissue the injury is located (Lock, 2010).

1.2. Kidney Injury Molecule 1 (KIM-1)

1.2.1. Structural and Functional Features of KIM-1

Kidney Injury Molecule 1 (designated KIM-1 in humans, Kim-1 in rodents) was first isolated and characterized by Joe Bonventre's group at Harvard Medical School (Ichimura *et al.*, 1998). It is one of the genes identified from post-ischemic rat kidney by a method called Representational Difference Analysis (RDA) (Hubank and Schatz, 1994). Human KIM-1 protein is conserved across species with 38% and 42% identity to rat and mouse, respectively (Vaidya *et al*., 2006).

KIM-1 is an immunoglobulin superfamily cell surface protein which is highly up-regulated on the surface of dedifferentiated renal proximal tubule epithelial cells regenerating after toxic or ischemic injury (Bailly *et al*., 2002). KIM-1 is encoded by *TIMd1* gene on chromosome 5q33.2 (McIntire *et al*., 2001). The KIM/TIM family consists of eight members in mice, six in rats and three in humans (Kuchroo *et al.*, 2006). All three transcript/splice variants in human coding for the same polypeptide differ only in their 5'untranslated regions (5'UTRs).

According to the literature, KIM-1 knock-out or over-expression seem not to have any influence on the phenotype. Rather than kidney injury, mostly, the immune system related functions of *Kim-1* have been investigated. For instance, *Tim-1* deficient and *Tim-1* over-expressing transgenic mice, which were investigated for their type-2 immune responses, were healthy and displayed no phenotypic abnormalities. There was no significant difference in proliferative or differentiative capacity of naive T and B cells between wild type and *Tim-1* deficient mice. Moreover, no differences were found in the production of the Th2 cytokines like IL-4, IL-5 and IL-10 or the Th1 cytokine interferon-c between wild type and *Tim-1*-deficient mice (Wong *et al*., 2010). As a result, the effect of KIM-1 knocked-out still remains to be explored further.

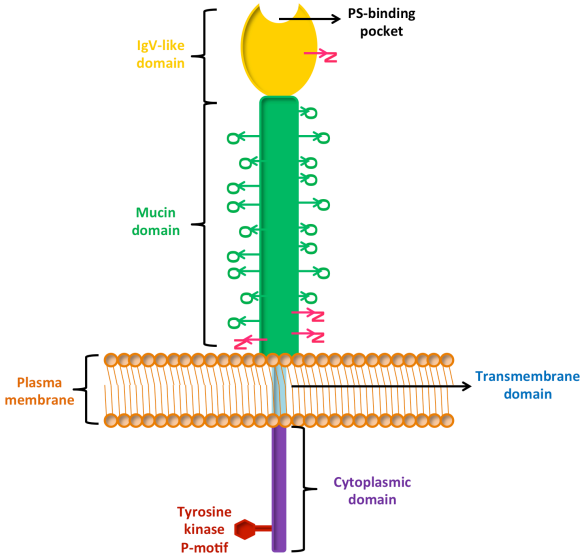

Figure 1.1. Schematic representation of Kidney Injury Molecule 1 (KIM-1) protein (Adapted from Huo *et al*., 2010).

KIM-1 is a type I cell membrane glycoprotein consisting of a unique six-cysteine immunoglobulin variable region (IgV)-like domain and a long Thr/Ser/Pro-rich mucin domain in its extracellular portion, which possesses a highly repetitive sequence containing *O*-linked glycosylation sites (Ichimura *et al*., 1998) (Figure 1.1). There are putative *N*-linked glycosylation sites between the mucin domain and the transmembrane region (Ichimura *et al*., 1998). KIM-1 also possesses a relatively short, C-terminal cytoplasmic domain containing a putative phosphorylation site, which is thought to be involved in cell signaling (Binne *et al*., 2007). The immunoglobulin-like domain is thought to mediate protein-protein interactions thereby allowing interactions between cell-cell or cell-extracellular matrix at the cell surface (Barclay, 1999). KIM-1 is also thought to function as an extracellular receptor for cell signaling or adhesion between cells, or between cells and pathogens. Specifically, the mucin domain is thought to modulate cell adhesion because of its homology to several known adhesion proteins. The mucin domain can also act as a structural domain to expose adjacent domains and it can have protective function on the cell surface of regenerating epithelial cells by isolating them from an environment that is filled with dead cells and their cellular contents (Jentoft, 1990). Additionally, KIM-1 contains a pocket in the IgV-like domain for the recognition of phosphatidylserine (PS) molecules.

A protein found in African Green Monkey kidney cells (COS7) that was reported to be the receptor for hepatitis A virus (HAVCR-1 / hepatitis A virus cellular receptor-1) was the first identified and cloned homolog of human KIM-1 with 85.3% amino acid sequence similarity (Kaplan *et al*., 1996). Two human homologs were then cloned from the kidney and liver (Figure 1.2). The one, mainly expressed in liver, was cloned as KIM-1 (KIM-1a, 334 aa) and the other that is expressed from the kidney was cloned as the homolog of HAVCR-1 (KIM-1b, 359 aa). Having identical extracellular domains, KIM-1a and KIM-1b differ only in their C-terminal portion of the cytoplasmic domain. For the two homologs, a common polypeptide sequence is produced up to the residue 323 followed by 11 amino acids in the case of KIM-1a making it a 334 aa long polypeptide, and followed by 36 amino acids in the case of KIM-1b ending up in a 359 aa long polypeptide structure (Bailly *et al*., 2002). Furthermore, genomic structure analysis and cDNA products of these two homologs indicate that they are splice variants.

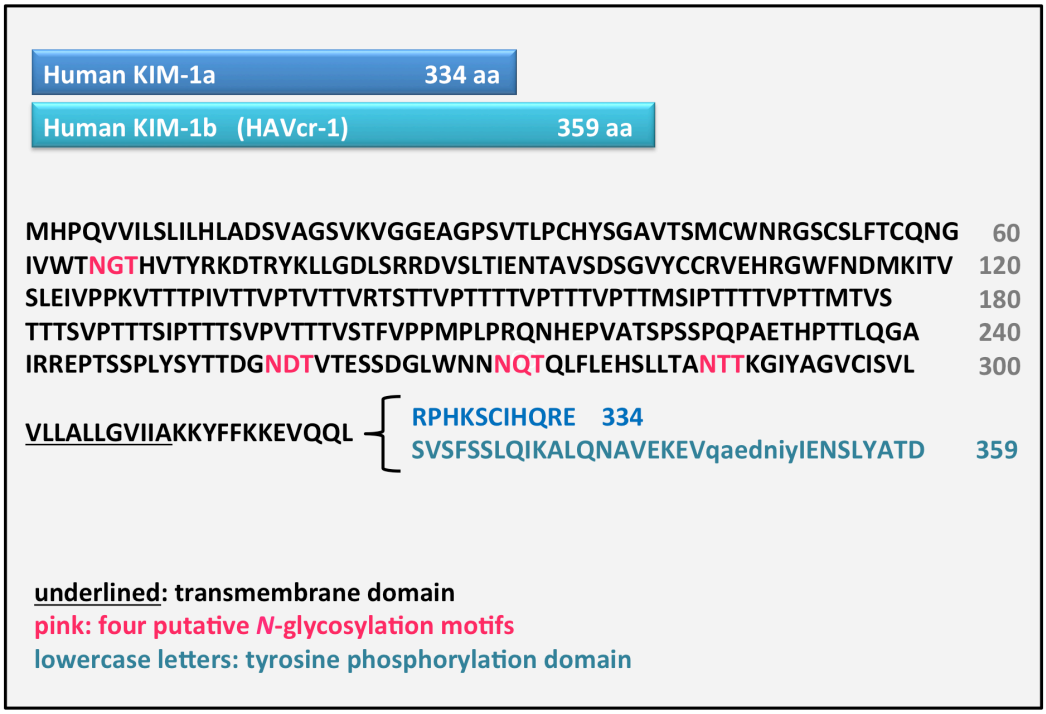

Figure 1.2. Two homologs of human KIM-1 protein (Adapted from Bailly *et al*., 2002).

Some membrane proteins like cytokines, growth factors, enzymes, receptors or cell adhesion proteins have soluble forms that are released into the extracellular space for different reasons. These soluble forms can be the products of alternative splicing or the result of proteolytic cleavage. It has been shown that the cells expressing endogenous or recombinant human KIM-1b constitutively shed the KIM-1 ectodomain into the extracellular space (Huo *et al*., 2010) (Figure 1.3). Release of soluble KIM-1 is a metalloproteinase-mediated process, which is proven by the fact that KIM-1 release can be blocked by two different matrix metalloproteinase (MMP) inhibitors Batimastat (BB-94) and Ilomastat (GM6001) (Bailly *et al*., 2002). Mature form of rodent KIM-1 located on the cell surface that runs at 104 kDa molecular mass on SDS-PAGE gel is shed during the kidney injury appearing in the urine, whereas the human counterpart is about 90 kDa (Bailly *et al*., 2002). As a summary, ectodomain of KIM-1 is shed from cells by the action of a metalloproteinases *in vitro* (Bailly *et al*., 2002) and *in vivo* into the urine in rodents (Vaidya *et al*., 2006) and humans (Han *et al*., 2002) after proximal tubular injury. This shedding process is partly regulated by Mitogen Activated Protein Kinase (MAPK) pathways, which are activated by stress (Zhang *et al*., 2007). Moreover, maintenance of the helical structure in the juxtamembrane region of the KIM-1 molecule is crucial for its recognition by metalloproteinases (Zhang *et al*., 2007). As a result of the shedding process, soluble KIM-1 is released into the tubule but its role remains unknown.

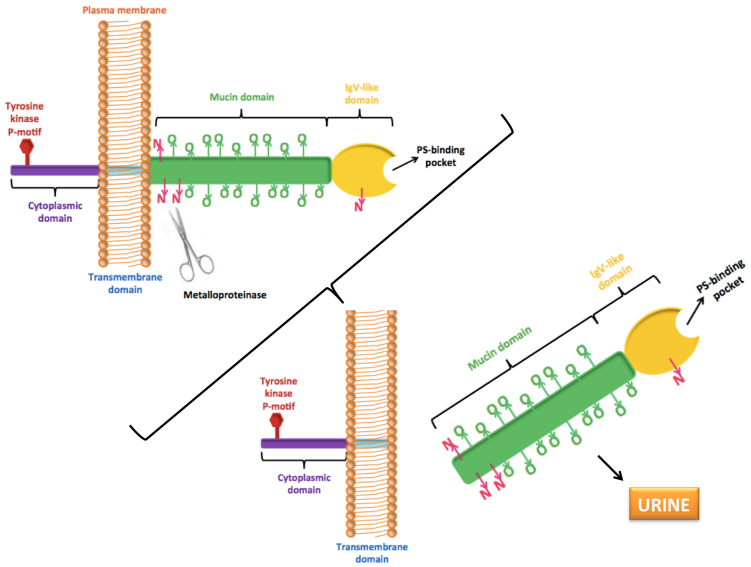

Figure 1.3. Shedding of KIM-1 protein (Adapted from Huo *et al*., 2010).

1.2.2. KIM-1 as a Kidney Injury Biomarker

KIM-1 is known as one of the most sensitive biomarkers of kidney injury approved by FDA (US Food and Drug Administration) for preclinical safety studies in the year of 2008 (Rached *et al.*, 2008). There are several characteristics of KIM-1 that make it an ideal biomarker for the detection of kidney injury. First, it is nearly absent in normal kidney, but upregulated upon injury and also it is present in the injured epithelial cells until complete recovery (Rached *et al.*, 2008). Moreover, the shed ectodomain that appears in the urine is stable at room temperature. It was demonstrated that there is an increase in the expression of KIM-1 in kidney biopsy specimens from patients with AKI, and KIM-1 ectodomain in the urine of these patients is at very high levels. In less severe cases of AKI, there is less KIM-1 ectodomain in the urine (Rached *et al.*, 2008). Another study has shown that, in a subset of patients whose urine was collected near the time of biopsy, the urinary KIM-1 levels correlated well with the tissue expression of KIM-1 (van Timmeren *et al*., 2007). Thus, the presence of KIM-1 ectodomain in the urine is a very specific and early diagnostic indicator of kidney injury.

1.2.3. Role of *Kim-1* in the Immune System

KIM-1 is also designated as TIM-1 (T cell immunoglobulin mucin protein 1), which regulates T-cell activation and tolerance. The TIM gene family includes three members (TIM-1, -3, -4) in human and eight members in mice. The size of mucin domain and the number of *O*-linked glycosylation sites are variable among the TIM family proteins. TIM-1 and TIM-4 have been reported to be expressed on mature macrophages and dendritic cells in both human and mice, and they can bind phosphatidylserine on apoptotic cells (Kobayashi *et al*., 2007). When a cell undergoes apoptosis, phosphatidylserine molecules are no longer restricted to the cytosolic part of the plasma membrane but become exposed to the external surface, and this phenomenon is a key signal for recognition of apoptotic cells by phagocytes. Rapid removal of the apoptotic cells is critical for the maintenance of tolerance against intracellular antigens released from dying cells. Therefore, inflammation and autoimmune responses are also prevented (Kobayashi *et al.,* 2007).

1.2.4. Role of *Kim-1* in Kidney Injury

The kidney plays a crucial role in the organism as it eliminates toxic compounds and other metabolic waste products. The renal proximal tubule epithelium is very prone to injury because of the localization of processes like active transport associated with the re-absorption and the biotransformation. After exposure to a toxic chemical or drug, the renal proximal tubule epithelial cells start to lose their polarity and cytoskeletal integrity undergoing apoptotic or necrotic cell death. Surviving cells start to dedifferentiate and proliferate by undergoing mitogenesis together with the repopulation and redifferentiation of regenerating cells (Bailly *et al*., 2002, Lim *et al.*, 2013). Dedifferentiation is thought to play an important role in spreading and migration of cells so that, at the end, a normal functional epithelium is reestablished rapidly and extensively in the place of the denuded basement membrane (Bonventre, 2003) (Figure 1.4). The pathological condition associated with this event is the accumulation of apoptotic and necrotic cell debris inside the tissue (Thadhani *et al*., 1996). Thus, the rapid removal of apoptotic cells prevents further necrosis, therefore preventing leakage of cytotoxic or pro-inflammatory intracellular contents into the tissue. This event also prevents an immune response against the proteins of the phagocytosed apoptotic cell, because otherwise there can be persistent inflammation and signs of autoimmunity (Duffield, 2003).

The role of KIM-1 in cell survival after kidney injury is unclear. Previous studies have identified rat and human KIM-1 cDNAs encoding Kidney Injury Molecule 1 as a gene important during the repair process with a high level of sequence homology across species. The PCR-based method used in these studies was called representational difference analysis (RDA) that helped analyze the difference in the mRNA populations between post-ischemic and normal kidney tissue (Hubank and Schatz, 1994). *Kim-1* was found to be one of the most highly upregulated genes in this screen.

Northern Blot analysis and RNA *in situ* hybridization in rat kidney revealed that KIM-1 mRNA expression is detectable in 48h post-ischemic kidney. These KIM-1 mRNA positive cells, which are the dedifferentiated proximal tubular epithelial cells, reside predominantly in the outer stripe of outer medulla and in the medullary rays of the cortex. On

the other hand, KIM-1 mRNA is almost undetectable in normal kidney (Ichimura *et al*., 1998).

Western blot analysis of rat kidney homogenates identified three proteins after ischemic injury. These proteins were absent in normal kidney tissue. The molecular masses of these three proteins were 40, 50 and 70-80 kDa. Therefore, Western analysis confirmed that, KIM-1 protein is upregulated in post-ischemic kidney *in vivo* (Ichimura *et al*., 1998). In another study, Western blot analyses using the extracts from HK2 cell line and murine monoclonal antibodies raised against the extracellular domain of KIM-1 protein showed one major band at about 100 kDa with two other bands at 70 and 50 kDa (Bailly *et al*., 2002). The appearance of the three protein sizes was thought to represent the *N*- or *O*-linked glycosylations of the same protein.

By immunohistochemistry, KIM-1 expression was shown to be apparent in the S3 segment of the proximal tubule in the outer stripe of the outer medulla which is the region highly susceptible to injury upon ischemia or other damage inducing agents like toxins (Ichimura *et al*., 1998). In addition, KIM-1 expressing cells also express markers of dedifferentiation (Vimentin) and proliferation (BrdU-Bromodeoxyuridine incorporation). Therefore, KIM-1 is proposed to be involved in alteration of differentiation status of kidney epithelial cells and remodeling during repair of the injured tissue (Ichimura *et al*., 1998). KIM-1 is also expressed at high levels in patients with clear cell-type renal cell carcinoma (RCC) which is a disease associated with proximal tubule cell differentiation (Han *et al*., 2002).

Although it is clear that the expression of KIM-1 is upregulated in the post-ischemic kidney, it is still unclear if KIM-1 acts as a positive or negative regulator during the repair process. It is predicted that it functions as a cell surface receptor for proximal tubule epithelial cells during regeneration. Normally, in both animals and humans, KIM-1 is expressed at the apical membrane of proximal tubule epithelial cells (PTECs); and if it is involved in cell-cell interactions, its potential binding/interacting partner should reside in the lumen side of injured and regenerating PTECs. It has been found that KIM-1 is a phosphatidylserine receptor, which recognizes apoptotic cells and direct them to the lysosomes

for clearance. Therefore, it might act as a receptor that transforms epithelial cells into residential phagocytes (Ichimura *et al.*, 2008). Ig variable domain of KIM-1 has been found to bind and internalize oxidized lipid molecules (Humphreys *et al.*, 2013). KIM-1 is also thought to be involved in covering the denuded area of the basement membrane after injury (Ichimura *et al.*, 1998). Moreover, it might play an important role in suppressing the immune response after renal injury. Clearance of apoptotic cells is such a mechanism for limiting the pro-inflammatory response (Kobayashi *et al.*, 2007).

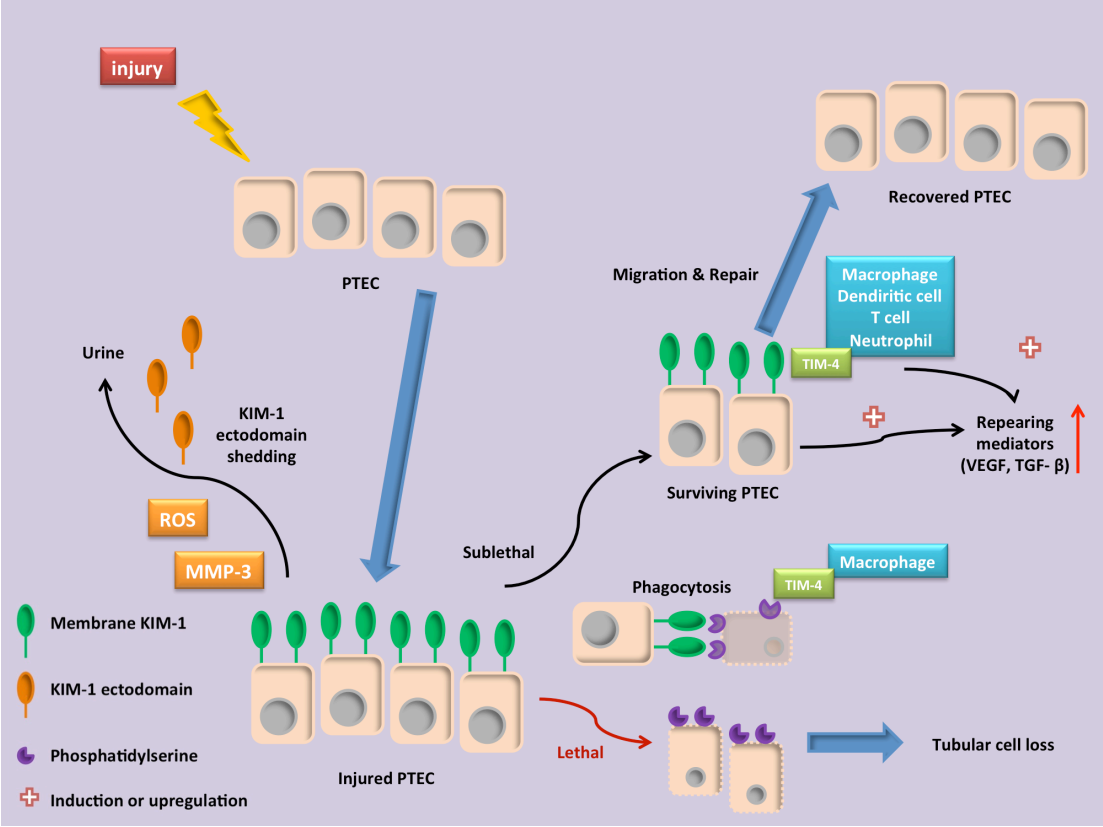

Figure 1.4. Role of KIM-1 in kidney injury (Adapted from Lim *et al.*, 2013).

1.3. Toxicity Inducers

Different drugs or xenobiotics can induce acute or chronic nephrotoxicity as their side effect. The effect might emerge in two ways: either dose-dependent or dose-independent (Bacchetta *et al.*, 2009). In this study, OTA, GM and CP were used as chemo-

toxic stress inducers in an *in vitro* cell culture system utilizing a human-derived proximal tubule epithelial cell line, Human Kidney Cell Line 2 (HK2).

1.3.1. Ochratoxin A (OTA)

OTA is a mycotoxin, produced by fungi of the species *Aspergillus ochraeus* or *Penicillium verrucosum*. It is found as a contaminant in many grains and other products such as cereals, coffee, nuts, red wine (Walker, 2002). Furthermore, it has been shown to cause porcine nephropathy and Balkan Endemic Nephropathy (BEN) in humans (Hald, 1991).

OTA is a phenylalanine-dihydroisocoumarine derivative that is very resistant to both high temperature and hydrolysis (Luhe *et al*., 2003). By its resemblance to the amino acid phenylalanine, it can inhibit protein synthesis by inhibiting phenylalanine tRNA synthetase (Dirheimer and Creppy, 1991). The kidney is the main target organ for OTA toxicity but the mechanism by which OTA contributes to nephrotoxicity, is not known. However, it has been suggested that OTA's mode of action could include formation of DNA adducts or single strand DNA breaks due to oxidative stress (Luhe *et al*., 2003).

OTA is thought to be genotoxic and has been shown to be involved in many cancer types like renal adenocarcinoma (Castegnaro and Dirheimer, 1998). It has been shown that the toxic effect of OTA might be due to the formation of reactive oxygen species (ROS) (Gillman *et al*., 1999). Observation of lipid peroxidation provides evidence for the induction of oxidative stress caused by OTA (Baudrimont *et al*., 1994).

1.3.2. Gentamicin

GM is an aminoglycoside antibiotic used against gram-negative bacterial infections. It can bind the 30S subunit of the prokaryotic ribosome so that the ribosomal initiation complex is blocked, leading to mistranslation. Thus, bacterial death occurs as a result of inhibition of protein synthesis (Sundin *et al*., 2001).

Aminoglycoside antibiotics like GM lead to some side effects such as nephrotoxicity or ototoxicity (Sundin *et al*., 2001). Nephrotoxic effects start to appear after a short period of treatment. However, the precise mechanism of the nephrotoxic effect of GM remains unclear. At elevated concentrations, aminoglycosides are thought to inhibit protein synthesis in eukaryotic cells by blocking incorporation of amino acids by ribosomes *in vitro*. It has been demonstrated that protein synthesis is significantly inhibited after two days of GM treatment in rats (Sundin *et al*., 2001). Accumulation of phospholipids due to inhibition of lysosomal phospholipases, and formation of lysosomal myeloid bodies are also implicated as mechanisms of nephrotoxicity (Beauchamp *et al*., 1990).

1.3.3. Cisplatin

CP is a chemotherapeutic drug used for the treatment of solid tumors. It crosslinks DNA so that cytotoxic lesions form in tumors and other normal, dividing cells subsequently leading to apoptotic cell death (Arany and Safistein, 2003).

