Role of basic region leucine zipper transcription factors in controlling gene transcription in mammalian cells

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To my mum and dad

"very special and successful persons"

whose love and influence will remain with me wherever I am

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Abbreviations

AP-1	Activating Protein-1
APS	Ammoniumpersulfate
Arg	Arginine
AS	Asparagine Synthetase
ATF	Activating Transcription Factor
ATP	Adenosine Tri Phosphate
BCA	Bicinchoninic acid
bZIP	Basic region leucine zipper
CAD	Constitutive Active Domain
cAMP	Cyclic Adenosine Mono Phosphate
CBP	CREB Binding Protein
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/Enhancer Binding Protein
CHOP	C/EBP Homologous Protein
CMV	Cytomegalo Virus
CNS	Central Nervous System
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
cRNA	Complementary ribonucleic acid
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.coli	Escherichia coli
EDTA	Ethylenediamintetraacetic acid
e.g.	For example
Egr-1	Early Growth Response protein-1
EPAC	Exchange Proteins directly Activated by cAMP

ERK	Extracellular Signal-Regulated Protein Kinase
FCS	Fetal Calf Serum
G418	Gentamycin 418 sulfate
GAL4	DNA-binding domains of the sacchromyces cerevisiae-transcription factors
GAPDH	Glycerinaldehyde-3-phosphate-dehydrogenase
GnRH	Gonadotropin-releasing hormone
G6P	Glucose-6-phosphatase
GPCR	G-Protein-Coupled Receptor
GST	Glutathione S-Transferase
GTP	Guanosine Tri Phosphate
GTPCHI	GTP cyclohydrolase I
h	Hour
HBSS	Hepes-Buffered Saline Solution
HEPES	[4-(2-hydroxyethyl)-1piperazinmethan]-sulfonic acid
HIV	Human Immunodeficiency Virus
IFN	Interferon
iNOS	Inducible Nitric Oxide Synthase
JNK	c-Jun-N-Terminal Kinase
KID	Kinase Inducible domain
KRB	Krebs-Ringer-bicarbonate buffer
LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
Lys	Lysine
MAP	Mitogen-Activated Protein
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-activated, ERK-activating Kinase
MEKK	Mitogen-activated, ERK-activating Kinase Kinase
min	Minute
MKP-1	MAP Kinase Phosphatase-1
mRNA	messenger RNA
MSK	Mitogen- and Stress-activated protein Kinase
NaF	Sodium Fluoride
NaN_3	Sodium Azide
Na_3VO_4	Sodium Ortho-Vanadate

NF-κB	Nuclear Factor-κB
NLS	Nuclear Localization Signal
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSRE	Nutrient-Sensing Response Element
nt	Nucleotide
Q	Glutamine
OD_{405}	Optic Density of 405 nM
ONPG	O-nitrophenyl-2-D-galactopyranoside
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma cells
PKA	Protein Kinase A, also called cAMP-dependent protein kinase
PMSF	Phenylmethylsulfonylfluoride
RPA	RNAse Protection Assay
RNA	Ribonucleic acid
RSV	Rous Sarcoma Virus
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
SAPK	Stress-Activated Protein Kinase
SD	Standard Deviation
SDS	Sodiumdodecylphosphate
Sec	Second
Ser	Serine
SgII	Secretogranin II
SRE	Serum Response Element
SV40	Simian Virus 40
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween 20
TEMED	Tetramethylethylendiamin
TH	Tyrosine Hydroxylase
Thr	Threonine

TLR4	Toll-Like Receptor 4
TNFα	Tumor Necrosis Factor α
TPA	12-O-Tetradecanoyl phorbol-13-acetate
TRE	TPA Response Element
Tricin	N-[Tris(hydroxymethyl)methyl]glycine
Tris	Tris-(hydroxymethyl)-aminomethan
U	Unit
UAS	Upstream Activating Sequence
UV	Ultraviolet
v/v	Volume/Volume
Vol.	Volume

A. Introduction

Each cell in a multicellular animal is programmed during development to respond to a specific set of signals that act in various combinations to regulate the behavior of the cell and to determine whether the cell lives or dies and whether it proliferates or stays quiescent.