The kidney is the responsible organ for excretion of CP through glomerular filtration. Thus, CP accumulates in the kidney to a greater degree than the other organs resulting in nephrotoxicity (Arany and Safirstein, 2003). Specifically, CP is accumulated in both the proximal and distal nephrons of the proximal tubule. The organic cation transporter 2 (OCT2) is known to be responsible for CP uptake in the proximal tubule in both animals and humans. After the uptake, it is conjugated to glutathione and metabolized into a potent nephrotoxin reactive thiol, thereby leading to nephrotoxicity (Kroning *et al*., 2000).

CP-induced nephrotoxicity is thought to be mediated by MAPK signaling pathways because it was shown that CP activates all three MAPKs (ERKs, p38 and JNKs) in the kidney tissue both in *in vitro* and *in vivo* (Arany *et al*., 2004). Formation of reactive oxygen species (ROS), hypoxia and mitochondrial injury have been shown to be involved in CP-induced nephrotoxicity. Moreover, a large pharmaceutical company consortium evaluated *Kim-1* as the most upregulated gene among the other 30000 genes under the test upon CP treatment (Amin *et al*., 2004).

1.4. Activator Protein 1 (AP1) and Gene Regulation

Each cell in an organism contains the same genomic information. Depending on the tissue type and other conditions like stress or developmental stage, a cell utilizes a subset of its genes at a time. The process of controlling gene expression is called gene regulation (Ptashne, 1986).

Regulation of a gene can occur at different levels such as transcription, mRNA processing, translation or post-translational modifications being primarily at the transcriptional level. The core promoter, which is the binding site for the basal transcriptional machinery usually lies immediately upstream of the transcription initiation site. Transcription factors (TFs) modify the pre-initiation complex assembly thus transcription of the target genes by binding to the distal control elements on the promoter region. The short sequences on the promoter region, onto which the TFs bind, are called *motif*s. TFs can regulate different genes by either acting as an activator or a repressor on those motifs. As their names imply, activators increase the transcription level of the regulated genes whereas the repressors do the opposite.

A variety of mechanisms are utilized for the regulation of gene expression. These mechanisms can work by regulating the activity of RNA polymerase through remodeling the chromatin structure or recruiting the coactivator/corepressor proteins to the area of transcription. As a summary, concept of gene regulation determines the fate of each cell in every tissue. Therefore, it is very important to understand how each gene is controlled at the molecular level (Ptashne, 1986).

In the literature, there is very little information on the transcriptional regulation of *Kim-1* gene. In one study concerning the role of *Kim-1* (TIM-1) in asthma, two promoter polymorphisms, -416G>C and -1454G>A were genotyped in 409 unrelated asthma patients and 305 healthy controls using PCR and RFLP (restriction fragment length polymorphism) techniques. The polymorphism at -416G>C has been found to be associated with asthma susceptibility in the Chinese Han Population. Also, there was an increased transcriptional activity of the *Kim-1* gene, when “C” is present instead of “G” at position -416. Furthermore, when the potential functional role of that substitution was explored using the MatInspector Software, it was found that “G” to “C” change leads to the generation of a

Yin and Yang 1 (YY1) transcription factor binding site, which might have regulatory roles in the subject of asthma for people carrying that substitution (Liu *et al*., 2007). Thus, promoter regions are thought to play a very crucial role on their target genes for regulation. These promoters can be regulated by utilization of differential *cis*-regulatory regions or differential binding of *trans*-acting factors like transcription factors or a combination of all of these events under different physiological conditions or different tissues. However, there is no detailed information on the *Kim-1* gene promoter and its corresponding transcription factors. Thus, identifying the promoter region of *Kim-1* and the role of potential transcription factors binding to that region or nearby regions are critical for the understanding of its regulation and role in the kidney injury.

According to the 5' RACE (Rapid Amplification of cDNA Ends) experiments of our group, *Kim-1* gene transcription start site was mapped to the -470 to -400 bp upstream region of the gene from the $A^{+1}TG$, the start codon for the translation. For the discovery of *cis*-acting sequences and *trans*-acting factors located in the promoter region of *Kim-1* gene, the 2000 bp upstream region of the gene was examined by bioinformatics tools such as Genomatix MatInspector. Based on the bioinformatic analyses and the knowledge on the transcription factors that are already present in other gene promoters responding to chemotoxic or other stress conditions, Activator Protein 1 (AP1) was thought to be one of the putative factors that might be involved in the transcriptional regulation of *Kim-1* gene.

1.4.1. Structural Features of Activator Protein 1 (AP1)

AP1 is one of the first mammalian, sequence-specific transcription factors recognized (Bohmann *et al.*, 1987). It is a member of the dimeric transcription factor family composed of Jun (c-Jun, JunB and JunD), Fos (Fos, FosB, Fra1, Fra2) or ATF (Activating Transcription Factor) families. These proteins are all basic region-leucine zipper (bZIP) proteins, which can form homo- or heterodimers in the AP1 transcription factor complex (Zenz *et al.*, 2008) (Figure 1.5).

While ATF family members can only homodimerize, Jun proteins not only homodimerize among themselves but also form heterodimers with Fos and ATF family

members. Jun-Jun and Jun-Fos dimers bind phorbol 12-O-tetradecanoate-13-acetate (TPA)-responsive element, which has the palindromic sequence "TGACTCA". However, Jun-ATF and ATF-ATF dimers prefer to bind cAMP-responsive element (CRE), which has a different sequence "TGACGTCA". Both of these sequences contain the same AP1 half-site (Zenz *et al.*, 2008). Moreover, as the number of known bZIP proteins increases, number of possible combinations of dimers that are able to interact with AP1 binding site also increases. This shows the complexity of transcriptional regulation of AP1 target genes in the cells (Karin *et al.*, 1997).

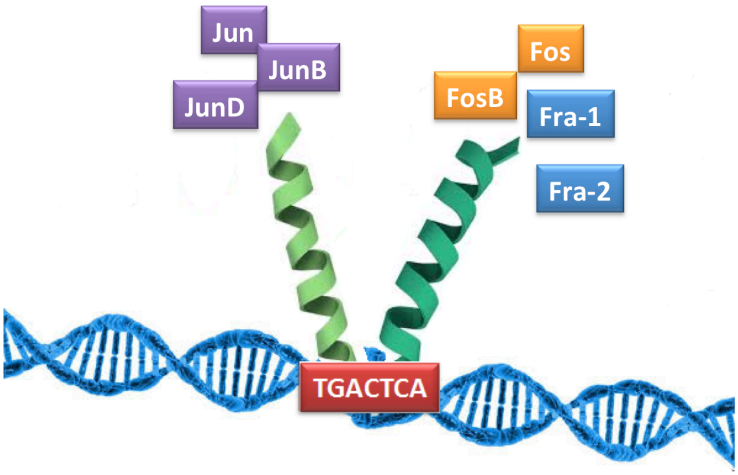

Figure 1.5. Activator Protein 1 (AP1) (Adapted from Zenz *et al.*, 2008).

c-Jun is the best characterized component of the AP1 transcription complex (Bohmann *et al.*, 1987). It was isolated from Avian Sarcoma Virus 17 in the year of 1987 as a cellular homolog of the retroviral oncogene *v-jun* (Maki *et al.*, 1987). It is associated with both cell proliferation and differentiation (Karin *et al.*, 1997).

1.4.2. Functional Features of Activator Protein 1 (AP1)

AP1 is one of the best-studied targets of MAPK signaling (Karin *et al*., 1997). The MAPK family consists of three subfamilies known as the extracellular signal-regulated kinases (ERKs) (Cobb *et al*., 1991), the c-Jun N-terminal kinases (JNKs) (Kyriakis *et al*., 1994) and the p38 MAPKs (Han *et al*., 1995). Being a series of parallel cascades of serine/threonine kinases, MAPK pathways are activated by various extracellular physical or chemical stress factors (Karin *et al*., 1997).

AP1 is known to have roles in many processes dictated by the cellular context such as the dimerization status, type and differentiation stage of the cell and type of the incoming extracellular stimulus having influence on the choice of cell proliferation, differentiation or apoptosis (Hess *et al*., 2004). The main role of AP1 is cell proliferation. For instance, its component c-Jun acts as a positive regulator of cell proliferation demonstrated in *c-jun-/-* fibroblasts having a proliferation defect due to defective cell cycle progression and undergoing premature senescence (Wisdom *et al*., 1999). AP1 has also a role in differentiation established by the knock-out studies and phenotypes of Jun and Fos knock-out animals (Eferl *et al.*, 1999, Hilberg *et al*., 1993). The role of AP1 in apoptosis depends on the cellular context resulting in different and sometimes even opposite responses (Bossy-Wetzel *et al*., 1997, Estus *et al*., 1994, Ham *et al*., 1995).

1.4.3. Activator Protein 1 (AP1) as a Potential Transcriptional Factor of *Kim-1* Gene Regulation

Many genes containing AP1 binding sites in their promoter regions are considered as AP1 target genes. In general, Jun proteins play role in the regulation of genes involved in cellular proliferation and apoptosis but the putative target genes for Fos proteins are generally associated with angiogenesis and tumor invasion (Hess *et al*., 2004). However, to date, AP1 target genes are poorly characterized.

It has been shown that AP1 plays a pivotal role in various renal diseases (Asai *et al*., 2003) suggesting that it might be a putative upstream regulator of *Kim-1* during kidney

injury. Moreover, CP was shown to promote increased production of reactive oxygen species (ROS) which can then activate JNK as a mediator of apoptosis (Francescato *et al*., 2007). JNK itself has already been shown to be activated by CP *in vitro* and *in vivo* (Sanchez-Perez *et al*., 1998). As apoptotic cell death is very prominent during kidney injury, JNK, being the upstream regulator of *c-Jun,* might affect *Kim-1* gene regulation when cells are exposed to CP. In addition, it has been shown that CP treatment with Tumor Necrosis Factor α (TNF-α) results in a switch of a more pro-apoptotic and inflammatory program in renal cells by altering their NFκ-B/JNK/c-Jun balance (Benedetti *et al*., 2013). It has been shown that KIM-1 protein expression increases upon treatment with CP *in vivo* (Vinken *et al.*, 2012), therefore, *Kim-1* gene might be a downstream target gene of AP1 transcription factor complex including c-Jun whose expression was found to be enhanced by CP/TNF-α treatment in proximal tubule epithelial cells (Benedetti *et al*., 2013).

Furthermore, there is no detailed information on the signaling pathways which regulate KIM-1 shedding. However, an *in vitro* culture study has shown the constitutive release of shed KIM-1 from 769-P cell line mediated by the activation of ERK. The same study showed that the activation of p38 MAPK upregulates the soluble KIM-1 release (Zhang *et al*., 2007). All these kinases are upstream regulators of AP1 as mentioned earlier. Moreover, KIM-1 expressing cells also express markers of proliferation, indicating that AP1, which is known to have a role in proliferation mechanisms, might be an upstream regulator of *Kim-1* gene during cell proliferation after chemotoxic injury.

2. PURPOSE

Expression of *Kim-1* gene is upregulated at both mRNA and protein levels upon kidney injury (Ichimura *et al*., 1998), and it is known to be the most sensitive biomarker of kidney injury approved by FDA for preclinical safety studies in 2008 (Rached, 2008). It has been shown to act as a receptor, which transforms epithelial cells into residential phagocytes (Ichimura *et al*., 2008) thereby having a positive effect on the injury. However, it is still unclear by which factors or pathways *Kim-1* gene is regulated during the repair process. Therefore, it is very crucial to examine how it is regulated at transcriptional level by the activity of *trans*-acting factors and *cis*-regulatory sequences.

Transciptional factors like AP1 are the main regulators of gene expression. They act by binding to their consensus sequences on their target genes. AP1 is known to have roles in cell proliferation and differentiation (Hilberg *et al.*, 1993). It also plays a pivotal role in various renal diseases (Asai *et al.*, 2003). Furthermore, shedding of KIM-1 is mediated upon the activation of ERK (Zhang *et al*., 2007), which is an upstream regulator of AP1. In addition, transcription factor binding prediction softwares such as Alibaba2 and Genomatix Genome Analyzer have shown that there are potential AP1 binding sites in the upstream region of *Kim-1* gene. As a result, we hypothesized that AP1 is a transcription factor candidate that may have roles in *Kim-1* gene regulation during the repair process after kidney injury.

Therefore, the main purpose of this thesis project is to characterize the promoter region of *Kim-1* gene in order to better understand the regulation of the human *Kim-1* gene at transcriptional level under chemotoxic stress conditions. Moreover, the binding capacity of AP1 to the upstream region of *Kim-1* gene as a possible regulator of its promoter activity was investigated.

3. MATERIALS

3.1. General Kits, Enzymes and Reagents

Table 3.1. List of kits, enzymes and reagents.

BCA Protein Assay Kit	Pierce, USA
Biotin Detection Kit (20148)	Thermo Scientific, USA
Cell Proliferation Kit II (XTT)	Roche, Switzerland
DMSO	Sigma-Aldrich, USA
DNase1 (#14785300)	Roche, Switzerland
Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) (Catalog No: 11039047)	GibcoBRL, USA
DNA Molecular Weight Marker	GeneRuler 1 kb DNA Ladder, Fermentas, USA 2-log DNA ladder, New England Biolabs, UK Lambda DNA/PstI Marker, 24, Fermentas, USA
Dual Luciferase Assay	Promega, USA
Dyna1 Protein G and Protein A magnetic beads	Invitrogen, USA
ChIP DNA Isolation Kit	Qiagen, Netherlands
3' End Labeling Kit (89818)	Thermo Scientific, USA
Fast Digest Green Buffer (10X)	Thermo Scientific, USA
Fast Digest Enzymes	Thermo Scientific, USA
Fetal bovine serum (FBS)	HyClone, USA
High Fidelity PCR Enzyme Mix	Thermo Scientific, USA
GeneJET Plasmid MiniPrep Kit (00117731)	Thermo Scientific, USA
Glycine Solution	Thermo Scientific, USA
Greenstar Mastermix (2X)	Bioneer, Denmark
Lumi-Glo chemiluminescent substrate	Cell Signaling Technologies, USA
NucleoBond Xtra Midi Kit (740410.2)	Macherey-Nagel, Germany
NucleoSpin Gel and PCR Clean-up Kit (740609.50)	Macherey-Nagel, Germany
Page Ruler Prestained Protein Ladder	Fermentas, USA
Penicillin-Streptomycin Solution	HyClone, USA

Table 3.1. List of kits, enzymes and reagents (cont.).

Phosphate buffered saline (PBS) – Cell Culture Grade	GibcoBRL, USA
Protease Inhibitor Cocktail (50X)	Roche, Switzerland
Proteinase K	Invitrogen, USA
Transcriptor High Fidelity cDNA Synthesis Kit	Roche, Switzerland
Transfection Reagent (Lipofec-tamine-2000)	Invitrogen, USA
Tripure Isolation Reagent	Roche, Switzerland
0.25% Trypsin-0.913 mM EDTA (1X), Phenol Red	GibcoBRL, USA
T4 ligase	New England Biolabs, UK
T4 DNA ligase buffer (10X) (#B69)	New England Biolabs, UK
Western Blotting Luminol Reagent	Santa Cruz, USA

3.2. Biological Materials

3.2.1. Bacterial Strains

Competent *Escherichia coli* DH5α and Rosetta (DE3) pLysS cell strains were used during the transformation experiments. The genotype of *E. coli* DH5α was "F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1" and the genotype of Rosetta strain was "F^- ompT $hsdS_B(R_B^- m_B^-)$ gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)".

3.2.2. Cell Lines

Human Kidney Cell Line 2 (HK2) was used during the cell culture experiments. The cell line was purchased from ATCC (American Type Culture Collection, Manassas, USA).

3.2.3. Plasmids

pCLucBasic2 (NEB, UK), pSV40-CLuc Control (NEB, UK), pTK-Gluc (NEB, UK), pGL3Basic (Promega, USA), pGL3Control (Promega, USA), pRL-TK (Promega, USA), pMEV2HA (Biomyx, USA) and pET30a(+) plasmids were used in the study.

3.2.4. Primers

Table 3.2. Primers used in this study.

Primer ID	**Sequence (5' to 3')**	**Tm (°C)**	**Application**
KIM1_2000_F1	ATAGATATCGCAGTGG-CACAATCATAGCCTCCAAC	70.1	SDM
KIM1_UTR_R1	AAACCATGGTATGG-GATCAGCCTGAAGGAAAATGAGCAGAC	66.2	SDM
AP1_1010_Mut_S	CAGCATTGTGAGGTGCAGAGCCTG	68.7	EMSA, ChIP, SDM
AP1_1010_Mut_A	AGGCTCTGCACCTCACAATGCTGG	68.7	EMSA, ChIP, SDM
KIM1_RT_F1	AACGAGCGTTCCAACGACAACG	62.8	RT-PCR
KIM1_RT_R1	AGATGGTGAAGTGGCTACTGGTTC	57.3	RT-PCR
HPRT1_RT_F1	CCTGGCGTCGTGATTAGTGATG	58.9	RT-PCR
HPRT1_RT_R1	ATCTCGAGCAAGACGTTCAGTCC	58.4	RT-PCR
KIM1-2000UP_F1	ATAGATATCGCAGTGGCACAATCATAGCCT-CCAAC	70.1	Promoter bashing
KIM1-1800UP_F2	ATAGATATCCAAAGTGTTGGGATTACAGGCGT	65	Promoter bashing
KIM1-1500UP_F3	ATAGATATCGGCCTCCCAAAGTGCTGGGGTTA	70.5	Promoter bashing
KIM1-1100UP_F4	ATAGATATCTTGGTGGGAGATAGAGGAAG-CATTTTTAG	65.6	Promoter bashing
KIM1-900UP_F5	ATAGATATCTTGTACAGGAGCATGAAAGGT-TAGGCA	65.9	Promoter bashing

Table 3.2. Primers used in this study (cont.).

KIM1-700UP_F6	ATAGATATCGCATTTGCTCTCGTGCTGCCATT-GA	72	Promoter bashing
KIM1-505UP_F7	ATAGATATCTGGGACTACAGGCGCCAGTGA	66.1	Promoter bashing
KIM1-TRX_R1	ATAGGTACCACGAAGTCAGGAGATCGAGAC-CAT	67.7	Promoter bashing
KIM1_TRX_R2	ATAGGTACCTCAATGGCAGCACGAGAGCAA-ATGC	72.4	Promoter bashing
Kim1_ORF_F	ATAGGATCCATGCATCCTCAAGTGGTCATCTTA AGCCTCA	74.2	Cloning of ORF
Kim1_ORF_XhoI_ Rev	ATACTCGAGTTAGTCCGTGGCATAAAGAC-TATTCTCAATG	74.8	Cloning of ORF

3.3. Chemicals, Plastic and Glassware

Table 3.3. Chemicals used in this study.

Acrylamide:Bisacrylamide (30% 37.5:1)	BioRad, USA
Ammonium Persulfate 10% (w/v)	Sigma-Aldrich, USA
Ampicillin	AppliChem, Germany
Chloroform	Sigma-Aldrich, USA
Cisplatin	Santa Cruz, USA
Ethanol	Sigma-Aldrich, USA
Ethidium bromide	Sigma-Aldrich, USA
Isopropanol	Sigma-Aldrich, USA
Formaldehyde (37%, 12.3M)	Sigma-Aldrich, USA
Gentamicin	HyClone, USA
Glycine	Sigma-Aldrich, USA

Table 3.3. Chemicals used in this study (cont.).

20X LumiGLO® Reagent & Peroxide	Cell Signaling, USA
N, N, N', N'-tetramethylethylenediamine(TEMED)	Sigma-Aldrich, USA
Ochratoxin A	Sigma-Aldrich, USA
PCIAA (acidic phenol:chloroform:isoamylalcohol 125:24:1, pH 4.5-5)	Fluka, USA
Phenol-chloroform (pH 7.7-8.3)	Sigma-Aldrich, USA
Phosphatase Inhibitor	Roche, Germany
Protease Inhibitor Cocktail	Roche, Germany
SDS (Sodium Dodecyl Sulfate)	Sigma-Aldrich, USA
Skimmilk Powder	Sigma-Aldrich, USA
Sodium acetate (NaAc pH 5.2)	Sigma-Aldrich, USA
Trisma Base	Sigma-Aldrich, USA
Tween-20	Roche, Germany

3.4. Buffers and Solutions

Table 3.4. Buffers and solutions used in this study.

Blocking Solution	5% skimmilk (w/v) in TBST
Coomasie Blue Staining Solution	1 g Coomasie Blue R250 100 ml glacial acetic acid 400 ml methanol 500 ml dH_2O
Destaining Solution	200 ml methanol 100 ml glacial acetic acid 700 ml dH_2O

Table 3.4. Buffers and solutions used in this study (cont.).

RIPA buffer	150 mM NaCl2 1% NP40 0.5% sodium deoxycolate 0.1% SDS 50 mM Tris pH 7.4
LB Agar	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast Extract 15 g/l Agar
Lysogeny Broth (LB)	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast Extract
Hypotonic Buffer Solution	10 mM HEPES 10 mM KCl 1 mM EDTA 1 mM DTT 1mM PMSF Protease inhibitor
Extraction Buffer Solution	20 mM HEPES 400 mM NaCl 1 mM EDTA 1 mM DTT 1 mM PMSF 0.1% NP40 Protease inhibitor
ChIP Lysis Buffer	50 mM HEPES 150 mM NaCl %1 Triton-X 100 %0.1 Na-deoxycholate 1 mM EDTA
PBS-T	1X PBS 0.02% Tween-20

Table 3.4. Buffers and solutions used in this study (cont.).

Primary Antibody Solution	5% BSA 0.02% Sodium Azide TBS-T
Protein Ladder	PageRuler Prestained (SM0671) Fermentas
4X Protein Loading Dye	2 ml 1M TrisHCl pH 6.8 0.8 g SDS 4 ml 100% Glycerol 0.4 ml β-mercaptoethanol 1 ml 0.5 M EDTA 8 mg Bromophenol Blue 2.6 ml dd H2O
12% Resolving gel (15 ml)	5 ml 1.5 M TrisHCl pH:8.8 75 µl 20% (w/v) SDS 6 ml Acrylamide:Bisacrylamide (30%/0.8% w/v) 75 µl 10% (w/v) APS 25 µl TEMED 3.9 ml dH2O
10X SDS Buffer	10 g SDS 30.3 g Tris Base 144.1 g Glycine dH2O up to 1L
Secondary Antibody Solution	5% (w/v) Skim milk powder TBS-T

Table 3.4. Buffers and solutions used in this study (cont.).

Stacking gel (4%) (5ml)	0.62 ml 0.5 M TrisHCl pH:6.8 25 µl 20% (w/v) SDS 0.833 ml Acrylamide:Bisacrylamide (30%/0.8% w/v) 50 µl 10% (w/v) APS 5 µl TEMED 3.817 ml dH2O
TBS-T	50 mM TrisHCl pH:7.4 150 mM NaCl %0.1 Tween-20 dH2O
1X Transfer Buffer	10% 10X Transfer Buffer (v/v), 20% Methanol, 70% dH2O
10X Transfer Buffer	1.92 M Glycine, 250 mM Tris-Base
10X FA gel buffer (pH 7.0)	200 mM 3[N-morpholino]propanesulfonic acid (MOPS) (free acid) 50 mM Sodium Acetate 10 mM EDTA
1X FA gel running buffer	100 ml 10X FA gel buffer 20 ml 37% (12.3 M) Formaldehyde 880 ml RNase-free dH_2O
4X RNA loading dye	16 µl Saturated Bromophenol Blue Solution 80 µl 500 mM EDTA, pH 8.0 720 µl 37% (12.3 M) Formaldehyde 2 ml 100% Glycerol 3084 µl Formamide 4 ml 10X FA Gel Buffer RNase-free dH_2O up to 10 ml

3.5. Antibodies

Table 3.5. Antibodies used in this study.

Name	**Species**	**Dilution**	**Source**
Anti-β-Actin	Mouse (mAb)	1:3000	Cell Signaling
Anti-c-Jun (AP1)	Rabbit (pAb)	1:1000	Santa Cruz Biotechnologies
Anti-Kim1	Rabbit (pAb)	1:1000	Pierce/Thermo Scientific
Anti-HA	Rabbit (pAb)	1:1000	Santa Cruz Biotechnologies
Anti-rabbit or mouse IgG, HRP-linked	Horse	1:2000	Cell Signaling

3.6. Disposable Labware

Table 3.6. List of disposable labware used in this study.

Cell Scraper	TPP, Switzerland
Cell culture plates	TPP, Switzerland
Eppendorf tubes (0.5 ml, 1.5 ml and 2 ml)	Axygen, Corning lifesciences, US
Filtered tips	Axygen, Corning lifesciences, US
Insulin syringes	Set Medikal, Turkey
Micropipettes (5 ml, 10 ml, 25 ml)	Axygen, Corning lifesciences, US

3.7. Equipments

Table 3.7. List of equipments used in this study.

Autoclaves	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Cell Culture Incubator	Sanyo, JAPAN
Centrifuges	Allegra X-22, Beckman, USA J2-MC Centrifuge, Beckman, USA J2-21 Centrifuge, Beckman, USA
Chemiluminescent Visualization System	Stella, Molecular Devices
XL-1000 UV Crosslinker	Spectrolinker™, Spectronics Corporation, USA
Agarose Imaging	GelDoc XR System, Bio-Doc, Italy
ELISA reader	Model 680, Microplate reader, Bio-Rad, USA
Deep Freezers	-20°C, Uğur, Turkey -80°C, Thermo Scientific, USA
Dish Washer	Mielabor G7783, Miele, Germany
Heat Block	DRI-Block DB-2A, Techne, UK
Ice Machine	Scotsman Inc. AF20, Italy
Microwave	MD554, Arçelik, Turkey
PCR Machine	T100™ Thermal Cycler, Bio-Rad, USA
Power Supply	Bio-Rad, USA
RT-PCR machine	Exicycler 96, Bioneer, South Korea
SDS Gel Electrophoresis	Biorad, USA
Semi-dry blotting machine	Biorad, USA
Shaker	VIB Orbital Shaker, Intermed, Denmark
Sonicator	Bioruptor
Stella	Raytest, Germany
Spectrophotometer	NanoDrop 1000, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK

4. METHODS

4.1. Cell Culture Techniques

4.1.1. Growth Conditions of Cells

An *in vitro* cell culture system using Human Kidney Cell Line 2 (HK2) was utilized during the experiments. HK2 is a proximal tubule epithelial cell line derived from human kidney. It was immortalized by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes (Ryan *et al*., 1994). The cell line was purchased from ATCC (American Type Culture Collection, Manassas, USA), and cultured in 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomicin containing DMEM/F12 medium (Gibco, Catalog No: 11039047) in a humidified 5% CO_2 atmosphere at 37°C.

4.1.2. Subculturing

Routine passaging was performed with 72-hour intervals by discarding DMEM/F12 medium from the 10-cm plate and washing the cells with 5 ml PBS. Then, 1.5 ml of 0.25% Trypsin-0.913 mM EDTA (1X) was added and the plate was incubated at 37°C for 5 minutes in order to detach cells from the surface. After that, fresh medium was added in order to block the activity of trypsin and cells were transferred into a 15 ml falcon tubes by pipetting. Then, cells were centrifuged at 2000 rpm for 2 minutes, and the supernatants were removed by vacuum. Finally, the cell pellets were dissolved in fresh medium and appropriate amount of cell suspension containing about $5x10^5$ cells was seeded into a new culture plate with sufficient amount of medium.

4.1.3. Storage

The cells were washed with PBS once. After trypsinization with 0.25% Trypsin-0.913 mM EDTA (1X) for 5 minutes at 37^{o}C, the cells were collected in a 15 ml falcon tube with growth medium. Then, the cells were centrifuged at 300 g for 5 min. Supernatant was discarded and the cell pellet was resuspended with FBS. Then, appropriate amount of DMSO was added to the final concentration of 10%. Finally the cells were transferred into cryovials and stored at -150 ^{o}C.

4.1.4. Thawing

The vials containing HK2 cells were frozen in a -150°C freezer. After a vial was incubated at 37^{o}C for complete melting of the cell suspension, cells were immediately transferred into 15 ml falcon tubes and centrifuged at 300 g for 5 minutes. After supernatant was discarded, the pellet was resuspended with growth medium and transferred into a plate.

4.2. XTT Cell Viability Assays

XTT Cell Viability Assays were performed using the XTT Cell Proliferation Kit II (Roche). This assay is based on the formation of the orange formazan dye from the yellow tetrazolium salt XTT (sodium 3´-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) by the mitochondrial dehydrogenase that was only produced in the metabolically active cells.

XTT measurements were done in triplets using 96-well cell culture plates with 7.5x10^{3} cells/well. HK2 cells were grown and treated with various amounts of OTA (10 mM stock dissolved in ethanol) (Sigma, Catalog No: O1877), GM (50 mg/ml stock dissolved in dH_2O) (HyClone, Catalog No: SV30080.01) and CP (2 mM stock dissolved in 0.9% saline) (Santa Cruz, Catalog No: 200896A) in DMEM/F12 medium containing 5%FBS, 1% Penicillin-Streptomicin for 24 hours. Then, previously thawed XTT labeling

reagent was mixed with the Electron Coupling Reagent (ECR) (50µl total volume) to form the XTT labeling mixture which was then added to wells and incubated for 4 hours at 37°C (for 96 wells; 5 ml XTT labeling reagent mixed with 0.1ml ECR). Finally, absorbances were measured at 490 nm and 655 nm (background reading) wavelengths using an ELISA reader (Model 680, Bio-Rad, USA). The results were expressed as the percentage of viability (%) relative to the vehicle control according to the formula; [(absorbance from treated cells-absorbance from blank)/(absorbance from control cells-absorbance from blank)] x 100.

4.3. Quantitative RT-PCR

4.3.1. Total RNA Isolation

Total RNAs were isolated from adherent HK2 cells grown in a monolayer on 10-cm cell culture plates. Cells were treated with 10 µM OTA, 2 mg/ml GM and 5 µM CP in DMEM/F12 medium containing 5% FBS, 1% Penicillin-Streptomicin for 24 hours along with an untreated group (control). Next day, medium was removed from the plates and 500 µl of Tripure Isolation Reagent (Roche) was added. Cells were scraped for lysis using a plastic scraper. After pipetting several times for homogenization, the lysates were transferred into a 2-ml Eppendorf tubes and left for 5 minutes at room temperature (RT).

Chloroform was added to each sample (200 µl for 1 ml Tripure Isolation Reagent) and tubes were shaken vigorously for 15 seconds. After incubation of the tubes at RT for another 5 minutes, samples were centrifuged at 12000 g for 15 minutes at 4°C until three phases form. Colorless aqueous phase was transferred into a new tube. Isopropanol was added (500 µl for 1 ml Tripure Isolation Reagent) and samples were inverted for several times in order to precipitate the RNA. After incubation for 5-10 minutes at RT, samples were centrifuged at 12000 g for 10 minutes at 4°C and the supernatants were discarded. Then, 75% ethanol was added (1 ml for 1 ml Tripure Isolation Reagent) to the samples which were then vortexed and centrifuged at 7500 g for 5 minutes at 4°C. After removing the supernatants, samples were placed on a paper towel for the removal of excess ethanol

by air-drying. The RNA pellets were resuspended in nuclease-free dH_2O by pipetting. RNA samples (85 µl) were mixed with 10 µl of 10X DNase1 Buffer (#14785300, Roche) and 5µl (5U) of DNaseI (#14785300, Roche). After that, tubes were incubated at 37°C for 30 minutes. For RNA clean-up, 260 µl nuclease-free dH_2O, 40 µl 3M NaAc (pH 5.2) and an equal volume (400 µl) of PCIAA (acidic phenol:chloroform:isoamylalcohol 125:24:1, pH 4.5-5) was mixed with the DNaseI treated 100 µl reactions. The tubes were quickly mixed by inverting and then centrifuged at 12000 g for 12 minutes at 4°C. About 350 µl of the aqueous phase of each sample was then transferred into a new tube. To minimize the RNA loss, 100 µl nuclease-free dH_2O was added to each sample and mixed. Then, the tubes were centrifuged at 12000 g for 12 minutes at 4°C. Aqueous phase (130 µl) was transferred into the previous tubes (total volume 480 µl). Equal volume of isopropanol (480 µl) was added to the tubes and centrifugation was repeated at 12000 g for 12 minutes at 4°C. After the supernatants were discarded, the pellets were washed with 1 ml of 75% ethanol and vortexed. The tubes were then centrifuged at 12000 g for 12 minutes at 4°C again and the pellets were dissolved in 30 µl nuclease-free dH_2O. Samples were kept at -80°C until use.

The RNA samples were diluted to 250 ng/µl in nuclease-free dH_2O. Prepared samples (1 µg) were run on a Formaldehyde (FA) gel, which was prepared by mixing 1.2 g agarose, 10 ml 10X FA gel buffer and 90 ml DEPC-treated dH_2O. After the mixture was heated in a microwave and cooled down to 65°C, 1.8 ml formaldehyde (37%, 12.3M) and 1 µl (0.5 µg/ml) ethidium bromide (EtBr) were added. The gel mixture was poured into the gel support left for polymerization. The gel was equilibrated in 1X FA gel running buffer for 30 minutes at 100V. RNA samples were mixed with 4X RNA loading dye and incubated at 65°C for 3-5 minutes. After that, samples were loaded into the FA gel, which was run at 100V for 1 hour in 1X FA gel running buffer. Finally, the gel was visualized under UV light.

4.3.2. cDNA Synthesis

Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used for cDNA synthesis. The RNA sample (1 µg) was mixed with random hexamer primers (12 µM) or oligo dT (0.5 µM), nuclease-free dH_2O, Reverse Transcriptase reaction buffer (1X), RNase inhibitor (40 U/µl), dNTPs (1 mM), DTT (5 mM) and reverse transcriptase (10 U) (RT samples) in a total volume of 20 µl. As a negative control, mixes were prepared without the reverse transcriptase. Prepared tubes were incubated at temperatures shown as in Table 4.1. The cDNA samples were further diluted to 100 µl with nuclease-free dH_2O and stored in -80 °C until use.

Table 4.1. Reaction conditions for cDNA synthesis.

Temperature	**Incubation Time**
29°C	10 minutes
48°C	55 minutes
85°C	5 minutes

4.3.3. qRT-PCR

Specific primers for KIM-1 and HPRT1 (normalizer) (Table 3.2) were used in the PCR. 2X Greenstar Mastermix (10 µl) (Bioneer), forward primer (0.25 µM), reverse primer (0.25 µM), 2.5 µl diluted cDNA template and nuclease-free dH_2O were mixed in a total volume of 20 µl for one reaction. The amounts of the ingredients were adjusted according to the triplicate number. After the mastermixes were deployed into the tubes of a 96-well plate, the plate was covered with a transparent cover and spinned down by centrifugation at 3000 rpm for 2 minutes. Then, the plate was placed into the Exicycler 96 RT-PCR machine (Bioneer). According to the protocol, first denaturation was done at 95°C for 10 minutes, then denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, scanning and extension at 72°C for 15 seconds was done for a total of 35 cycles. Finally, melting curve analysis was performed to examine the specificity of the amplifications by heat-

ing the samples from 60°C to 94°C and recording fluorescent signal at every 1°C temperature change. Relative quantitative measurements of KIM-1 mRNA was performed using the $2^{\Delta\Delta Ct}$ method.

4.4. Biochemical Methods

4.4.1. Cell Lysis and Protein Extraction from HK2 cells

HK2 cells were exposed to 10 μM OTA, 2 mg/ml GM or 5 μM CP along with an untreated (control) group. Cells were washed with PBS and then lysed with 400 μl, phosphatase and protease containing RIPA (Radio Immuno Precipitation Assay) buffer in 10-cm plates. After incubation for 5 minutes on ice, lysates were collected into 1.5-ml Eppendorf tubes using a plastic scraper. Samples were homogenized by passing through 25-gauge syringes 5-6 times. Then, samples were centrifuged at 14000 g at 4°C for 15 minutes and supernatants were transferred into new 1.5-ml Eppendorf tubes.

4.4.2. Quantification and Preparation of Protein Lysates

Protein concentrations were measured using BCA Protein Assay (Pierce) according to the manufacturer's protocol. Different concentrations of bovine serum albumin (BSA) were used to construct the standard curves in order to measure protein concentrations in the samples. After incubation, absorbance of the samples was measured at 570 nm in an ELISA Reader (Model 680, Bio-Rad, USA). After calculating protein concentrations, 15 μg sample was mixed with appropriate amount of 4X protein loading dye and incubated at 95°C for 5 minutes. The samples were loaded into the 8-12% SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) gel.

4.4.3. SDS-PAGE

Resolving gel (12%) was prepared in a falcon tube and it was poured between the glass plates. After about 15 minutes, polymerization was complete. Stacking gel (4%) was prepared and poured on top of the polymerized resolving gel using 1.5 mm thick comb for well formation. After stacking gel polymerization was complete, the gels were placed in the Western blotting tank filled with 1X SDS running buffer. Finally, combs were removed and previously prepared samples were loaded into the wells along with the protein ladder.

4.4.4. Western Blotting

Samples were run at 100V in the stacking gel and then at 150V in the separating gel. After that, samples were transferred onto PVDF (polyvinylidene fluoride) membranes in 1X Transfer Buffer at 100V for 1.5 hour. Membranes were blocked for 1 hour on orbital shaker at RT in 5% skim milk (w/v) containing TBS-T. Primary antibodies were prepared in TBS-T/5% BSA/0.02% sodium azide. Membranes were incubated overnight at 4°C with the primary antibodies. Next day, membranes were washed three times for 5 minutes with TBS-T. Membranes were then incubated in HRP-linked anti-mouse or anti-rabbit secondary antibodies (1:2000) prepared in 5% skim milk (w/v) containing TBS-T. Again, membranes were washed three times for 5 minutes using TBS-T and incubated in Lumi-Glo chemiluminescent substrate (Cell Signaling Technologies) for 5 minutes. Finally, protein bands on the membranes were detected using a chemiluminescent visualization system (Stella, Raytest). β-Actin was used as a loading control in Western blot analyses.

4.5. Molecular Biological Techniques

4.5.1. Preparation of Chemically Competent *Escherichia coli* Cells

Escherichia coli DH5α cells were grown in 5 ml lysogeny broth (LB) at 37°C overnight. Next day, grown cells were diluted in a 1/100 ratio in fresh LB. The culture was grown until its optical density (OD) at 595 nm reached to 0.4. Then, the culture was incubated on ice for 20 minutes and centrifuged at 4500 rpm for 10 minutes at 4°C. After the supernatant was discarded, the pellet was resuspended in 12.5 ml 50 mM $CaCl_2$ and incubated on ice for 20 minutes. Centrifugation was performed at 4500 rpm for 10 minutes at 4°C and supernatant was discarded. Pellet was resuspended in 1600 µl of 0.1 M $CaCl_2$ containing 15% glycerol solution. Finally, aliquots were prepared by dispensing 50 µl of competent cells into 1.5-ml Eppendorf tubes which were then immediately frozen in liquid nitrogen and kept at -80°C until use.

4.5.2. Transformation of Competent *E. coli* DH5α

Ligation products (10 µl) were transferred into the competent cell mixture (50 µl) and mixed gently by pipetting. The tubes were incubated on ice for 30 minutes. For heat shock, the tubes were incubated at 42°C for 90 seconds. Then, the tubes were incubated on ice for 5 minutes for DNA take-up. LB (900 µl) was added to the cells and left for incubation at 37°C on a shaker for 1 hour to recover. Finally, 100 µl of cells were spread onto LB agar plates containing the appropriate antibiotic.

4.5.3. PCR Purification of DNA Samples

PCR products were purified using NucleoSpin Gel and PCR Clean-up Kit (740609.50, Macherey-Nagel) according to the manufacturer's protocol.

4.5.4. Agarose Gel Extraction of DNA Samples

A clean scalpel was used to excise the DNA fragments of interest from the agarose gel under UV light. Excised products were purified using NucleoSpin Gel and PCR Clean-up Kit (740609.50, Macherey-Nagel) following the manufacturer's protocol.

4.5.5. Restriction Digestion of DNA

Digestion reactions were performed in the presence of 1X FastDigest Green Buffer (Thermo Scientific), the appropriate FastDigest enzymes (Thermo Scientific) and the DNA to be digested (up to 1 µg for each reaction) according to the manufacturer's protocol.

4.5.6. Ligation

Ligation reactions were performed at a 1:3 or 1:4 molar ratio considering the sizes of the used insert and vectors to be ligated. Each reaction (10 µl) contained 100 ng of the plasmid, the appropriate amount of the insert, 1 µl of T4 ligase (400000 units/ml) (NEB) and 1X T4 DNA ligase buffer (NEB). The reactions were incubated either at RT for 1 hour or 16°C overnight.

4.5.7. Plasmid Isolation

Mini and midi plasmid isolations were performed using the GeneJET Plasmid Miniprep Kit (00117731, Thermo Scientific) and NucleoBond Xtra Midi Kit (740410.2, Macherey-Nagel) according to the manufacturer's protocol, respectively.

4.6. Transient Transfections and Luciferase Reporter Assays

HK2 cells were grown in DMEM/F12 medium in either 6-cm plates ($5x10^5$ cells/plate) or 12-well plates ($7.5x10^4$ cells/plate) for 24 hours. Plate type and cell number varied according to the specific purpose of the experiment. DNA (1 μg) was mixed with 2.5 μl Lipofectamine-2000 transfection reagent (Invitrogen) in 250 μl or 100 μl FBS- and antibiotics-free medium for 6-cm plates and for 12-well plates, respectively. Then, transfection reagent-DNA mixtures were incubated at RT for 30 minutes for complex formation. DMEM/F12 medium was added to the FBS- and antibiotics-free medium in which transfection reagent-DNA complexes have formed, and then the mixture was layered onto the cells. After 6 hours, the complex including medium was replaced with growth medium, and luciferase measurements were performed on the day after.

The luciferase reporter assays were performed using the Dual Luciferase Assay (Promega) according to the manufacturer's protocol. Briefly, the medium was vacuumed and the cells were rinsed with PBS. Passive Lysis Buffer (250 μl) was added to each well and the plates were incubated at RT on orbital shaker for 15 minutes. Lysates (100 μl) were taken into the 96-well white opaque plates and the plates were inserted into Fluoroskan Ascent FL Luminometer (Thermo Scientific). The luminometer was adjusted to dispense 100 μl firefly luciferase substrate and measure the luminescence with 1-second integration time. After the measurement, 100 μl Stop & Glo® Reagent was added into the wells, incubated for 15 minutes and the luminescence from the Renilla luciferase was measured again with 1-second integration time.

The luciferase reporter assays performed for the promoter bashing analyses were done using the pClucBasic2 vector (NEB), which contained the Cypridina luciferase whose product is excreted into the medium by the transfected mammalian cells. pTK-Gluc (NEB) plasmid which contained the Herpes Simplex Virus thymidine kinase (TK) promoter driven Gaussia luciferase was used as the transfection control. Luciferase measurements were performed following the manufacturer's protocol.

4.7. Electrophoretic Mobility Shift Assay (EMSA)

4.7.1. Preparation of Nuclear Protein Extract

HK2 cells exposed to either 10 μM OTA, 2 mg/ml GM or 5 μM CP along with an untreated (control) group for 24 hours were collected by scraping into 3 ml PBS/0.5mM EDTA. Collected cells were centrifuged at 300g for 2 minutes. The cell pellets were dissolved in Hypotonic Buffer Solution and incubated on ice for 30 minutes. NP-40 (IPGAL) was added to the mixture to the final concentration of 0.5%, then, the mixture was centrifuged at 12000 rpm for 5 minutes. Supernatant (cytoplasmic extract) was transferred into another tube for later use. The nuclear pellets were dissolved in the Extraction Buffer Solution and incubated on ice for 30 minutes. As a last step, centrifugation was done at 12000 rpm for 5 minutes and the nuclear protein extract was obtained. Finally, glycerol was added to be 10% as the final concentration and the extracts were stored at -80 °C until use.

4.7.2. Designing and Biotinylation of the Probes

About 20-30 nucleotide long wild type or mutant probes (with 2 or 3 base changes in the consensus regions) flanking the putative AP1 binding sites were designed and ordered from Macrogen (Korea). These probes were designed to have a 3' overhanging nucleotide when annealed for easier biotinylation process. Single-stranded oligonucleotides were mixed at equal concentrations in annealing buffer. Then the oligonucleotide mixtures were heated up to 95°C for 2 minutes and cooled down slowly to RT so that they were annealed as double helix probes. Finally, those double-stranded probes were biotinylated using 3' End Labeling Kit (Thermo Scientific, 89818) following the manufacturer's protocol. Wild type and the mutant probes designed for the EMSA experiments were shown in Table 4.2.

Table 4.2. Wild type and mutant probes for AP1 binding region at -1010 and -1303 from the translation start site of *Kim-1*. Wt, wild type; mut, mutant. Bold lowercases indicate the substituted nucleotides for the mutant probes.

Probe Name	**Probe Sequence**
AP1_1010_Wt	5'- CAGCATTGTGAGTGACAGAGCCTG -3'(+) 5'- AGGCTCTGTCACTCACAATGCTGG -3'(-)
AP1_1010_Mut	5'- CAGCATTGTGAG**gtg**CAGAGCCTG -3'(+) 5'- AGGCTCTGCAC**ctc**ACAATGCTGG -3'(-)
AP1_1303_Wt	5'-AGTACTAGGCAAGCTTACTCATGTTTGTATG-3'(+) 5'-ATACAAACATGAGTAAGCTTGCCTAGTACTT-3'(-)
AP1_1303_Mut	5'-AGTACTAGGCAAGC**cac**CTCATGTTTGTATG-3'(+) 5'-ATACAAACATGAG**gtg**GCTTGCCTAGTACTT-3'(-)

4.7.3. Nuclear Extract (NE)-Probe-Antibody (Ab) Binding Reaction

The reactions shown in Table 4.3 were established in order to obtain NE-probe or NE-probe-Ab complexes.

Unlabeled wild type or mutant competitors were added, when necessary, in order to check the specificity of the shifted complexes. In some reactions, mutant probes were used to check the specificity of the probe.

Native polyacrylamide gels were prepared using 0.5X Tris Borate EDTA (TBE) and run for 30 minutes for equilibriation. Prepared complexes were loaded on the gel and run at 100V in 0.5X TBE for 45 minutes.

Table 4.3. Reactions established for the EMSA experiments. BB, binding buffer; ssDNA, salmon sperm DNA; NE, nuclear extract, Ab; antibody; Comp, competitor.

Ingredient	**Probe**	**Probe NE**	**Probe NE Ab**	**Probe Comp 50x**	**Probe Comp 100x**
dH_2O	11 µl	11 µl	9 µl	8,5 µl	6 µl
Binding Buffer (5X)	2 µl	2 µl	2 µl	2 µl	2 µl
ssDNA (1 µg)	1 µl	1 µl	1 µl	1 µl	1 µl
NE (5 µg)	-	5 µl	5 µl	5 µl	5 µl
Extraction Buffer Solution	5 µl	-	-	-	-
Ab (2 µg)	-	-	2 µl	-	-
Probe (50 fmol)	1 µl	1 µl	1 µl	1 µl	1 µl
Competitor	-	-	-	2.5µl (2.5 pmol)	5µl (5 pmol)

4.7.4. Detection of DNA Probe-Protein Complexes

Using the semi-dry blotting equipment (Bio-Rad, USA), complexes on the gel were transferred to the positively charged nylon membrane (Zeta-Probe Membrane) applying 3.55 mA/cm^2 current for 30 minutes. After that, membranes were exposed to UV light (120 mjoule/cm^2) for crosslinking (Spectronics Corporation, USA). EMSA bands on the membrane were visualized using Biotin Detection Kit (Thermo Scientific, 20148) according to the manufacturer's protocol.