A.1. Stimulus-transcription coupling

Cell signaling requires both extracellular signaling molecules and a complementary set of receptor proteins in each cell that enable it to bind and respond to extracellular stimuli in a programmed and characteristic way. Through cascades of highly regulated protein phosphorylations, elaborate sets of interacting proteins relay most signals from the cell surface to the nucleus, thereby altering the gene expression pattern of the cell and, as a consequence, its behavior. G-protein-coupled receptors (GPCR) are the largest family of cell-surface receptors. Most G-protein-coupled receptors activate a chain of events that alter the concentration of one or more small intracellular signaling molecules. These small molecules, often referred to as second messengers, in turn pass the signal in by altering the behavior of selected cellular proteins. One of the most widely used intracellular mediators is cyclic AMP (cAMP), which is synthesized from ATP by the plasma-membrane-bound enzyme adenylyl cyclase, and it is rapidly and continuously destroyed by cAMP phosphodiesterases, which hydrolyze cAMP to adenosine 5'-monophosphate. cAMP exerts its effects by activating the enzyme cAMP-dependent protein kinase, also called protein kinase A (PKA), which catalyzes the transfer of the terminal phosphate group from ATP to specific serine or threonine residues of selected proteins. The proteins phosphorylated by PKA are marked by the presence of two or more basic amino acids on their amino-terminal side. Phosphorylation of the appropriate amino acids in turn regulates the activity of the target protein. In the inactive state PKA consists of a complex of two catalytic subunits and two regulatory subunits that bind cyclic AMP. The binding of cAMP alters the conformation of the regulatory subunits, causing them to dissociate from the complex. The released catalytic subunits are thereby activated to phosphorylate specific substrate protein molecule (Fig. 1). In some cells an increase in cAMP

activates the transcription of specific genes. In cells that secrete the peptide hormone somatostatin, for example, cAMP turns on the gene that encodes this hormone. The regulatory region of the somatostatin gene contains a short DNA consensus sequence (5`-TGACGTCA-3`), called cAMP response element (CRE), that is also found in the regulatory region of other genes that are activated by cAMP. This sequence is recognized by a specific gene regulatory protein called CRE-binding (CREB) protein. CREB belongs to the family of basic region leucine zipper proteins and was first discovered through its ability to bind to the CRE in the promoter of the somatostatin (Gonzalez et al., 1989; Montminy and Bilezikjian, 1987; Montminy et al., 1986).

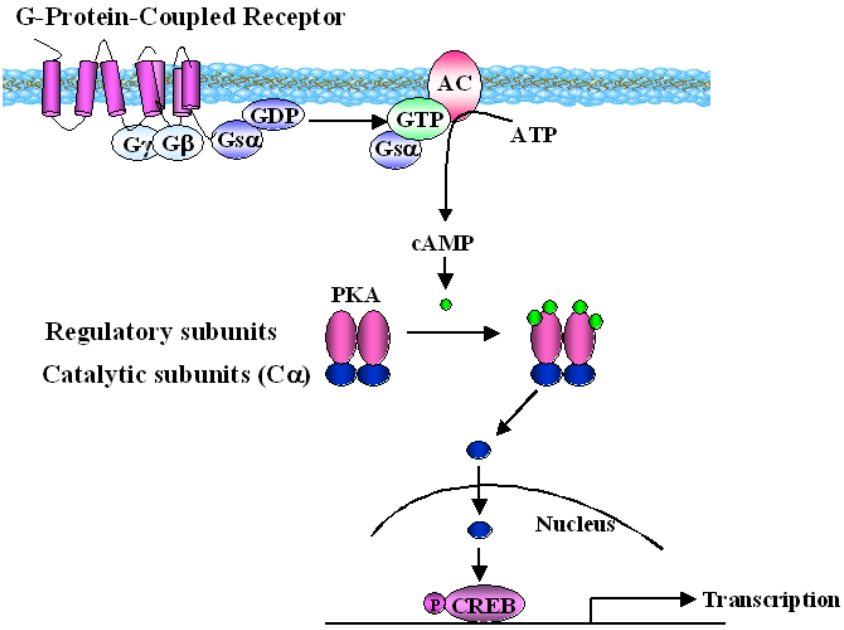


Fig. 1. cAMP and PKA signaling pathway.
Activation of G-protein-coupled receptors leads to the activation of adenylyl cyclase which will synthesize cAMP from ATP. When cAMP binds to the regulatory subunits of PKA, the catalytic subunits will be released, will translocate to the nucleus, phosphorylating and subsequently activating the transcription factor CREB.