4.8. Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation assays were performed according to the literature as described with minor modifications (Aparicio et al., 2005, Kuo and Allis, 1999, Nelson et al., 2006).

4.8.1. Lysate Preparation

HK2 cells were grown in 15-cm plates to 100% confluency (about $5x10^6$ cells per experiment). Formaldehyde (Sigma) was added to a final concentration of 1% (v/v) into the 20 ml medium containing plate and the cells were incubated 10 minutes at RT with gentle shaking. In order to quench the reaction, glycine solution (Fisher Scientific) was added to a final concentration of 0.125M. Cells were further incubated for 5 minutes at RT with gentle shaking and then they were scraped and transferred into a 50-ml falcon tube followed by centifugation at 2000 rpm for 2 minutes. After the supernatant was discarded, the pellet was washed twice with 10 ml PBS and centrifuged in between. ChIP Lysis Buffer containing 0.25% SDS and protease inhibitor cocktail (Roche) was used to resuspend the cells (250 µl buffer for $1x10^6$ cells) and cells were incubated for 1 hour on ice. DNAs present in the lysates were sheared into 300-1000 bp fragments by using the sonicator (Bioruptor) as 15 cycles of 1-minute sonication with 60% power, and 2-minute incubations in between. After sonication, lysates were centrifuged at 13000 rpm for 5 minutes. Finally, the supernatant was transferred into new eppendorf tubes and kept at -80°C until use.

4.8.2. Preparation of Input DNA

Prepared lysates (430 µl of the total lysate) were incubated in the presence of 0.0625 µg/µl RNase A for 45 minutes at 39°C in order to get rid of RNA. Proteins were removed by incubating the samples with 0.2 µg/µl Proteinase K (Invitrogen) for 30 minutes at 55°C followed by boiling for 10 minutes. After that, an equal volume of phenol-chloroform (pH 7.7-8.3, Sigma) was added, mixed and centrifuged at 14000 rpm for 10 minutes at RT. Upper aqueous phase was transferred into a new tube and 2.5 volume of 100% Ethanol (Sigma), 1/10 volume of 3 M Sodium Acetate (Sigma) and 1 µl (5 mg/ml) glycogen were added. The mixtures were incubated at -20°C for 2 hours and then centrifuged at 14000 rpm for 10 minutes. After liquid phase removal, 500 µl of 70% ethanol was added and centrifuged at 14000 rpm for 5 minutes at 4°C. Again the liquid phase was removed and the pellets were air-dried. Finally, the pellets were dissolved in 20 µl nuclease-free dH_2O.

4.8.3. Immunoprecipitation of Transcription Factor-Bound DNA Fragments

For immunoprecipitation, 650 µl chromatin lysate (1-5 million cell equivalents of lysate per ChIP), c-Jun (AP1) antibody and rabbit IgG were used. Dyna1 Protein A/G magnetic bead (Invitrogen) mix (50 µl) was washed twice with PBS-T for each antibody. Antibodies (5 µg) were mixed with the washed magnetic beads and the total volume was brought up to 150 µl with PBS-T. The mixture was incubated at RT for 45 minutes on an orbital shaker. Magnetic beads were then washed with 500 µl PBS-T and 500 µl ChIP lysis buffer lacking SDS. Finally, magnetic beads were dissolved in 50 µl ChIP lysis buffer solution lacking SDS.

Previously prepared 650 µl chromatin lysate was diluted 3-fold with ChIP lysis buffer solution. After that, diluted lysate and antibody-bound magnetic beads (50 µl) were mixed together and incubated at RT for 45 minutes by shaking. Then, the magnetic beads were washed twice with ChIP lysis buffer solution, twice with ChIP lysis buffer solution supplemented with 1 M NaCl and twice with TE buffer solution. Later, the beads were dissolved in 100 µl TE buffer. After that, magnetic beads were boiled for 10 minutes and incubated on ice for 5 minutes. In order to remove the proteins, Proteinase K (200 ng/µl) (Invitrogen) treatment was performed for 30 minutes. Finally, mixture was boiled for 10 minutes. Immunoprecipitated DNAs were isolated from the mixtures using ChIP DNA Isolation Kit (Qiagen) by following the manufacturer's protocol. Each ChIP reaction yielded 16 µl immunoprecipitated DNA for each antibody (c-Jun and rabbit IgG).

4.8.4. Polymerase Chain Reaction

Input and immunoprecipitated DNAs were diluted in a 1/100 and 1/20 ratio, respectively. Primers were chosen from the previously designed probes of EMSA experiments. Two primer sets flanking the -1010 upstream region of *Kim-1* gene were used in the PCR reactions (Table 4.4). One set amplified the region between -1278 and -968 producing a 332 bp product (SET 1), and the other set amplified between -1322 and -968 yielding a 380 bp PCR product (SET 2). PCR reactions were performed taking the Tm values of the primers into consideration (Table 4.5 and 4.6).

Table 4.4. ChIP probe sets for PCR of AP1 binding reaction. Minus sign depicts the number of bases upstream of the $A^{+1}TG$ codon (translation start site) of the *Kim-1* gene.

Recognition site from the TSS	**Primer sequence (5' to 3')**	**Expected fragment size (bp)**
-1278/-968	TGTATGGTTTAATGATTAACAGCAGAAGT	332 (SET1)
	GAATGCTTCAGGAAACAGAAGTCTGGAGC	
-1322/-968	CCAGAAGAGGAGTTAATGAAGTACTAGGCA	380 (SET2)
	GAATGCTTCAGGAAACAGAAGTCTGGAGC	

Table 4.5. Ingredients of PCR Reactions for the amplification of DNAs. DNAs were captured with the input DNA and proteins along with a negative control (no template).

Ingredient	**Input DNA (µl)**	**Immunoprecipitated DNA (µl) (with specific Ab or rabbit IgG)**	**Negative Control (µl)**
DNA	2.5	1	-
Forward primer (10µM)	1.6	1.6	1.6
Reverse primer (10µM)	1.6	1.6	1.6
2X PCR mix	10	10	10
dH_2O	4.3	5.8	6.8

Table 4.6. PCR reaction protocol for the ChIP experiments.

Reaction Step	**Temperature**	**Incubation Time**	**Cycle**
Initial denaturation	95°C	10 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	66°C	30 seconds	
Elongation	72°C	30 seconds	

4.9. Cloning of 5'-Deleted Mutant Constructs of Human *Kim-1* Gene

Human *Kim-1* gene promoter was cloned by PCR amplifying the 2000 bp upstream region of *Kim-1* gene where the potential promoter was thought to be present. For this purpose, specific forward and reverse primers containing EcoRV and KpnI restriction sites, respectively, were designed in order to produce deletional mutant constructs of *Kim-1* gene promoter. The reverse primer was designed to anneal to the region 400 bp upstream of the translation start site as the 5' RACE data indicated this site to be the transcriptional initiation site.

Table 4.7. PCR reaction protocol for the production of *Kim-1* upstream fragments.

Reaction Step	**Temperature**	**Incubation Time**	**Cycle**
Initial denaturation	95°C	6 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	63°C	30 seconds	
Elongation	72°C	1:30 minutes	
Final elongation	72°C	15 minutes	1

After the PCR amplifications, the *Kim-1* upstream regions were digested and ligated into the pCLucBasic2 vector and transformed into *E. coli* DH5α competent cells.

4.10. Site-Directed Mutagenesis

In order to amplify the 2000 bp *Kim-1* upstream region with the mutant AP1 site at position -1010 relative to the $A^{+1}TG$ translation initiation codon, overlap-extension PCR was performed using the primers given in Table 3.2. The left (990 bp) and the right (1010 bp) regions were amplified with the indicated primers (Table 4.8). In the subsequent PCR reaction, the outer primers were used to amplify the whole region with AP1 mutant site (Table 4.9) (Figure 4.1, mutation is indicated with a star).

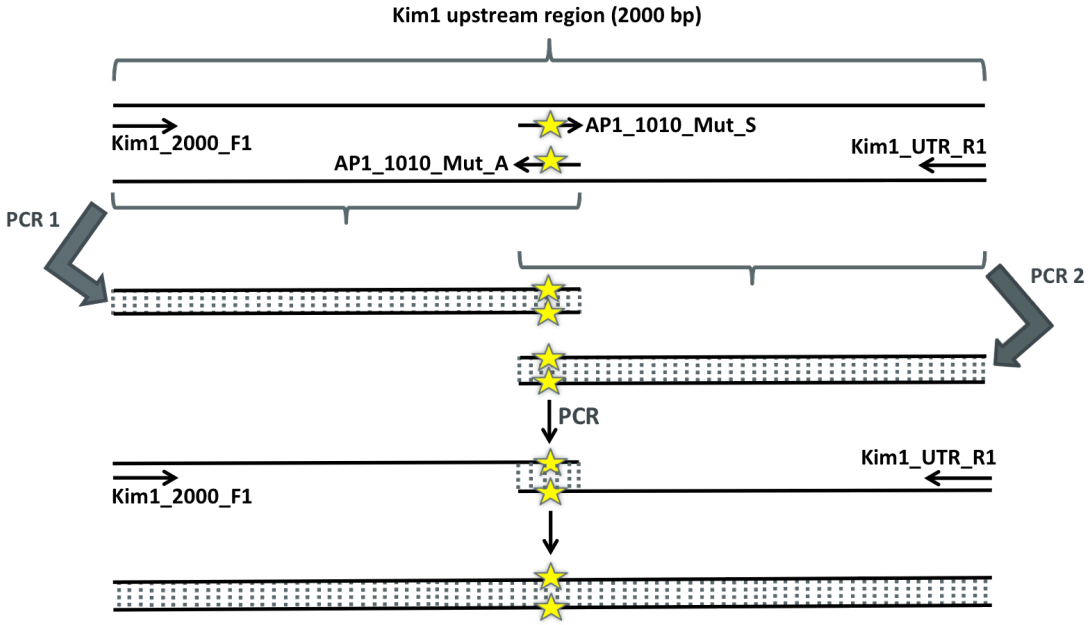

Figure 4.1. Schematic representation of overlap-extension PCR.

Table 4.8. Reaction protocol for overlap-extension PCR.

Reaction Step	**Temperature**	**Incubation Time**	**Cycle**
Initial denaturation	95°C	3 minutes	1
Denaturation	95°C	30 seconds	40
Annealing	68.5°C	30 seconds	
Elongation	72°C	1:30 minutes	
Final elongation	72°C	15 minutes	1

Table 4.9. PCR reaction protocol for the production of 2000 bp *Kim-1* upstream region. The region contains the mutant AP1 site at position -1010 relative to the $A^{+1}TG$ translation initiation codon.

Reaction Protocol	**Temperature**	**Incubation Time**	**Cycle**
Initial denaturation	95°C	2 minutes	1
Denaturation	94°C	1 minute	40
Annealing	55°C	1 minute	
Elongation	72°C	2 minutes	
Final elongation	72°C	7 minutes	1

As a result of overlap-extension PCR, the mutant product (AP1*) was obtained and cloned into pGEM®-T Easy (Promega) vector. After digesting the insert with EcoRV and NcoI enzymes (ThermoScientific), it was cloned into the NcoI-SmaI digested pGL3-Control vector (5256 bp). By digestion, the control vector lost its promoter region becoming pGL3E (pGL3_enhancer vector). Wild type and mutant -1010 AP1 site bearing vectors were transfected into HK2 cells and their luciferase activities were measured. Transient transfections and luciferase measurements were performed as described previously in Section 4.6 using 12-well plates.

4.11. Cloning of KIM-1 Open Reading Frame Region into Expression Vectors

In order to clone the KIM-1 ORF into protein expression vectors, total RNA was extracted from HK2 cells (Section 4.3.1) and converted into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) (Section 4.3.2). Using indicated primers in Table 3.2, KIM-1 open reading frame (ORF) was amplified and cloned into pGEM®-T Easy (Promega) vector. After dropping the insert with BamHI and XhoI enzymes (ThermoScientific), it was cloned into pET30a(+) and pMEV2HA vectors digested with the same restriction enzymes.

In order to produce the recombinant KIM1 protein, a culture was started from the Rosetta bacterial strain colony previously transformed with pET30a(+)_ORF using 5 ml LB containing 30 µg/ml Kanamycin and incubated on an orbital shaker at 37°C overnight. Next day, a fresh culture was started from the overnight cultures (1/10 dilution) in 25 ml LB containing 30 µg/ml Kanamycin, and the culture was incubated at 37°C. After the bacterial culture reached its log phase (about 3 hours later from the dilution), it was divided into two 50-ml falcon tubes; one was induced with 1 mM IPTG and the other one was left uninduced. Then, both cultures were incubated on a shaker at 22°C or 37°C for induction to occur. Culture samples were collected with one-hour intervals over 4 hours and a sample was taken from the uninduced culture as negative control. Each sample was centrifuged at 4500 rpm for 10 minutes and dissolved in 100 µl RIPA buffer for lysis. Sonication was performed for 1 or 2 cycles in order to reinforce the lysis of the cells. After the lysates lost their viscosity, 45 µl of the each total lysate was taken as an aliquot and mixed with the 6X protein loading dye. Then, the remaining lysates were centrifuged at 14000 rpm for 5 minutes and 45 µl of the resulting supernatant was also taken as an aliquot into another tube, which was mixed with the 6X protein loading dye. Finally, total lysate and supernatant samples were incubated at 95°C for 5 minutes for denaturation, and loaded on 12% SDS-PAGE gel. After the protein ladder was visualized as separate bands, the gels were washed with dH_2O and incubated in destaining solution for 5 minutes on a shaker. Then, the gels were incubated in Coomasie Blue staining solution for 5 minutes on a shaker. In order to visualize the protein bands, stained gels were destained using destaining solution until the background was clear.

Cloning into pMEV2HA plasmid was performed following the usual cloning protocol (Section 4.5). Transient transfections were done for the obtained constructs and Western blotting was performed to see exogenous KIM-1 protein expression. Presence of recombinant protein was checked using anti-HA antibody as the vector tags the protein with Human influenza hemagglutinin (HA) from its amino end.

4.12. Statistical Analysis

Experiments were performed in at least three independent occasions in triplicates and their quantitative data were analyzed using Graphpad Prism v6. The data were presented as mean ±SEM. Standard deviations (±SD) were shown for each parameter for the experiments that were performed only once but in triplicates and statistical significance among the compared groups was evaluated by Student's T-test.

5. RESULTS

5.1. Effect of Chemical Treatment on Cell Viability

In order to determine the appropriate chemical concentrations of OTA, GM and CP at which HK2 cells do not undergo extensive necrosis or apoptosis but show the stress responses mimicking nephrotoxicity *in vivo*, XTT cell viability assays were performed using the Cell Proliferation Kit II (Roche).

Although this kit is a proliferation kit, under chemotoxic stress conditions it is used to measure cell viability. Therefore, the experiments were designed for various concentrations of OTA, GM and CP for 24 hours in DMEM/F12 medium containing 5% FBS, 1% Penicillin-Streptomicin.

Significant chemotoxic response with the decrease in cell number was observed at 10 μM OTA (75.9% cell viability) (Figure 5.1), 2000 μg/ml GM (63.9% cell viability) (Figure 5.2), and 5 μM CP treatment (59.7% cell viability) (Figure 5.3).

For our experimental design, most of the cells should be viable and show chemotoxic responses; therefore, the concentrations with approximately 60% cell viability were selected for further experiments.

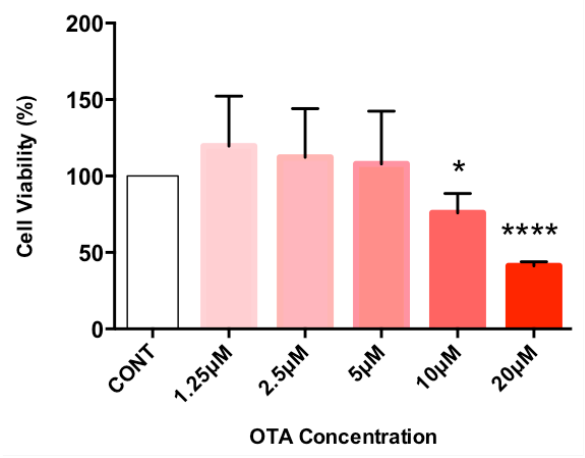

Figure 5.1. Effect of OTA on HK2 cell viability. Treatments were performed with various concentrations of OTA for 24 hours. CONT; vehicle (EtOH). The data represent the mean ±SD of triplicates. ($P < 0.05$, *; $P < 0{,}0001$, ****).

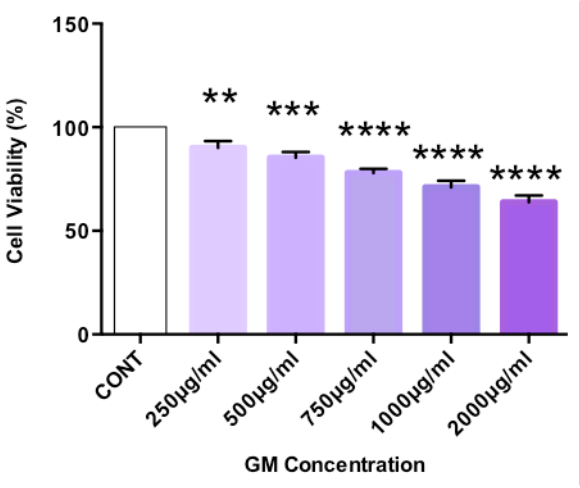

Figure 5.2. Effect of GM on HK2 cell viability. Treatments were performed with various concentrations of GM for 24 hours. CONT; vehicle (dH_2O). The data represent the mean ±SD of triplicates. ($P < 0.05$, **; $P < 0.05$, ***; $P < 0{,}0001$, ****).

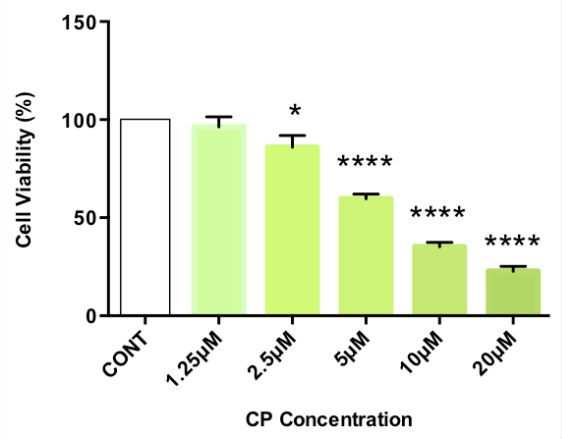

Figure 5.3. Effect of CP on HK2 cell viability. Treatments were performed with various concentrations of CP for 24 hours. CONT; vehicle (0.9% saline). The data represent the mean ±SD of triplicates. (P < 0.05, **; P < 0.05, ***; P < 0,0001, ****).

5.2. KIM-1 mRNA Expression in Response to Chemical Nephrotoxins

Determination of the changes in KIM-1 mRNA levels upon chemical treatments were crucial for understanding the regulation of *Kim-1* gene at transcriptional level. Therefore, KIM-1 mRNA expression levels were determined by quantitative real time PCR (qRT-PCR) in HK2 cells exposed to 10 µM OTA, 2 mg/ml GM and 5 µM CP or left untreated.

There was a significant decrease in KIM-1 mRNA levels upon treatment for 24 hours with OTA and CP; however, mRNA levels did not significantly change in GM treated cells (Figure 5.4).

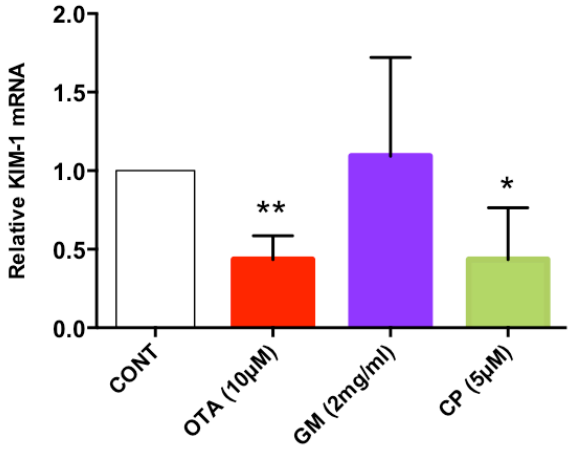

Figure 5.4. Relative quantification of KIM-1 mRNA. KIM-1 mRNA levels of HK2 cells treated with either 10 μM OTA, 2 mg/ml GM or 5 μM CP for 24 hours were measured. HPRT1 was used as reference gene and the expression values were normalized to the untreated group. Relative quantitative measurements of KIM-1 mRNA was performed using the $2^{\Delta\Delta Ct}$ method. The data represent the mean±SEM of replicates ($P < 0.05$, *; **).

5.3. Effect of Cell Confluence on the Expression of KIM-1 protein

It was previously shown by immunohistochemistry that, in regenerating epithelial cells of post-ischemic rat kidney, expression of KIM-1 protein colocolized with BrdUrd-labeled nuclei, which was an indication of cell proliferation (Ichimura *et al*., 1998). The behavior of surviving cells to proliferate by undergoing mitogenesis in order to fill and repair the injured kidney tissue *in vivo* could be similar to the behavior of cells seeded at less denser *in vitro* culture conditions used in the experimental setup. Therefore, we reasoned to test this possibility by seeding the HK2 cells at various cell density and examining the expression of KIM-1 protein by Western blotting. For this reason, HK2 cell confluency was optimized for the Western blotting experiments. The results showed that the amount of KIM-1 protein inversely correlates with the number of HK2 cells seeded in the untreated (CONT) groups (Figure 5.5). Moreover, KIM-1 protein amount increased in OTA (10 μM) treated cells compared to the untreated cells. In summary, when HK2 cells were plated initially at 70% confluence and collected after 48 hours, KIM-1 protein levels were kept at minimum levels in the control groups similar to *in vivo* conditions.

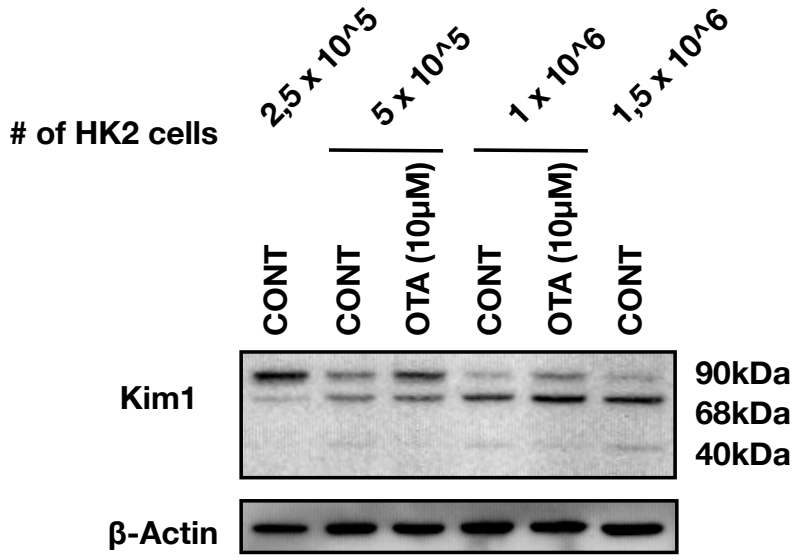

Figure 5.5. Effect of cell confluence on KIM-1 protein amount. HK2 cells were treated with 10 μM OTA or left untreated for 24 hours and followed by Western blotting for KIM-1 protein detection. β-Actin was used as loading control.

5.4. KIM-1 Protein Levels upon Treatment with Different Chemicals

According to the literature, KIM-1 protein amounts were expected to increase upon chemotoxic stress as shown in animal models and human specimens. In order to test if the chosen chemicals can induce similar responses in our *in vitro* cell culture system, HK2 cells were treated with 10 μM OTA, 2 mg/ml GM and 5 μM CP or left untreated when they were at 90% confluence for 24 hours. According to Western blot analyses, three different sizes of KIM-1 protein bands were observed at 90, 68 and 40 kDa. Ninety-kDa band was the major band, which was mainly influenced by the chemical treatment. KIM-1 protein was observed to increase approximately 3-fold upon OTA treatment (Figure 5.6). Although there was no significant change in the 90 kDa protein band, 68 and 40 kDa protein bands seemed to accumulate in GM or CP treated cells (Figure 5.6).

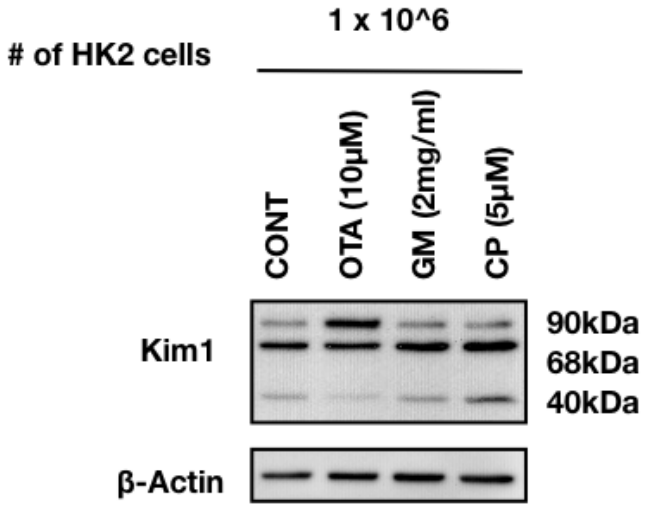

Figure 5.6. KIM-1 protein amounts in HK2 cells upon chemical treatment. HK2 cells were treated with 10 μM OTA, 2 mg/ml GM and 5 μM CP for 24 hours and Western blotting was performed for KIM-1 protein detection. β-Actin was used as loading control.

5.5. Characterization of Human *Kim-1* Gene Promoter Region

As a first step to characterize the human *Kim-1* gene promoter, the upstream region of human *Kim-1* gene was examined by deletional mutation analysis of the immediate 2000 bp upstream region where the potential promoter was thought to reside. Amplifications of the fragments with various sizes were achieved by PCR using forward primers specific to the -2000 (F7), -1800 (F6), -1500 (F5), -1100 (F4), -900 (F3), -700 (F2) and -505 (F1) with a reverse primer at -400 (R1) from the $A^{+1}TG$ –translation start site (Figure 5.8). All of the seven 5'-deleted *Kim-1* upstream fragments were successfully amplified for the subsequent cloning procedure.

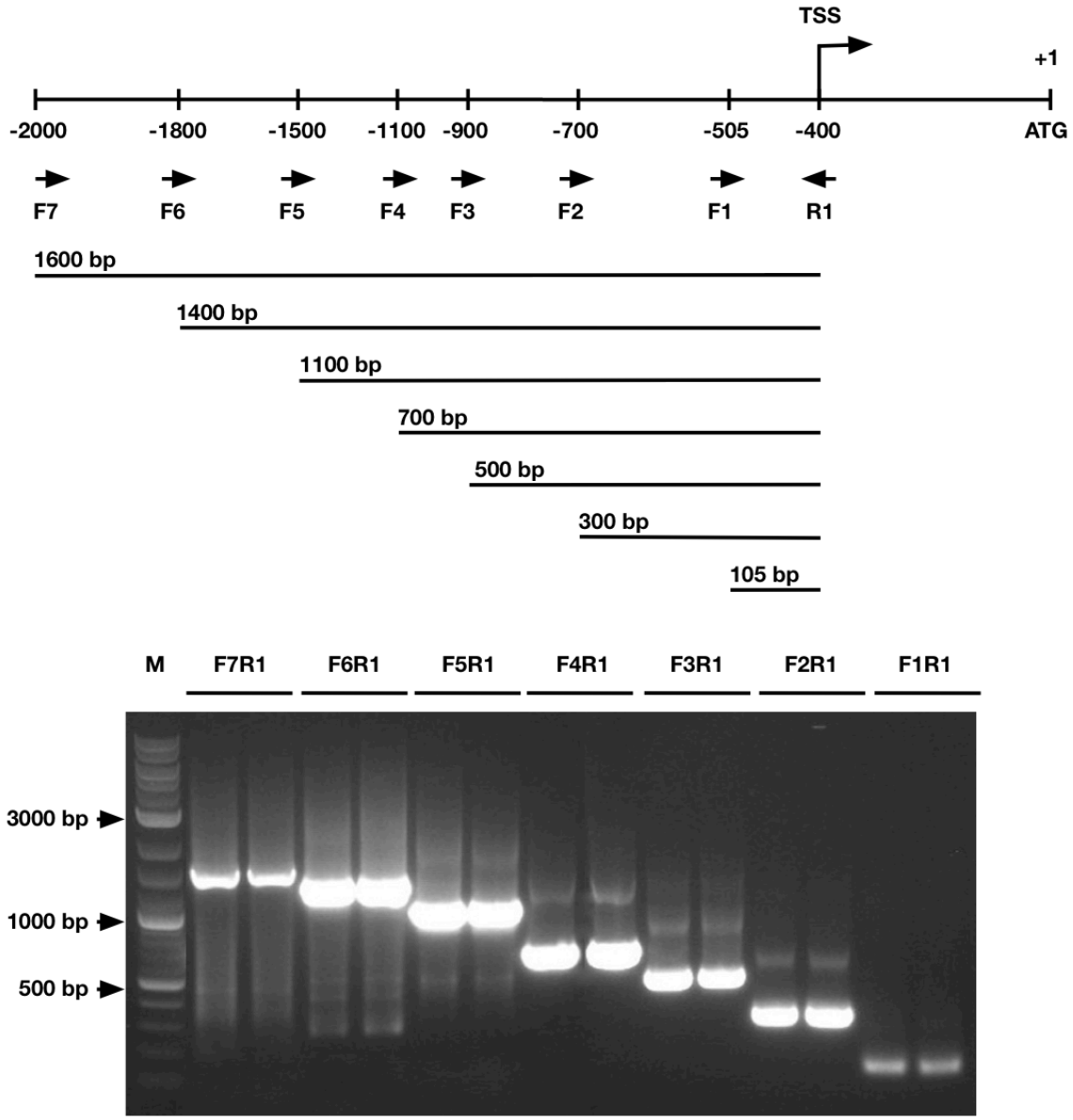

Figure 5.7. PCR amplification of 5'-deleted *Kim-1* upstream fragments for pCLucBasic cloning. F, forward primer; R, reverse primer; TSS, trancription start site; ATG, first codon at the translation start site.

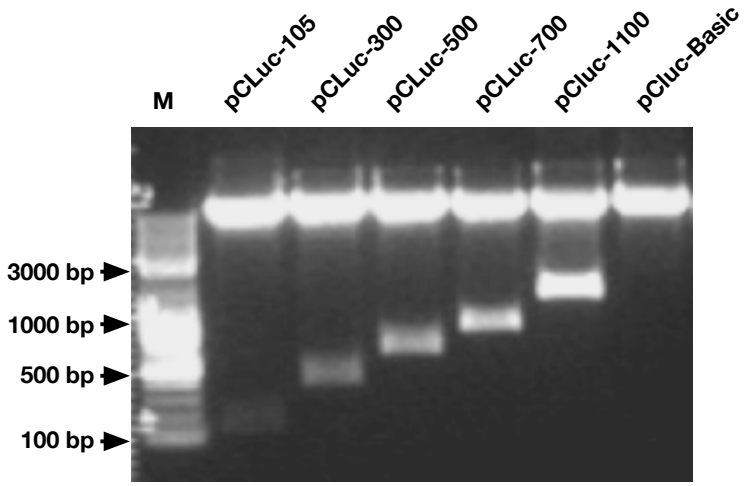

Figure 5.8. Analytic digestion of pCLuc plasmids containing the various 5' upstream regions of *Kim-1* gene. EcoRV and KpnI enzymes were used for the analytic digestion.

A series of 5'-deleted *Kim-1* upstream constructs (pCLuc) were generated in the PCR reactions where F1R1 (pCLuc-105), F2R1 (pCLuc-300), F3R1 (pCLuc-500), F4R1 (pCLuc-700), F5R1 (pCLuc-1100) primers were used. However, pCLuc-1400 and pCLuc-1600 constructs could not be obtained (Figure 5.9). Then, the 5'-deletion constructs were analyzed by sequencing followed by alignment to the reference genome sequence (Kim-1_2000UP) retrieved from the NCBI (National Center for Biotechnology Information) database (Figure 5.10).

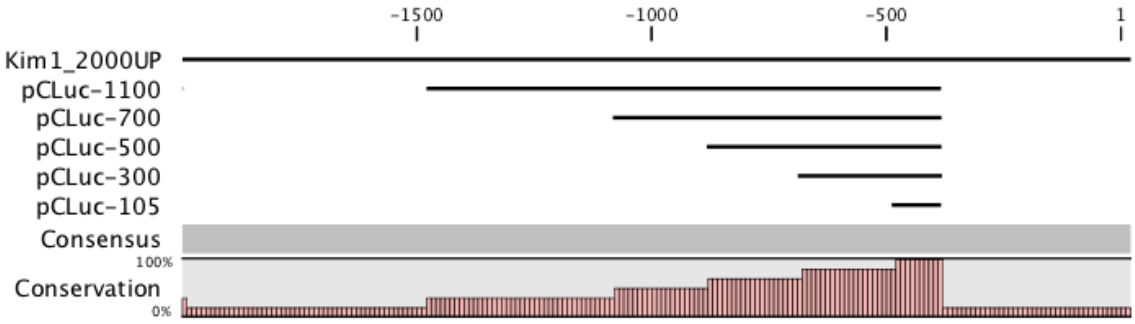

Figure 5.9. Alignment of the 5'-deletion constructs of the upstream region of *Kim-1* gene. Translation start site is indicated as "1".

In order to see the effect of each 5'-deleted region on the regulation of *Kim-1* gene activity, prepared promoter-reporter constructs were transiently transfected into HK2 cells and their relative luciferase activities were compared to the promoterless, empty vector. The maximum luciferase activity (1.8-fold) was observed for the -700 to -400 upstream region of *Kim-1* gene (pCLuc-300) (Figure 5.11). The -900 to -400 upstream region of *Kim-1* gene (pCLuc-500) also yielded a nominal increase in luciferase activity (1.2-fold) compared to the empty vector (pCLucBasic2). Therefore, the minimal promoter might reside within the -700 to -400 upstream region of *Kim-1* gene. The decrease in luciferase activity in further upstream parts of the analyzed region suggested that the regions between the -1100 to -700 might harbor inhibitory *cis*-acting sequences.

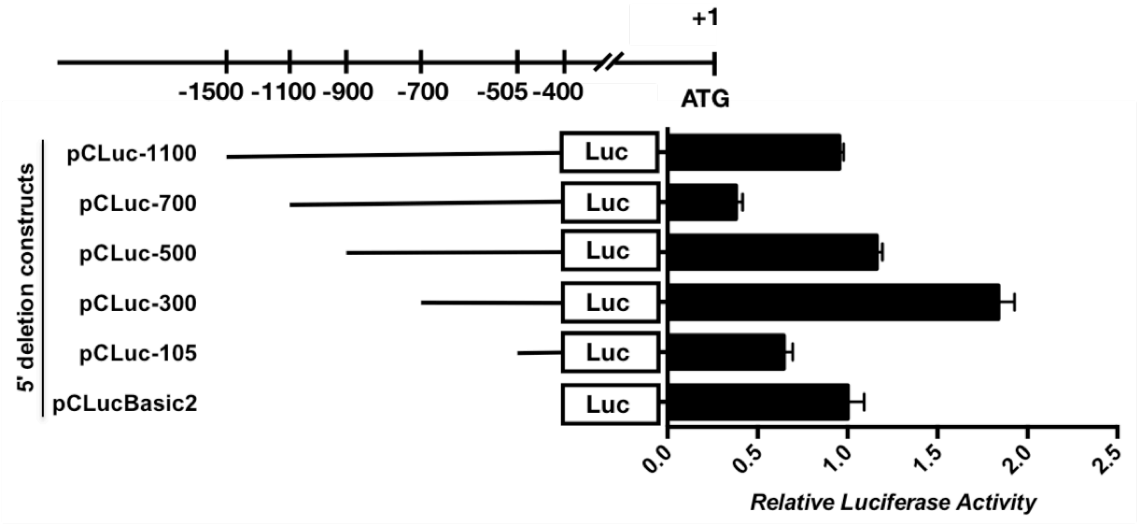

Figure 5.10. Relative luciferase activity of the *Kim-1* 5'-deletion constructs. Luciferase activities were measured after 24 hours for the untreated HK2 cells. The Cypridina/Gaussia luciferase activity of each construct was given relative to the activity of pCLucBasic2. Error bars represent the ±SEM of replicates.

In order to see the effect of chemotoxic stress on the regulation of *Kim-1* gene, the luciferase activities of the 5'-deletion constructs were also measured under chemical treatments. No significant change was observed in the luciferase activities of the constructs upon treatment with 10 μM OTA, 2 mg/ml GM and 5 μM CP for 24 hours (Figure 5.12). A similar pattern was observed for the untreated cells transfected with the 5'-deletion constructs of *Kim-1* gene upstream regions (Figure 5.11). The -700 to -400 upstream region of

Kim-1 gene (pCLuc-300) caused an 1.6-fold increase of the luciferase activity compared to pCLucBasic2 (Figure 5.12) and the -900 to -400 upstream region of *Kim-1* gene (pCLuc-500) caused an 1.3-fold increase of the luciferase activity compared to pCLucBasic2 (Figure 5.12).

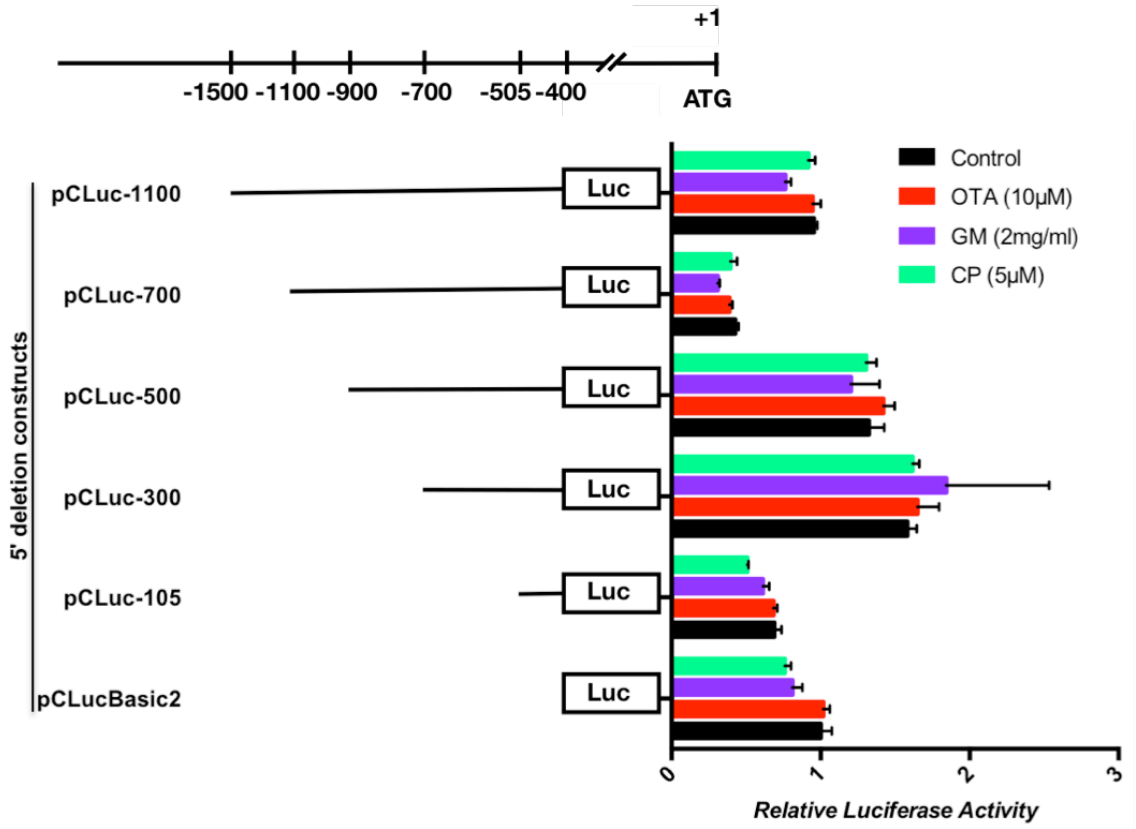

Figure 5.11. Relative luciferase activities of the *Kim-1* 5'-deletion constructs under chemotoxic stress. Luciferase activities were measured from the transfected HK2 cells treated with 10 μM OTA, 2 mg/ml GM and 5 μM CP for 24 hours. The ratios of Cypridina/Gaussia luciferase activities of each construct were given relative to the activity of pCLucBasic2. The data represent the mean ±SEM of replicates.

In addition to the previous luciferase assays, another luciferase system with pGL3Basic vector was used in order to analyze the promoter region of *Kim-1* gene. This was mainly due to the difficulties in cloning of the whole 2000 bp upstream region of *Kim-1* gene into the pCLucBasic2 vector. This procedure was especially necessary for the comparison of the luciferase activities of the mutant (AP1 site at -1010) and wild type luciferase-reporter constructs.

In this case, PCR amplifications of the 5'-deleted *Kim-1* upstream fragments were performed for the 2000 bp and 700 bp fragments (Figure 5.13). Moreover, the reverse primer was designed to be complementary to the translation start site ($A^{+1}TG$) in order to examine the effect of the region starting from +1 to -400 upstream of *Kim-1* gene, which was excluded in the previous system (Figure 5.8 and 5.13).

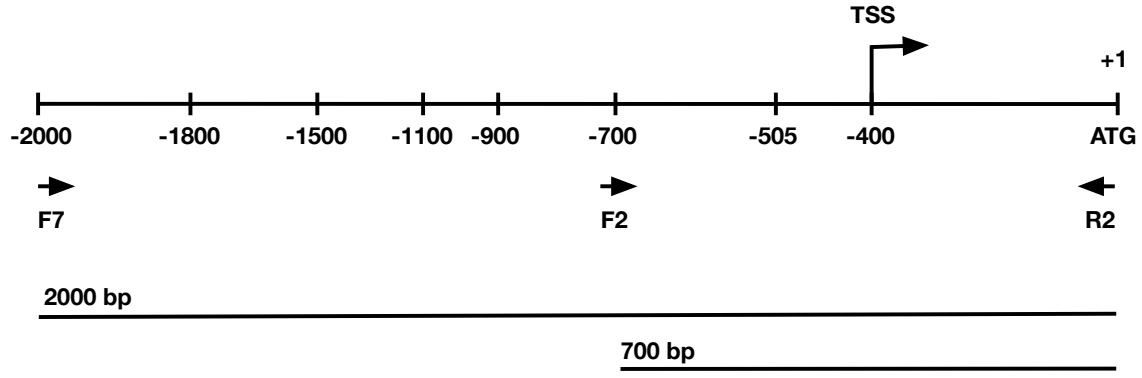

Figure 5.12. Schematic representation of PCR amplification of 5'-deleted *Kim-1* upstream fragments for cloning into pGL3 reporter plasmids. F, forward primer; R, reverse primer; TSS, trancription start site; ATG, first codon at the translation start site.

In order to see if the presence of an enhancer sequence would have any effect on *Kim-1* promoter activity and to have more reliable luciferase readings, the enhancer sequence from pGL3Control vector was cloned into the pGL3Basic vector yielding the pGL3Enhancer (pGL3E) vector. After cloning the 2000 bp (pGL3E_2000) and the 700 bp (pGL3E_700) upstream regions of *Kim-1* gene into pGL3E vector, the luciferase activity was determined to be about 2.5-fold for the pGL3E_700 construct compared to the empty

vector (pGL3E). The activity of pGL3E_2000 construct was 0.2-fold of the empty vector (pGL3E). This might indicate the presence of some inhibitory elements within the region between the -2000 to -700 upstream of *Kim-1* gene. Moreover, no significant change was observed for pGL3E_2000 or pGL3E_700 among the treatment groups (Figure 5.14).

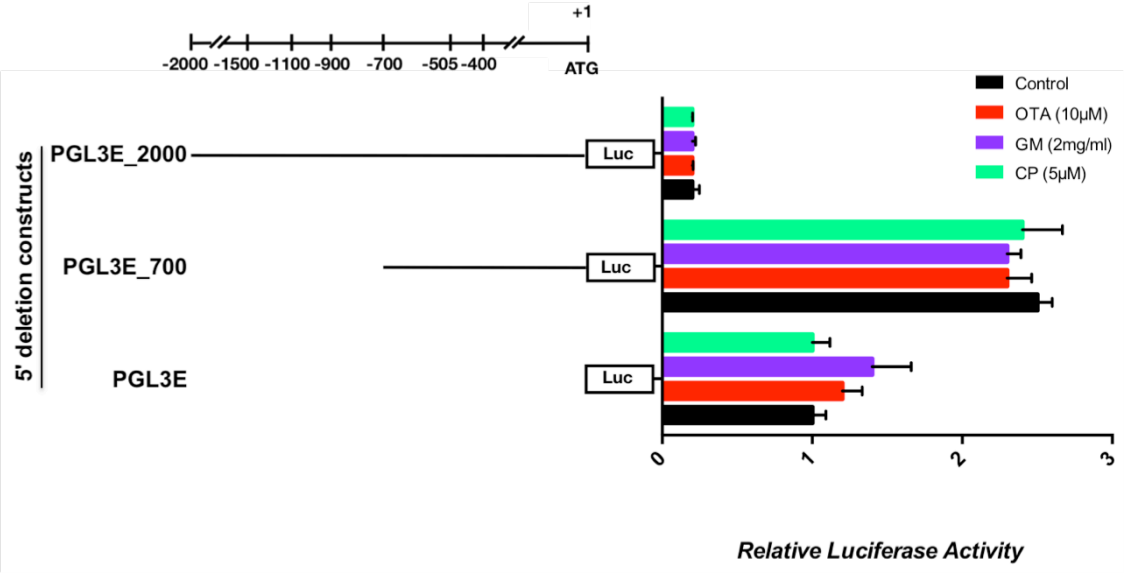

Figure 5.13. Relative luciferase activity of the pGL3E_2000 and pGL3E_700 constructs. pGL3E; promoterless vector with the enhancer. The Firefly/Renilla luciferase activity of each construct was given relative to the activity of pGL3E. The data represent the mean ±SEM of replicates.

5.6. Activator Protein 1 (AP1) Protein Levels upon Treatment with Different Chemicals

In addition to the information provided by the literature (Section 1.4.3), Alibaba2 and Genomatix Genome Analyzer softwares were utilized to identify the putative *trans*-acting factors that may interact with the promoter region of *Kim-1* gene. Both softwares identified potential AP1 binding sites as well as other putative binding sites for various transcription factors (TFs) within the upstream region of *Kim-1* gene (Figure 5.14).