Other well studied cell-surface receptors are the receptor tyrosine kinases (RTKs). All RTKs consist of a single transmembrane domain that separates the intracellular tyrosine kinase domain from the extracellular portion (Ullrich and Schlessinger, 1990). The intracellular catalytic domain includes the ATP-binding site that catalyzes receptor autophosphorylation and tyrosine phosphorylation of RTK substrates (Yarden and Ullrich, 1988). The

phosphotyrosyl residues are binding sites for adaptor proteins that interface with numerous downstream signaling pathways (Pawson, 1995). The mitogen-activated protein (MAP) kinases are components of the downstream signal transduction cascades initiated following binding of RTK cognate ligand. MAP kinases (MAPKs) are serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a wide array of extracellular stimuli. Three distinct groups of MAPKs have been identified in mammalian cells; extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Fig. 2). Although MAPKs regulate cytoplasmic targets, such as cytosolic phospholipase A_2 (Lin et al., 1993), a major role for MAPKs is to transmit extracellular signals to the nucleus, where the transcription of specific genes is induced by phosphorylation and activation of transcription factors (Hill and Treismann, 1995; Karin and Hunter, 1995). The transcription factor complex activating protein-1 (AP-1), which belongs to the family of basic region leucine zipper proteins, has been identified as a target of MAPK signaling pathway (Karin, 1995).

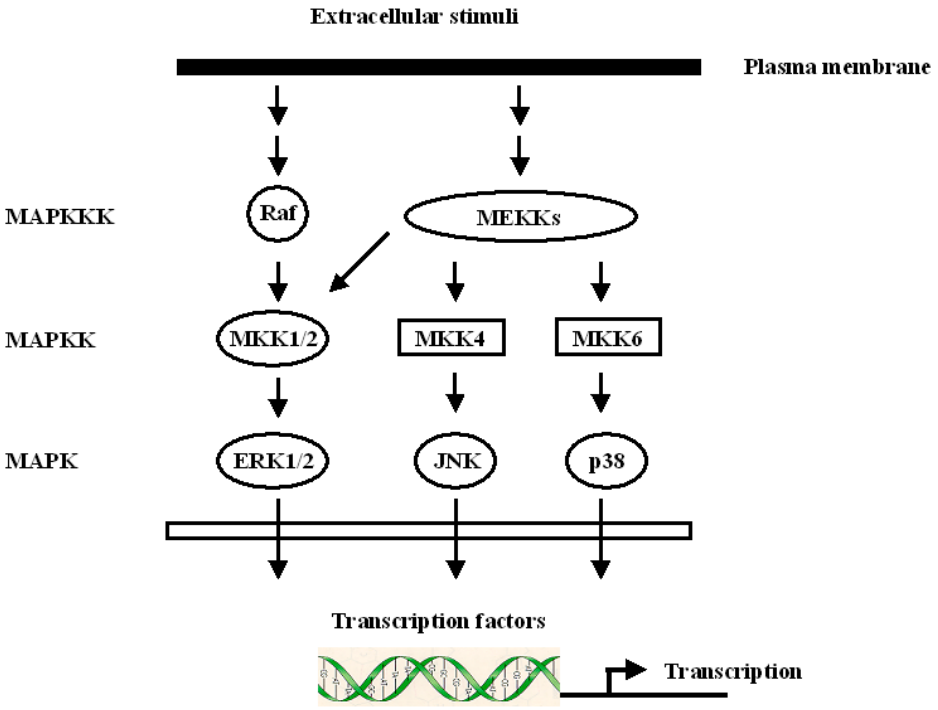


Fig. 2. The MAPK signaling cascade.
In response to extracellular stimuli, such as mitogen and stress, MAPKKK is activated, leading to the activation of MAPKK, which in turn activates the MAPK which will translocate to the nucleus to phosphorylate and subsequently activate specific transcription factors.