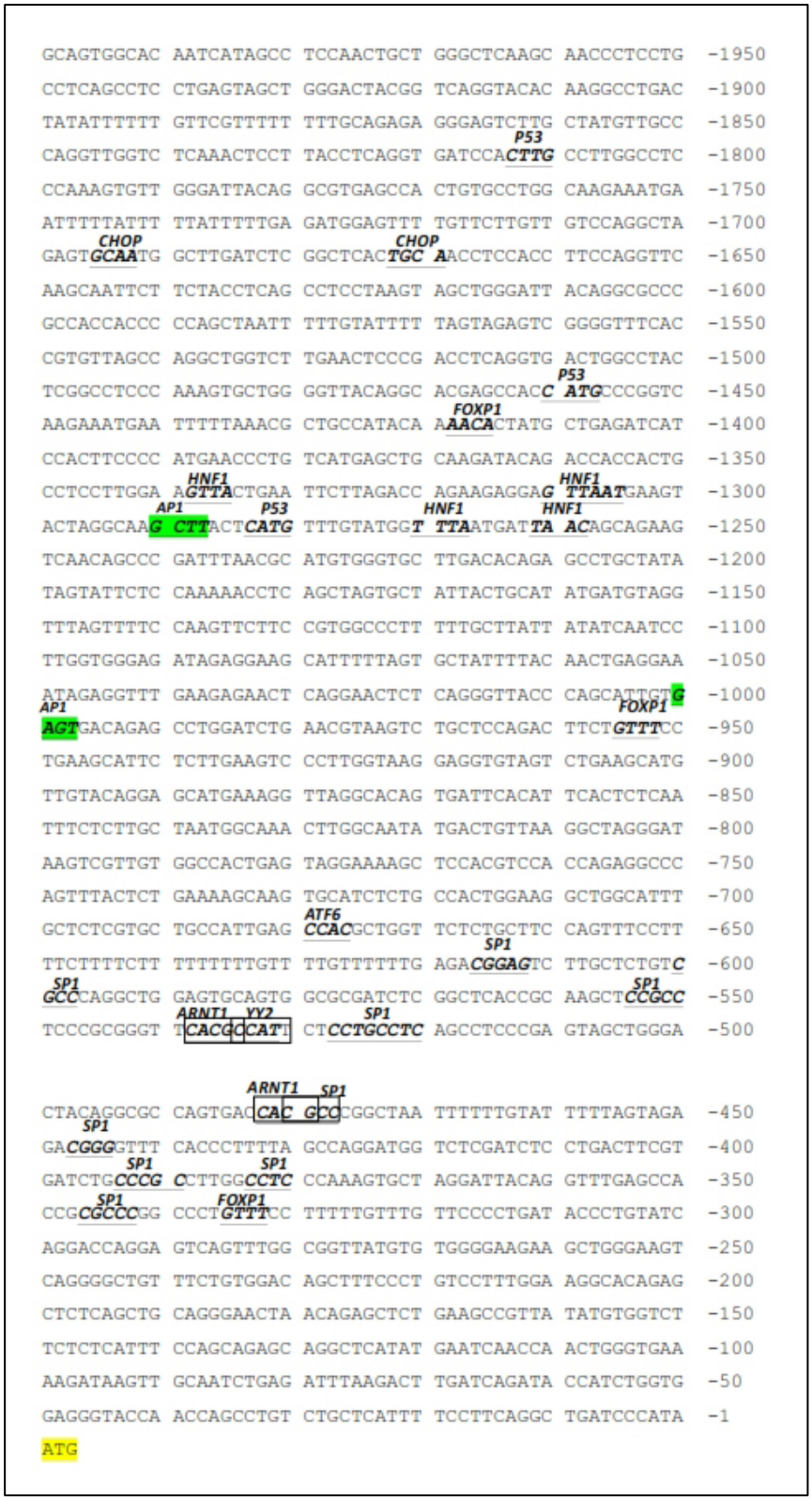

Figure 5.14. *In silico* analysis of *Kim-1* gene upstream region. *Kim-1* gene upstream region is shown from -2000 to the first $A^{+1}TG$ (highlighted in yellow). Sequences in bold, putative binding sites for TFs; green highlights, putative AP1 binding sites.

Presence of AP1 consensus binding sequences in the regulatory upstream region of *Kim-1* gene suggested that the levels of AP1 in the cell could influence the regulation of *Kim-1* gene under chemotoxic stress. Therefore, the levels of c-jun component of the AP1 complex were analyzed by Western blotting in extracts prepared from HK2 cells treated with 10 μM OTA, 2 mg/ml GM and 5 μM CP for 24 hours. c-Jun antibody was used for detection of AP1 during Western blotting experiments and the protein was observed at 42 kDa (Figure 5.15). Upon OTA treatment, there was a decrease in c-Jun protein amounts. However, GM and CP treatments caused an increase in c-Jun protein amounts (Figure 5.15). This observation might suggest that AP1 might work as a negative regulator of *Kim-1* gene because when KIM-1 protein was at high levels, AP1 protein levels were low, as in the case of OTA treatment.

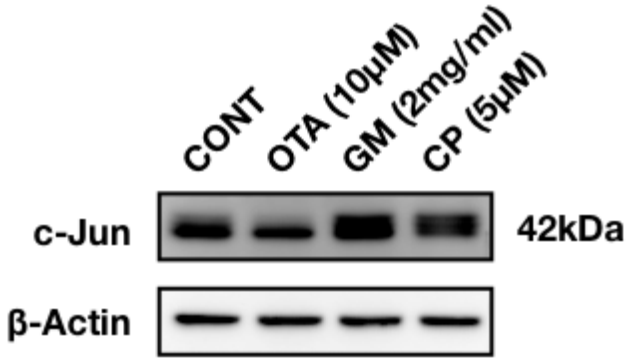

Figure 5.15. c-Jun protein amounts in HK2 cells upon chemical treatment. HK2 cells were treated with 10 μM OTA, 2 mg/ml GM and 5 μM CP or left untreated for 24 hours followed by Western blotting for c-Jun protein detection. β-Actin was used as loading control.

5.7. Interaction of AP1 Transcription Factor with *Kim-1* Gene Upstream Region

Transcription factors play a crucial role in gene regulation by binding to the promoter regions of their target genes. Identification of these interactions between transcription factors and their putative binding sites has importance in not only characterization of promoter regions but also understanding of gene regulation. Therefore, as a first step, the putative binding sites determined by *in silico* analyses should be confirmed by experimental methods. Thus, we utilized various experimental approaches to verify the physical interactions of AP1 and sequences within the upstream region of *Kim-1* gene predicted by *in silico* analyses.

5.7.1. Electrophoretic Mobility Shift Assay (EMSA)

In order to test if AP1 binds to the regulatory region of *Kim-1* gene, Electrophoretic Mobility Shift Assay (EMSA) was performed in various experimental conditions (Section 4.7). Probes designed for the EMSA experiments encompassed the -1010 (AP1_1010) and -1303 (AP1_1303) AP1 binding sites from the $A^{+1}TG$ of *Kim-1* gene.

According to the EMSA, the interaction of AP1_1010 probe comprising the putative AP1 binding site at -1010 resulted in specific protein-DNA complex (the shifted band) (Figure 5.16, lane 2). EMSA supershift analysis using the commercially available c-Jun antibody yielded no supershifted band but caused the fading of protein-DNA complex (Figure 5.16, lane 3). This confirmed AP1 binding to its consensus sequence at site -1010. Similarly, AP1_1303 probe comprising the putative AP1 binding site at -1303 resulted in a specific protein-DNA complex (Figure 5.16, lane 5), however, it did not yield any supershifted band or fading of the protein-DNA complexes (Figure 5.16, lane 6).

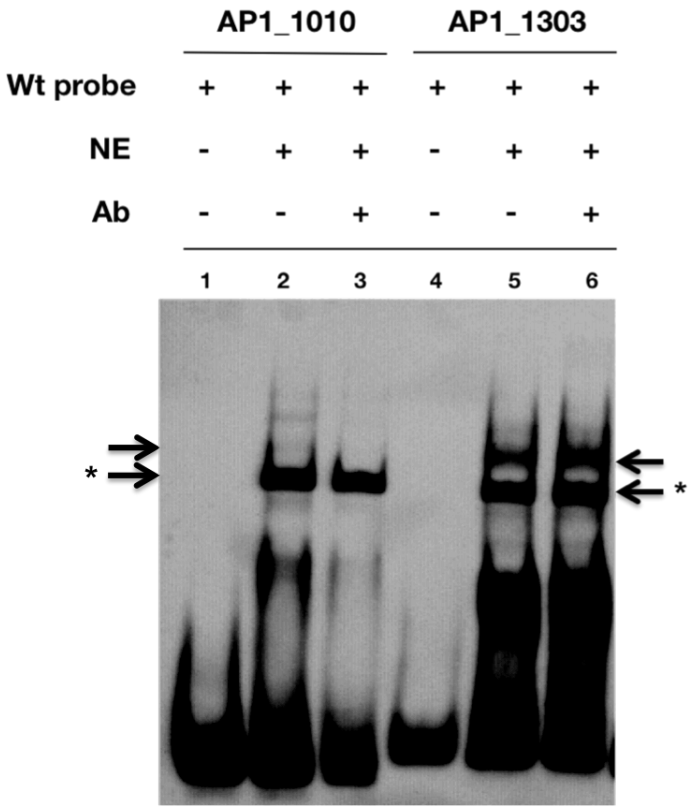

Figure 5.16. EMSA of nuclear protein interaction with probes AP1_1010 and AP1_1303. NE, nuclear extract; Ab, c-Jun antibody; +, present; -, absent from the reaction; wt, wild type; mut, mutant; comp; competitor; black arrow, shifted band; arrow with the asterix, non-specific band.

To confirm the specificity of the protein-DNA complex for the AP1_1010 probe, unlabeled wild type and mutant competitors were used at 50x and 100x fold in excess. When the wild type competitors were used, the shifted band disappeared indicating specific binding (Figure 5.17, lanes 4-5). However, the shifted band still appeared when the mutant unlabeled competitor was used (Figure 5.17, lanes 6-7).

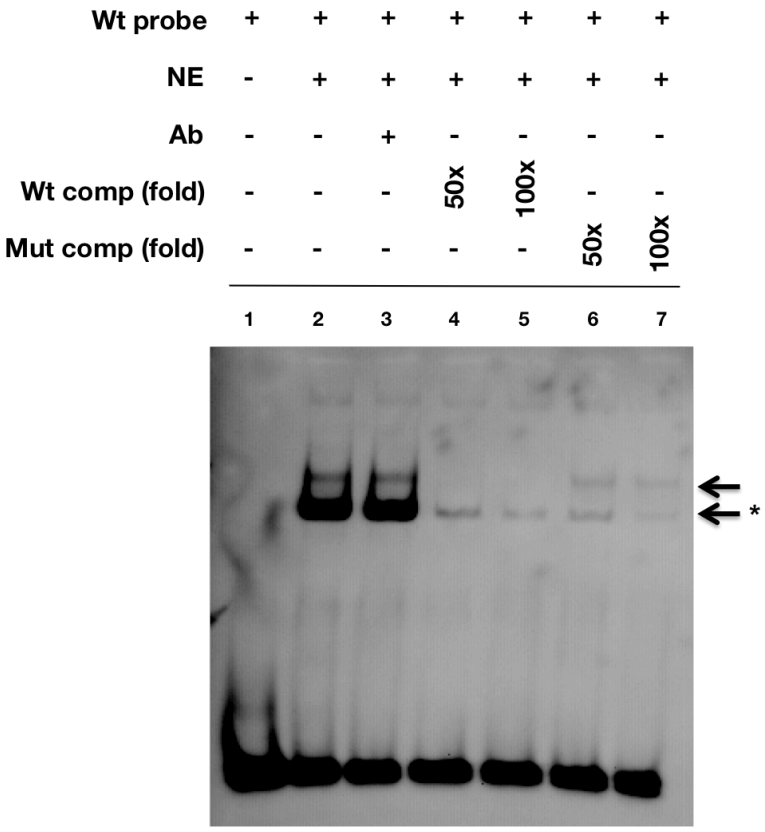

Figure 5.17. EMSA of nuclear protein interaction with probe AP1_1010. NE, nuclear extract; Ab, c-Jun antibody; +, present; -, absent from the reaction; wt, wild type; mut, mutant; comp; competitor; black arrow, shifted band; arrow with the asterix, non-specific band.

Commercially available c-Jun and Fra-2 antibodies were used in order to observe if those proteins were present in the protein-DNA complexes of HK2 cells. Previously, EMSA supershift analysis confirmed that c-Jun protein was present in protein-DNA complexes (Figure 5.18, lane 3); however, the supershifted band could not be observed. Similarly, the use of Fra-2 antibody also did not yield any supershifted band. Moreover, there was no fading of protein-DNA complexes in this case (Figure 5.18, lane 12), indicating that Fra-2 protein was absent from the complexes. This might indicate that Fra-2 was not a dimer component of AP1 transcription factor complex involved in *Kim-1* gene expression in HK2 cells.

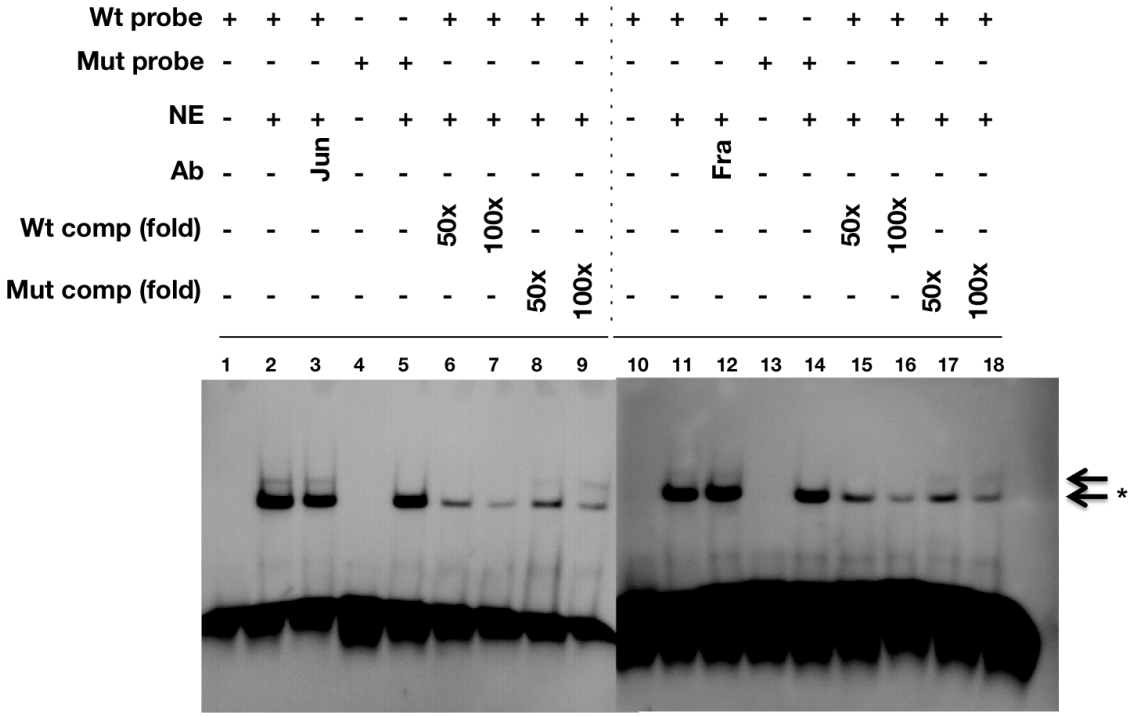

Figure 5.18. EMSA of nuclear protein interaction with probe AP1_1010 using c-Jun (Jun) and Fra-2 (Fra) antibodies. NE, nuclear extract; Ab, antibody; +, present; -, absent from the reaction; wt, wild type; mut, mutant; comp; competitor; black arrow, shifted band; arrow with the asterix, non-specific band; 50x and 100x, fold concentrations of the competitors.

To see if chemotoxic stress had any influence on AP1 protein binding on its consensus sequence at -1010 from the $A^{+1}TG$ of *Kim-1* gene, HK2 cells were treated with 10 μM OTA, 2 mg/ml GM and 5 μM CP or left untreated for 24 hours and nuclear proteins were extracted. Fading of the protein-DNA complexes (the shifted band) in the GM and OTA nuclear protein extracts was observed compared to the untreated nuclear protein extracts (Figure 5.19, lanes 2-3 and 5). However, the nuclear lysates of CP treated cells yielded a similar band with the untreated one (Figure 5.19, lanes 2-4). EMSA supershift analysis yielded no supershifted bands but fading of the protein-DNA complexes for both OTA treated (Figure 5.19, lanes 5-7) and untreated nuclear protein extracts (Figure 5.19, lanes 2-6) were observed. This might indicate that GM and CP treatment causes formation of AP1 or other complexes that do not contain c-Jun protein in contrast to control and OTA treated samples.

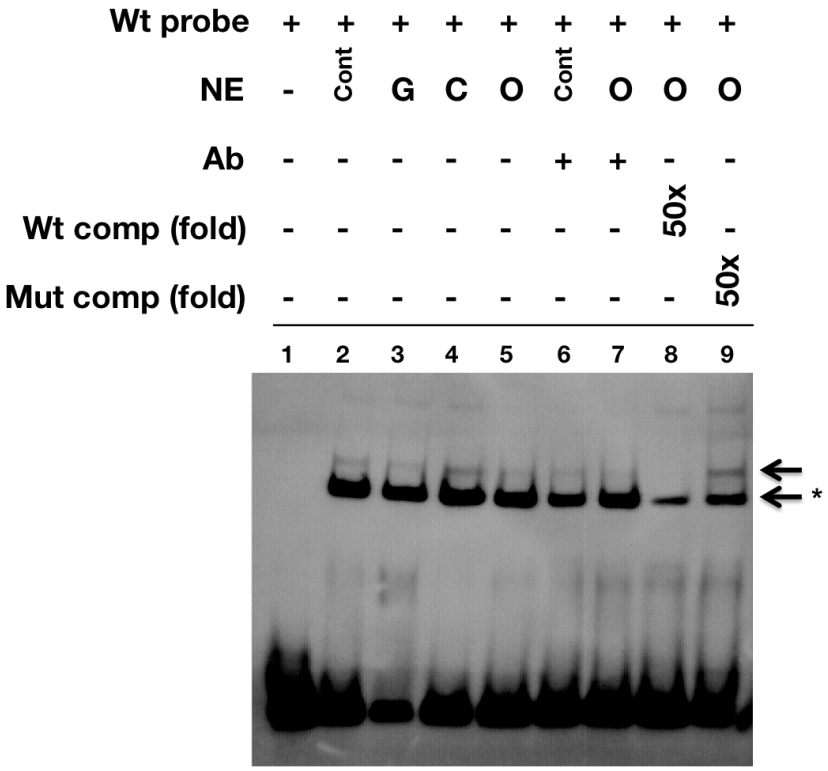

Figure 5.19. EMSA of chemically treated nuclear protein interaction with probe AP1_1010. Nuclear extracts were treated with 10 μM OTA (O), 2 mg/ml GM (G) and 5 μM CP (C) with a control NE (Cont). Ab, c-Jun antibody; +, present; -, absent from the reaction; wt, wild type; mut, mutant; comp; competitor; black arrow, shifted band; arrow with the asterix, non-specific band; 50x, fold concentration of the competitors.

5.7.2. Chromatin Immunoprecipitation (ChIP)

Interaction of AP1 with the regulatory region of *Kim-1* gene was also tested with chromatin immunoprecipitation (ChIP) assay. ChIP was performed and captured DNA was amplified using specifically designed primers flanking the AP1 binding regions.

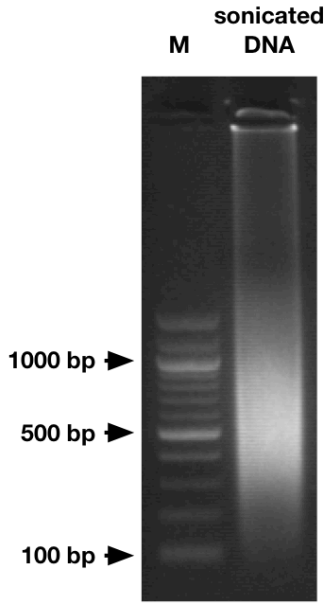

Figure 5.20. Sonicated DNA fragments from HK2 cells for the chromatin immunoprecipitation assay.

Two primer sets were designed and used to amplify the target regions; SET 1, amplifying between -1278 and -968 producing a 332 bp product and SET2, amplifying between -1322 and -968 yielding a 380 bp product.

Sonicated lysate from HK2 cells yielded DNA fragments between 300-1000 bp sizes (Figure 5.20) and immunoprecipitated DNA regions bound to c-Jun antibody were successfully amplified confirming the binding of c-Jun to the corresponding regions (Figure 5.21). Specificity of this binding was confirmed by the less and non-specific background seen in the reaction where the DNA was captured with rabbit IgG. The amplification in the Input reactions proved that the amplification in the "AP1" reaction was not due to any contamination but arised from the chromatin lysate. In the negative control (NTC) with no fragmented DNA, there was no amplification as expected.

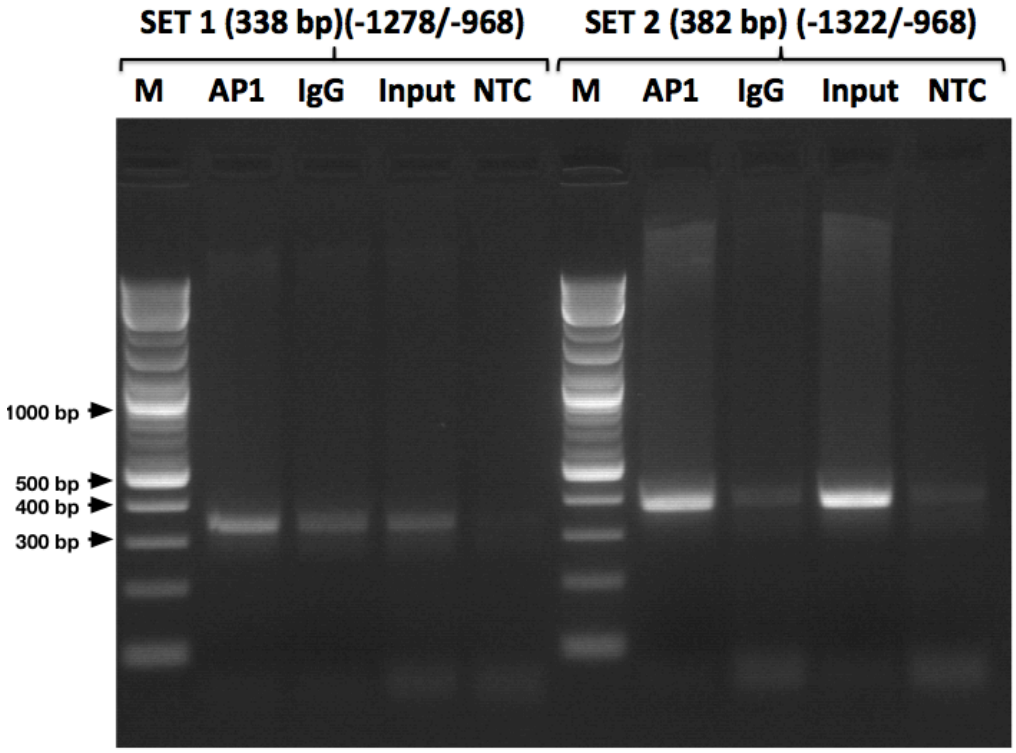

Figure 5.21. Analysis of AP1-*Kim-1* promoter interaction using ChIP Assay. AP1, DNA fragments that were captured with AP1 antibody (c-Jun); IgG, DNA fragments captured with rabbit IgG antibody; Input, the PCR reaction using the total fragmented DNA; NTC, the negative control reaction with no fragmented DNA.

In summary, AP1 binding to the -1010 region from the translation start site was confirmed by ChIP assays. However, chromatin lysates treated with OTA, GM or CP could also be used in order to better characterize the binding phenomenon of AP1 transcription factor on the promoter region of *Kim-1* gene because the binding affinity of this transcription factor might change under chemotoxic stress, which can therefore influence the gene activity.

5.8. Effect of AP1 Transcription Factor on *Kim-1* Gene Regulation

The impact of AP1 transcription factor binding to its consensus motifs within the promoter region of *Kim-1* gene was functionally examined by utilizing promoter-reporter constructs. For this purpose, the ability of the mutant (pGL3E_2000_AP1mut) and its wild type counterpart (pGL3E_2000) to drive the expression of *Kim-1* gene was compared by transient transfections into HK2 cells. The mutant vector was constructed by site-directed mutagenesis to carry a TGA to GTG substitution in the putative AP1 binding site at -1010 from the $A^{+1}TG$ (Section 4.10) (Figure 5.22, 5.23, 5.24 and 5.25).

Briefly, in order to obtain the mutant construct, overlap-extension PCR was employed using primers that have mutations corresponding to the putative AP1 binding site at position -1010 from the $A^{+1}TG$. As a first step, the left (990 bp) and the right (1010 bp) regions were amplified (Figure 5.22). In the second round of overlap-extension PCR, the outer primers were used to amplify the whole 2000 bp region with AP1 mutant site (AP1*) (Figure 5.23). Because of the limitations to obtain the mutant product (AP1*) due to the production of non-specific PCR products, it was extracted from the gel and cloned into pGEM®-T Easy (Promega) vector by T/A cloning (Figure 5.24). Colony 8 was thought to be positive as it yielded the expected bands after the screen with analytic digestion. The mutant insert was digested using EcoRV and NcoI restriction enzymes and cloned into the NcoI-SmaI digested pGL3-Control vector (5256 bp). Analytic digestion with KpnI and HindII enzymes revealed that the obtained clones (pGL3E_2000_AP1mut_1, pGL3E_2000_AP1mut_2 and pGL3E_2000_AP1mut_3) contained the insert (Figure 5.25).

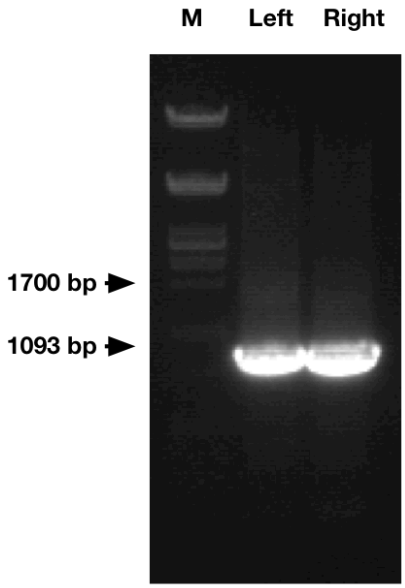

Figure 5.22. PCR amplification of the left and right regions for site-directed mutagenesis in overlap-extension PCR. M, marker; Left, 990 bp left PCR product; Right, 1010 bp right PCR product.

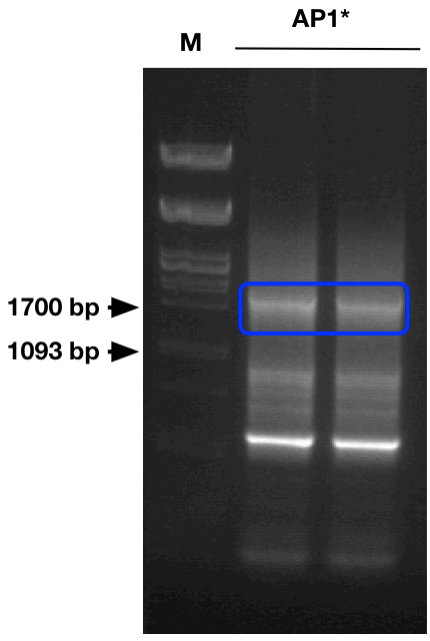

Figure 5.23. Amplification of the 2000 bp upstream region of *Kim-1* gene carrying mutation at putative AP1 binding site (AP1*) by overlap-extension PCR. The expected 2000 bp band is shown in the blue box.

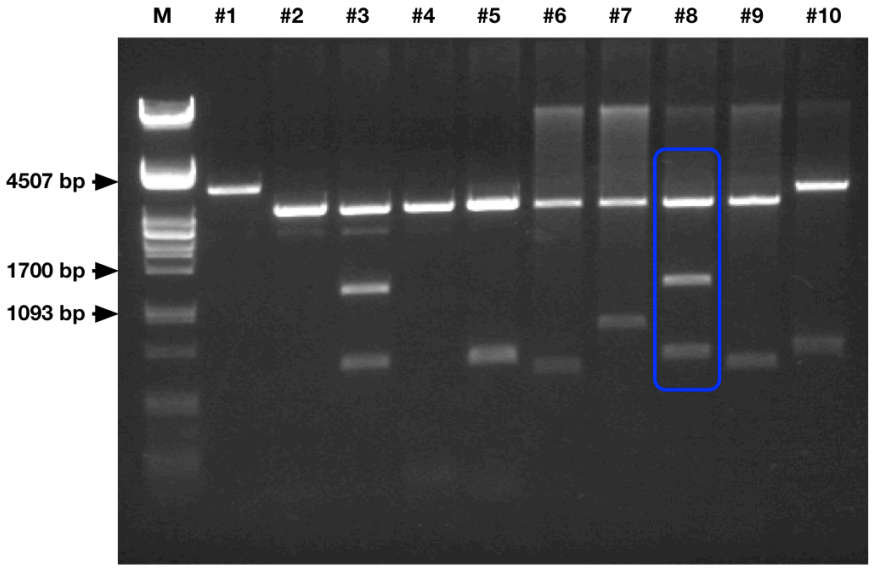

Figure 5.24. Analytic digestion for the plasmids obtained by T/A cloning. EcoRI restriction enzyme was used for the analytic digestion. Blue box; expected bands of the obtained clone.

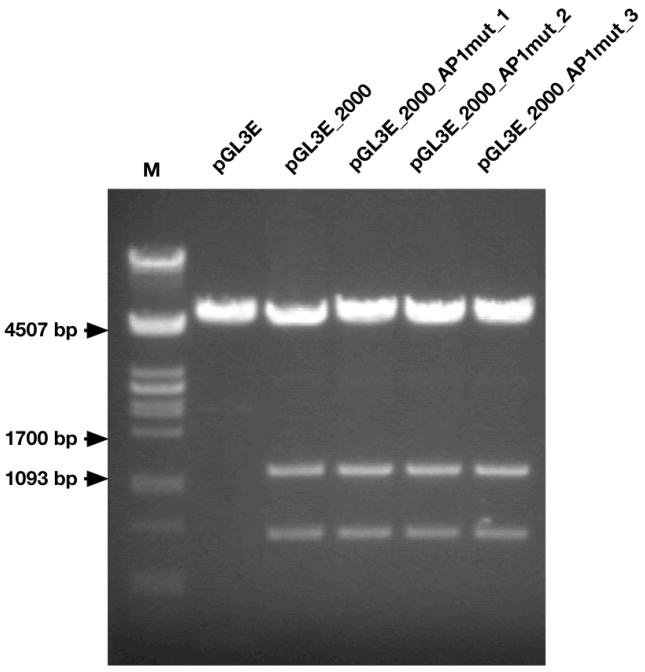

Figure 5.25. Analytic digestion of pGL3E_2000_AP1mut clones. KpnI-HindIII enzymes were used for the analytic digestion. pGL3E, empty plasmid; pGL3E_2000, wild type construct; pGL3E_2000_AP1mut_1/2/3, AP1 mutant constructs.

Obtained AP1 mutant clones (pGL3E_2000_AP1mut_1/2/3) were sequenced for the confirmation of the mutagenesis (TGA substitution into GTG at site -1010) (Figure 5.26 and 5.27) and they were also analyzed for the presence of any other point mutations. After the analysis, pGL3E_2000_AP1mut_1 was selected for use in transient transfection and luciferase activity assays, which were performed to examine the regulatory effect of this mutation on *Kim-1* gene.

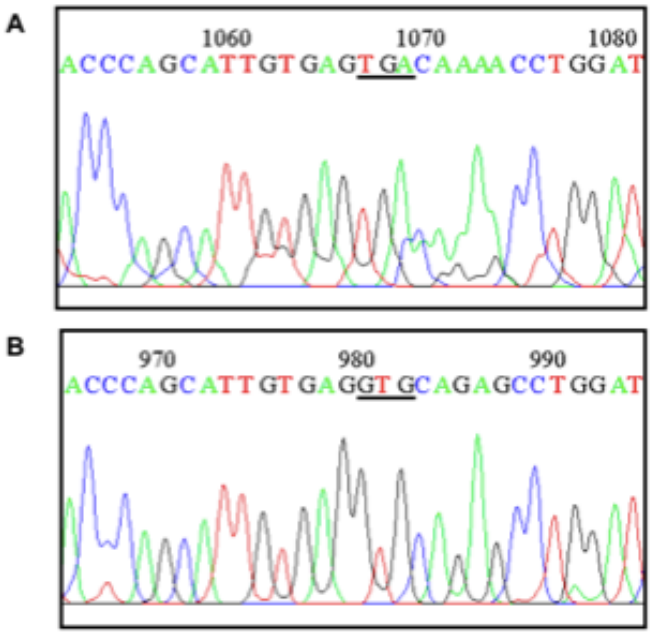

Figure 5.26. Sequence analysis of the TGA to GTG mutation for pGL3E_2000_AP1mut_1. A, the unmutated sequence (underlined TGA); B, same region (underlined GTG) after being mutated by site-directed mutagenesis.

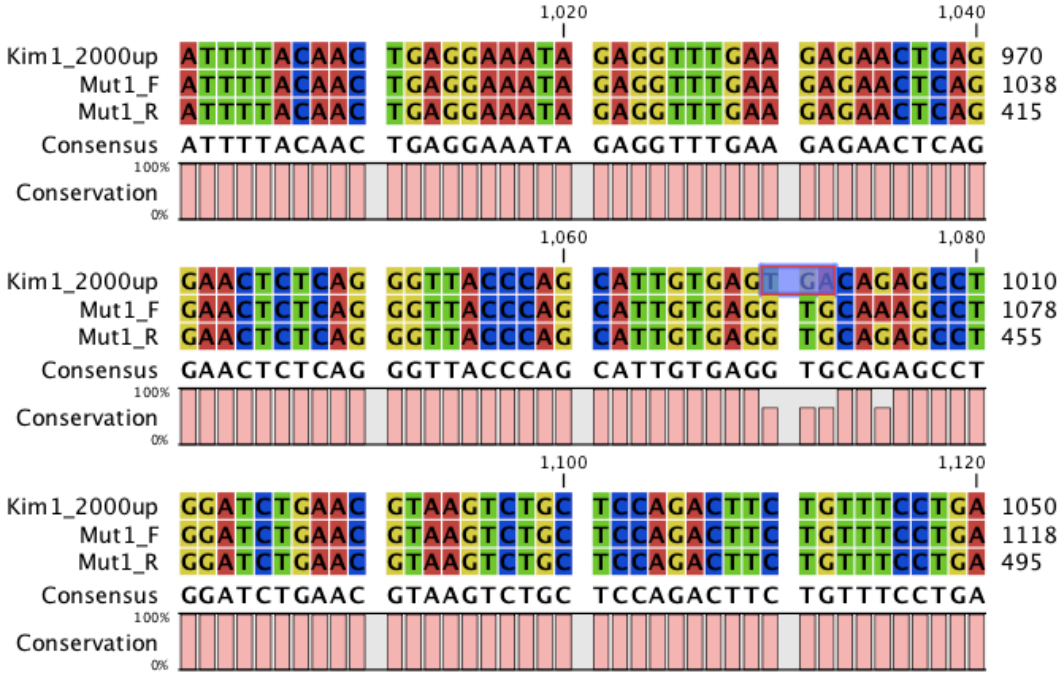

Figure 5.27. Alignment of forward (Mut1_F) and reverse (Mut1_R) sequence readings of pGL3E_2000_AP1mut_1 to the reference sequence (Kim-1_2000up).

According to the luciferase measurements after the transient transfection experiments, there was no significant difference in the luciferase activities of wild type (pGL3E_2000) and AP1 mutant (pGL3E_2000_AP1mut) luciferase-reporter constructs. OTA treatment (10 μM) for 24 hours led to an increase in the luciferase activity in both of the cell groups transfected with the wild type and AP1 mutant constructs. However, GM and CP did not have any significant effect on the luciferase activity compared to the untreated cells (Figure 5.28). Taken together, these results indicate that, AP1 transcription factor binding on -1010 from the $A^{+1}TG$ by itself is not likely to be responsible for driving the transcription of *Kim-1* gene under chemotoxic stress.

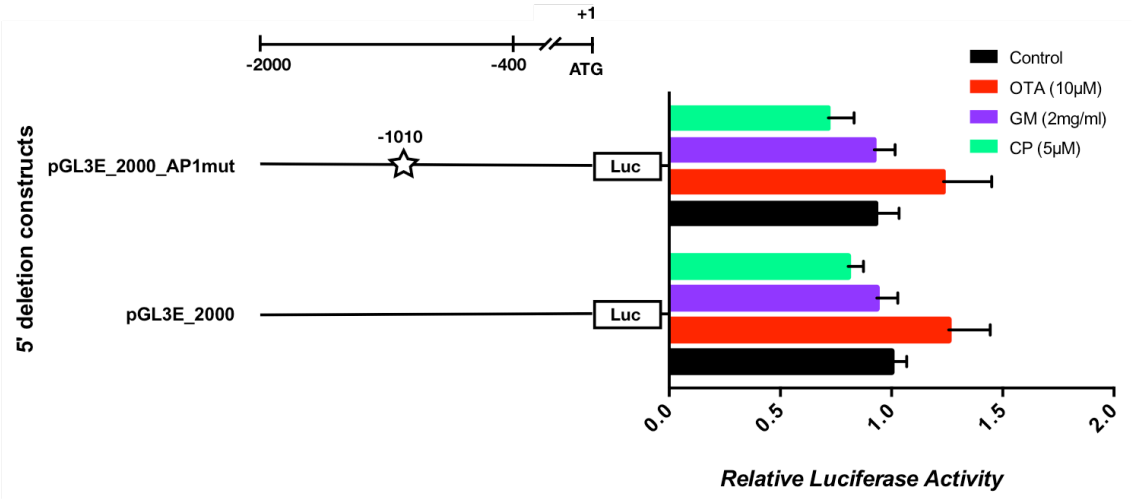

Figure 5.28. Mutation analysis. HK2 cells were transfected with wild type or AP1 mutant promoter-reporter constructs and analyzed for the luciferase activity. Firefly/Renilla normalized luciferase activity of each construct was normalized to the activity of the untreated group. Error bars represent the ±SEM of replicates.

5.9. Cloning of the KIM-1 Open Reading Frame (ORF) Region into Expression Vectors

In order to produce KIM-1 antibody and to study the functional role of KIM-1 protein in human cells, KIM-1 ORF region was cloned into bacterial and eukaryotic recombinant protein expression vector systems, respectively (Figure 5.28).

After cloning KIM-1 open reading frame sequence (1095 bp) into pGEM®-T Easy vector, the constructs were sequenced (Macrogen, Korea) for point mutation analysis. All the constructs had one or more point mutations which might alter protein expression.

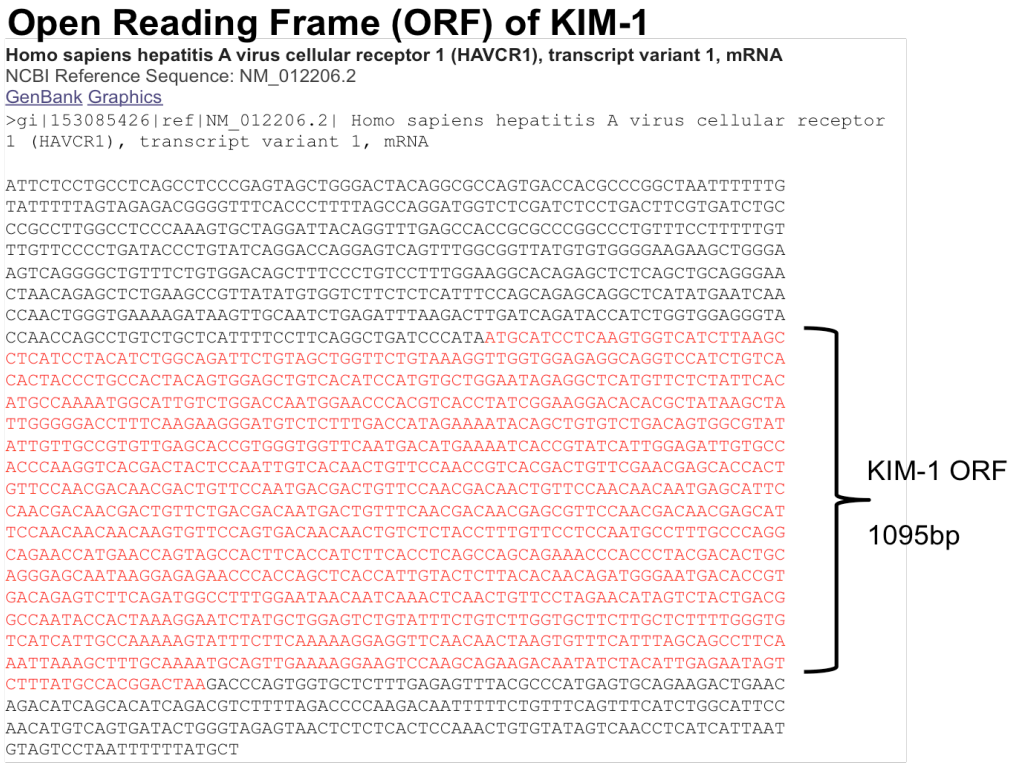

Figure 5.29. KIM-1 open reading frame (ORF) sequence. KIM-1 ORF was highlighted in red in between the 5'UTR and 3'UTRs. Sequence from NCBI database (Ref Seq NM_012206.2).

A detailed analysis of the sequences showed that the point mutations in Clone 1 did not seem to lead to any amino acid alterations. Furthermore, the sequences were aligned to the reference KIM-1 ORF sequence from NCBI database (Ref Seq NM_012206.2).

According to the alignment analysis, it was noticed that the 15 nucleotide region in the KIM-1 ORF was deleted from every clone including Clone 1 (Figure 5.29).

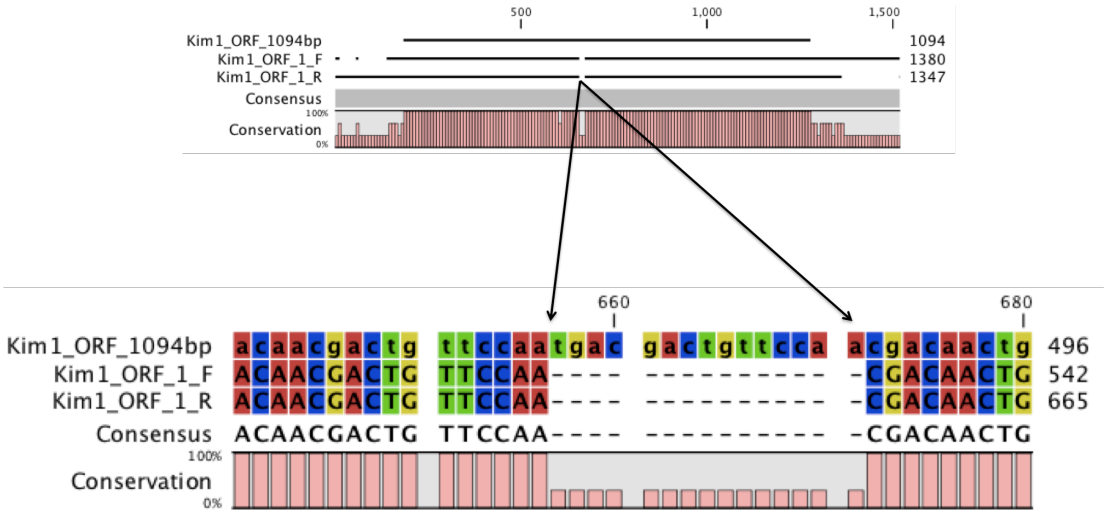

Figure 5.30. Alignment of forward and reverse readings of KIM-1 ORF sequences. Reference sequence was retrieved from NCBI Database.

After obtaining the KIM-1 ORF from pGEM®-T Easy vector, it was successfully cloned into target vectors, pET30a (+) and pMEV2HA, for bacterial and mammalian protein expressions, respectively (Figure 5.31 and 5.32).

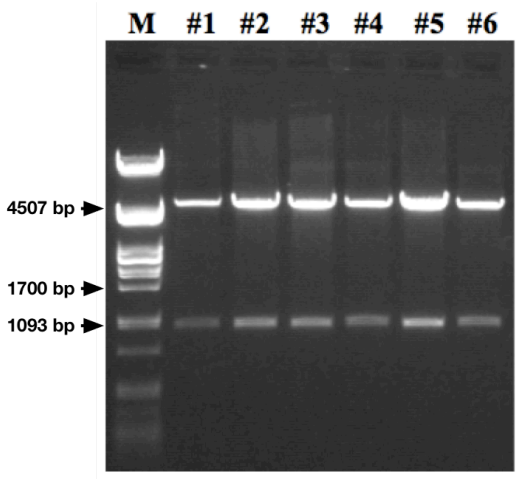

Figure 5.31. Cloning of KIM-1 ORF into the pET30 (+) plasmid. BamHI-XhoI enzymes were used for the analytic digestions. All clones contained an insert with the expected size of KIM-1 ORF (1095 bp).

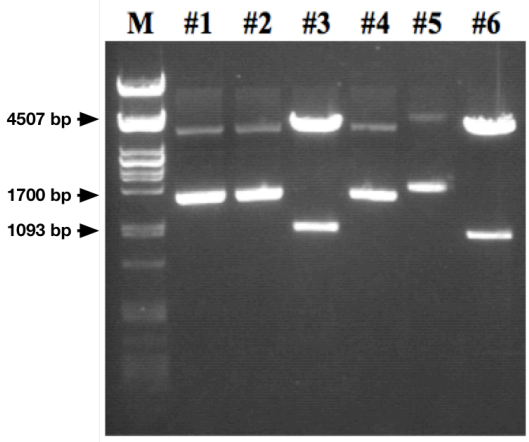

Figure 5.32. Cloning of KIM-1 ORF into the pMEV-2HA plasmid. BamHI-XhoI enzymes were used for the analytic digestion. Clones #3 and #6 contained an insert with the expected size of KIM-1 ORF (1095 bp).

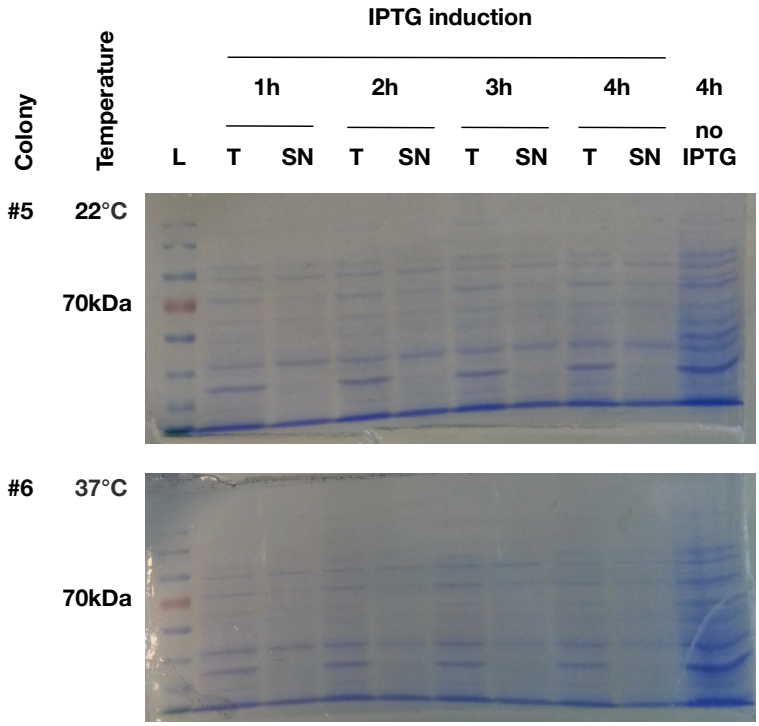

Figure 5.33. IPTG induction of pET30a (+)_ORF constructs. T, total lysate; SN, supernatant.

For recombinant bacterial expression of KIM-1 ORF, pET30a(+)_ORF constructs (#5 and #6) were transformed into Rosetta (BL21 derivative) competent cells. After transformation of Rosetta competent cells, IPTG induction was performed (Section 4.11). However, no protein production was observed as a burst on the gel for KIM-1 protein (Figure 5.33).

In order to see if KIM-1 can expressed endogenously in HK-2 cells, transient transfections were performed using only pMEV2HA vector and two pMEV2HA_ORF constructs (#3 and #6) along with no-transfection control. After the transfections, the cell lysates were prepared and then Western blot analyses were performed using antibodies against HA-tag and KIM-1. However, the HA-tag could not be observed on the blotting membrane (Figure 5.33). Interestingly, KIM-1 protein expression seemed to increase in all transfected HK2 cells suggesting that the transfection protocol increases the expression of endogenous KIM-1 protein levels.

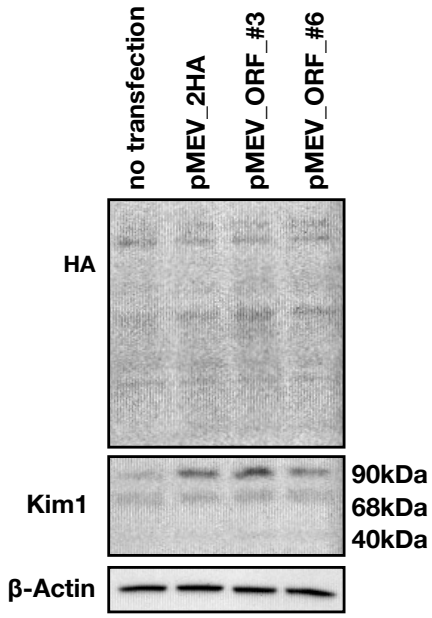

Figure 5.34. Western blotting analysis of recombinant and endogenous KIM-1 protein expression in HK2 cells. β-Actin was used as loading control.

6. DISCUSSION

In this thesis, the minimal promoter region of *Kim-1* gene was characterized using 5'-deleted luciferase reporter constructs and the binding capacity of AP1 transcription factor to the upstream region of *Kim-1* gene was examined. Moreover, the role of AP1 on the regulation of *Kim-1* gene at the transcriptional level was tested under chemotoxic stress using mutated or wild type AP1 binding site containing luciferase reporter constructs. A human derived proximal tubule epithelial cell line called Human Kidney Cell Line 2 (HK2) was utilized in these studies. OTA, GM and CP were used as chemotoxic stress inducers. Therefore, HK2 cells could mimic nephrotoxic effects similar to *in vivo* injury conditions.

As a first step, the appropriate concentrations of stress-inducing chemicals had to be determined since over-toxicity could mask the stress response pathways of the cells. To do this, XTT cell viability assays were performed for each chemical. Although XTT cell viability assay is generally used to determine proliferative profiles of the cells, it could be used to measure cell viability because of the implementation of chemotoxic stress on the cells. As a result, concentrations of each chemical was determined according to their toxicity and cell death for use in further experiments such as determination of KIM-1 mRNA or protein levels under chemotoxic stress.

Western blotting analysis of KIM-1 protein was performed under chemotoxic stress conditions for 24 hours. There were three distinct bands for KIM-1 protein, which were 90, 68 and 40 kDa in size. Actually, the calculated size of the human KIM-1 polypeptide is 36 kDa (Bailly *et al*., 2002). However, KIM-1 protein appears as three distinct bands because it contains four potential *N*-glycosylations and multiple *O*-glycosylation sites leading to changes in size (Bailly *et al.,* 2002) (Figure 1.2). However, the sizes in our findings were slightly different from the previous *in vitro* findings as in the case of the study where Western blotting analysis in HK2 cell line using murine monoclonal antibodies raised against the extracellular domain of KIM-1 protein yielded one major band at about 100 kDa with two other bands at 70 and 50 kDa (Bailly *et al*., 2002). In addition, *in vivo* stud-

ies with rat kidney yielded KIM-1 protein bands at 40, 50 and 70-80 kDa (Ichimura *et al.,* 1998). Thus, different sizes could represent the different number of glycosylations of KIM-1 protein influenced by the experimental conditions that were used during this study. Moreover, these differences between rat and human KIM-1 could be attributed to species differences. Nevertheless, the significant changes were always observable for the 90 kDa band and KIM-1 protein levels were observed to increase significantly by OTA treatment in 24 hours. On the other hand, GM or CP treatments did not give such similar increases in the amount of 90 kDa KIM-1 protein, however, we observed that the KIM-1 protein corresponding to the smaller sizes were increasing. These differences might be due to different characteristics of those three chemicals. For instance, OTA is known to be very resistant to both temperature and hydrolysis (Dirheimer and Creppy, 1991); therefore, it might exert its effects stronger compared to the other two chemicals. Another reason could be the differences in mode of actions for each chemotoxic agent. For example, GM and CP could inhibit glycosylation of proteins resulting in lower expression of KIM-1 species at 90 kDa. A not mutually exclusive possibility could also be the overactivation of matrix metalloproteinases (MMPs) that cleave the mature form of KIM-1 by GM and CP. In summary, at least for the case of OTA treatment, we faced similar results with a study in which HK2 cell line was exposed to oxalate (a metabolic end product excreted by the kidney) where KIM-1 protein amounts increased *in vitro* (Khandrika *et al.*, 2012). Also, in our *in vitro* cell culture system, we could mimic the *in vivo* kidney injury conditions, at least by OTA treatment, as in the study of Western blotting analysis of rat kidney homogenates where KIM-1 protein was upregulated in post-ischemic kidney *in vivo* (Ichimura *et al.*, 1998).

In contrast to KIM-1 protein, expression of c-Jun, which is a component of AP1 protein complex decreased by OTA treatment but it increased with GM or CP treatments. These observations suggest that AP1 might work as a negative regulator of *Kim-1* gene expression as its decreased levels lead to dissociation from its binding regions and increased KIM-1 expression. In one study where siRNAs designed for c-Jun were utilized, AP1 knockdown resulted in the silencing of the gene which codes for MMP-13 (Matrix-metalloproteinase-13) (Mak *et al*., 2011). Thus, AP1 seems to be a positive regulator for MMP activity. Not specifically MMP-13, but matrix metalloproteinases are known to be involved in the shedding process of KIM-1 protein (Bailly *et al*., 2002). Therefore, one can envision that the reduced levels of AP1 might decrease MMP activity preventing KIM-1

ectodomain shedding and leading to the detection of more KIM-1 protein by Western blotting. This could also explain the fact that the excretion of the shed KIM-1 ectodomain into the medium from the HK2 cells under chemotoxic stress *in vitro* prevents the detection of KIM-1 during Western blotting. Thus, observation of more KIM-1 protein (90 kDa) and less AP1 protein under 10 μM OTA treatment could be explained by the positive involvement of AP1 in MMP regulation and indirect prevention of KIM-1 shedding. This might suggest that AP1 might work as a negative regulator of *Kim-1* gene (Rajasekaran *et al.*, 2012).

In addition to protein expression, it was important to show the changes in the amounts of KIM-1 mRNA upon chemotoxic stress. Interestingly, no increase in KIM-1 mRNA levels was observed upon treatment with the chemicals that were selected to be the chemotoxic stress inducers in our experimental design. In contrast, significant decrease in KIM-1 mRNA levels was observed for OTA and CP treatments. As mentioned earlier, Northern Blot analysis and RNA *in situ* hybridization in rat kidney revealed that KIM-1 mRNA expression was detectable after 48h ischemic injury to the kidneys (Ichimura *et al*., 1998). Therefore, the absence of increase in mRNA levels was likely due to the time interval we had chosen or due to the difference between *in vitro* conditions from *in vivo*. Moreover, our preliminary data for KIM-1 mRNA levels for early time points (10 μM OTA treatment for 6 hours) yielded an increase in KIM-1 mRNA. Therefore, at early time points of stress, cells might use KIM-1 mRNA reserves to produce more protein but at later time points shedding of the already produced KIM-1 could be sufficient for the clearance of the apoptotic cells during kidney failure.

Analysis of the upstream regions of *Kim-1* gene was performed as those regions were thought to contain the potential promoter and other sequences necessary for *Kim-1* gene regulation. Based on the results of 5'-deletional mutation analysis in conjunction with the luciferase reporter assays, the minimal promoter region of *Kim-1* which confers the basal promoter activity was determined to lie within a 300 bp region between the -700 bp and -400 from the translation start site. During the transcriptional initiation process in general, assembly of the transcription initiation complex is facilitated by the TATA box which is a motif in the core promoter region (Xi *et al*., 2007). Interestingly, there was no consensus

TATA-box within the minimal promoter region we identified for *Kim-1* gene. This implies that the regulation of *Kim-1* gene transcription is more complex and might be different from that of the general transcription processes. Because of the decrease in the luciferase activity of the 5'-deletional constructs like pCLuc-1100 and pCLuc-700, there could be some inhibitory *cis*-acting sequences between -1100 and -700 upstream region. In addition, *Kim-1* gene is known to be expressed under stress conditions, however, there was no significant change in luciferase activity between the control and chemically treated groups after the transient transfection of the 5'-deletional constructs into HK2 cells. This could be due to the absence of gene-specific enhancer region(s) that *Kim-1* gene might need in order to be expressed under influence of chemotoxic stress.

After identification of the minimal promoter region of *Kim-1* gene, the 2000 bp upstream region was analyzed by the bioinformatic tool, Genomatix MatInspector. There were two different putative binding sites for AP1, residing at -1303 bp and -1010 bp from the translation start site. Both contained one mismatch but they mainly had the consensus sequence for the binding of AP1 (5'-TGA(G/C)TCA-3') and were considered as putative candidates for further research. The best candidate was the binding point at -1010 because it was on the positive strand, yielded a specific shifted protein-DNA complex fading in the presence of the c-Jun antibody in the EMSA analyses. The other site at -1303 yielded a shifted band but it did not show any supershifts or fading of the complexes in EMSA when antibody was present, so it was eliminated from the further research.

In order to observe whether c-Jun and Fra-2 proteins were present in the protein-DNA complexes formed between AP1 and the probes derived from the *Kim-1* promoter regions, c-Jun or Fra-2 antibodies were used as they are the dimer components of the AP1 transcription factor in EMSA analyses. As stated in the Introduction, in different tissues and cell types, AP1 transcription complex can be composed of various dimer components (Zenz *et al*., 2008). Moreover, the c-Jun protein has been associated with cellular proliferation and apoptosis, which are the processes occurring during kidney failure (Bohmann *et al*., 1987). Thus, c-Jun was a stronger candidate, which might yield a supershifted band during the EMSA experiments. Also, it was important to check the content of supershifted band with as many different antibodies as possible because the dimer could also be com-

posed of different proteins in HK2 cells leading to different functions of AP1 in those cells. However, the supershifted band was not observed in either case but there was a fading of complex formation with c-Jun antibody, which might be due to the inhibitory effect of the antibody for the formation of DNA-protein complexes when present in the reaction (Carey *et al*., 2012). In addition, chemical treatment was thought to influence the binding of AP1 on the regulatory region. For the antibody containing reactions, supershifted bands were not observed but fading of the complexes were obvious for both control and OTA treated nuclear extracts. This might indicate that chemical treatment did not significantly affect protein-DNA complex formation. Because of the inability to determine the supershifted band by EMSA experiments, AP1 binding on the regulatory region of *Kim-1* gene was also examined by ChIP assays. ChIP confirmed that AP1 binds the upstream regulatory region of *Kim-1* gene at the -1010 position from the translation start site *in vivo*. Due to this binding, AP1 was thought to have a potential function in the regulation of *Kim-1* gene under stress conditions.

Because of the difficulties in cloning of the whole 2000 bp upstream region of *Kim-1* gene into the pCLucBasic2 vector system, the pGL3 vector system was also utilized. This was especially necessary for the comparison of the luciferase activities of the AP1 mutant and wild type luciferase-reporter constructs. Parenthetically, the initial difficulty of cloning of the 2000 bp upstream region was thought to be due to the presence of some repeat sequences like Alu repeats (Figure A.5) which could undergo recombination in *E.coli* (Razin *et al*., 2001). Moreover, the sequences present in the particular plasmid vector could also affect cloning procedure. Nonetheless, comparisons of the luciferase reporter activities of AP1 mutant (pGL3E_2000_AP1mut) construct and its wild type counterpart (pGL3E_2000) yielded no significant difference between the two vectors. This might be due to the cooperative acting of AP1 proteins at -1010 and -1303 in *Kim-1* gene regulatory region. Furthermore, gene regulation is complex and might require the activity of more than one factor. Thus, not only AP1 proteins on the mentioned sites, but also the other transcription factors (*trans*-acting factors) (Kawana et al., 1995) or *cis*-acting sequences might work cooperatively with AP1 to regulate *Kim-1* gene expression. Further experiments involving mutation analyses of the -1303 and other sites should be performed in order investigate those possibilities. Additionally, instead of transient transfections, stable HK2 cell lines harboring the 5'-deletional constructs could be generated, especially the

constructs that contain the whole mutant and wild type AP1 site for the understanding of *Kim-1* promoter activity.

Apart from the promoter regulation studies, we tried to express and purify the recombinant KIM-1 protein to be used in the production of a KIM-1 antibody for the future studies. This was necessary because there is no information on the commercially available KIM-1 antibodies such as the recognition epitope of the antibody on the target protein. Therefore, we performed molecular cloning for the KIM-1 ORF into a bacterial and mammalian recombinant protein expression vectors. Unfortunately, KIM-1 protein could not be expressed in those systems, yet. Any point or frameshift mutation could influence the protein production; so further analysis is a must. However, if succeeded, we would not only be able to produce the antibody but also study the functional role(s) of KIM-1 in mammalian systems. Because of the presence of a putative phosphorylation site in the cytoplasmic domain of KIM-1 protein, this study could reveal many unknown facts about the role of KIM-1 molecule in signaling pathways during kidney injury (Binne *et al*., 2007).

All in all, our results suggest that, the 700 bp upstream region of *Kim-1* gene contains the minimal promoter region of *Kim-1* gene. AP1 transcription factor seems to be a crucial factor as it was shown to interact with the -1010 region from the translation start site of *Kim-1* gene. The binding of AP1 to only that site seemed to be insufficient to alter the activity of *Kim-1* gene promoter under chemotoxic stress. This finding suggests that AP1 might act cooperatively with other *trans*-acting or *cis*-regulatory elements for the control of this gene. Further research will unravel the unknowns about the regulation of *Kim-1* gene under stress conditions.

APPENDIX A: VECTORS

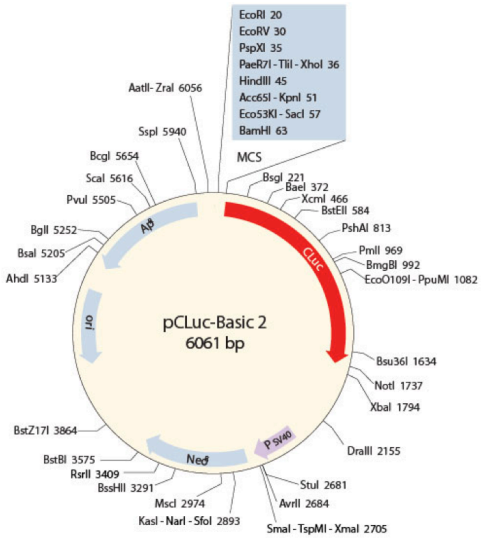

Figure A.1. Schematic representation of pCLucBasic2 plasmid. This plasmid was used for the measurement of luciferase activities of the *Kim-1* 5'-deletion constructs during luciferase reporter assays.

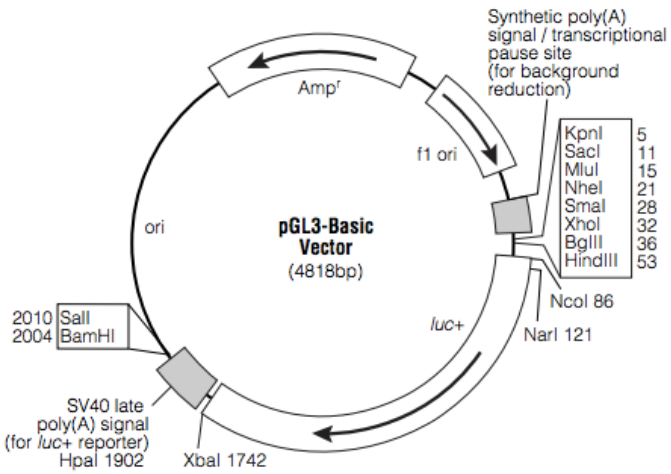

Figure A.2. Schematic representation of pGL3Basic plasmid. This plasmid was modified (addition of an enhancer) and then used during the luciferase measurements of pGL3E_2000_AP1mut.

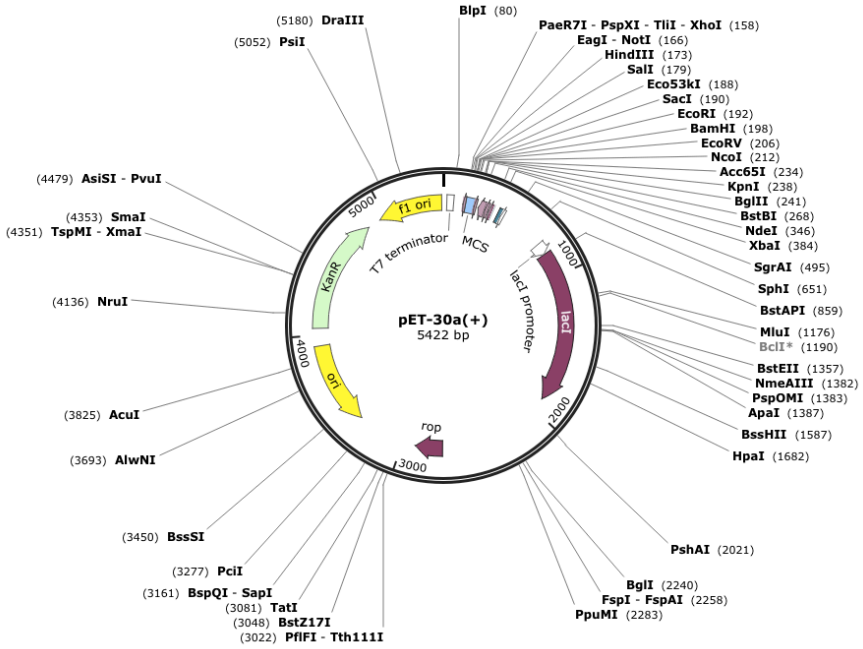

Figure A.3. Schematic representation of pET30a(+) plasmid. This plasmid was used for bacterial recombinant protein expression.

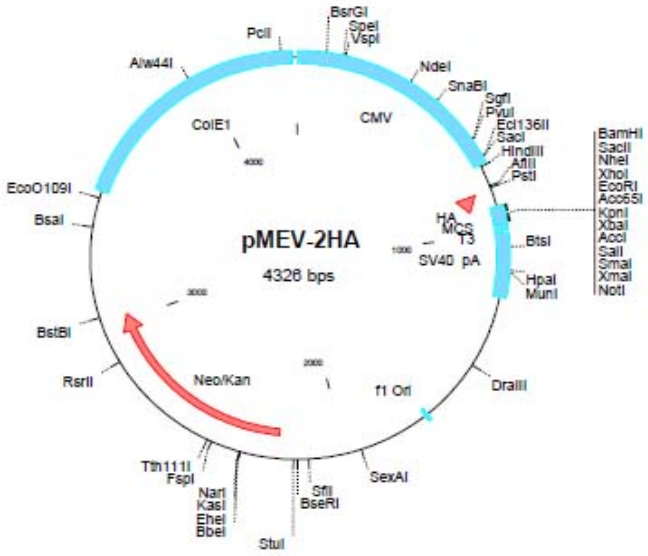

Figure A.4. Schematic representation of pMEV2HA plasmid. This plasmid was used for mammalian recombinant protein expression.

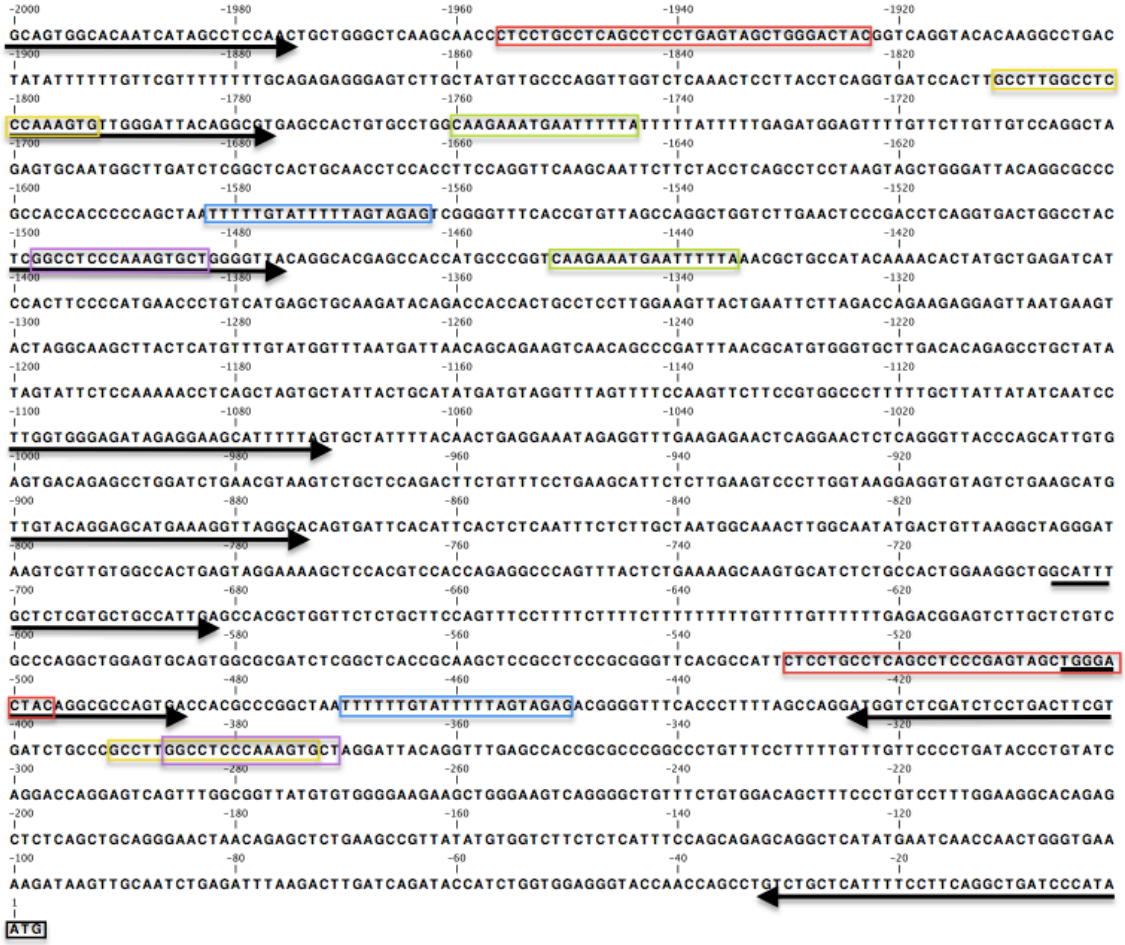

Figure A.5. Repeat sequences in the 2000 bp upstream region of *Kim-1* gene. (Black arrows represent the primers used during promoter bashing experiments. The translation start site is indicated by the first ATG codon in box. Colored boxes represent the repeating sequences.)

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