A.2. Basic region leucine zipper (bZIP) transcription factors

The bZIP transcription factors are proteins that bind to sequence-specific double-stranded DNA as homodimers or heterodimers to either activate or repress gene transcription (Hurt, 1995). The amino acid alignment of bZIP proteins allowed the identification of the bZIP motif, a long bipartite α-helix that is 60 to 80 amino acids long (Vinson et al., 1989). The N-terminal half contains two clusters of basic amino acids responsible for sequence-specific DNA-binding, while the C-terminal half contains an amphipathic protein sequence of variable length with a leucine every seven amino acids (Vinson et al., 2002). The DNA-binding domain consists of a high concentration of basic residues (Lys or Arg) that can interact with the negatively charged phosphates of the DNA backbone. The amphipathic sequence termed "leucine zipper" mediates homo- and heterodimerization of bZIP proteins (Landschulz et al., 1988). There are many types of leucine zipper proteins, and they cannot all form heterodimers with one another. Whether or not a particular heterodimer can be formed depends on how well the hydrophobic surfaces of the two leucine zippers α helices mesh with each other, which, in turn, depends on the exact amino acid sequences of the two zipper regions. Thus each leucine zipper protein in the cell can form dimers with only a small set of the other leucine zipper proteins (Vinson et al., 2002).

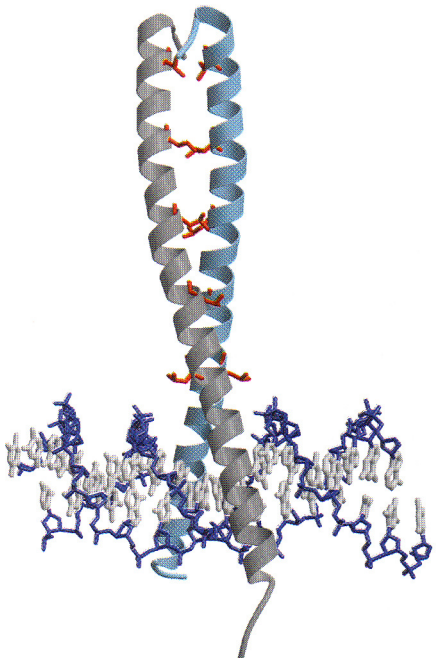


Fig. 3. Leucine zippers.
Leucine zipper of the yeast activator protein GCN4. Only the "zippered" α helices (white and light blue), derived from different subunits of the dimeric protein, are shown. The two helices wrap around each other in a gently coiled coil. The interacting leucine residues are shown in red. (Nelson and Cox, 2000).

In the late 1980s, several mammalian bZIP proteins were purified and the cDNAs were cloned (Vinson et al., 2002). Among the first bZIP proteins identified were the AP-1 (c-FOS and c-JUN) heterodimer (Bohmann et al., 1987), the CREB homodimer (Montminy and Bilezikjian, 1987) and the C/EBP homodimer (Johnson et al., 1987; Landschulz et al., 1988).

CREB

The transcription factor CREB is the prototype of the bZIP family of transcription factors. CREB binds to a conserved cAMP response element present within the 5`-flanking region of many cAMP-responsive genes (Montminy et al., 1986; Roesler et al., 1988). CREB contains a bZIP domain and two glutamine rich domains, referred to as Q1 and Q2/CAD (constitutive active domain) that are separated by the kinase inducible domain (KID). Within the KID resides the critical residue Ser-133, which once phosphorylated in a stimulus-inducible manner, makes the KID a binding target for the transcriptional coactivator, CREB binding protein (CBP) (Chrivia et al., 1993; Kwok et al., 1994). CBP binds to the Ser-133-phosphorylated KID domain via its KIX domain (Parker et al., 1996; Radhakrishnan et al., 1997), and it is the stimulus-dependent interaction between these two domains that is believed to function as the trigger for inducible gene expression. Q2/CAD interacts with components of the basal transcriptional machinery and may be responsible for facilitating stimulus-independent CRE-derived gene expression (Ferreri et al., 1994; Quinn, 1993; Xing et al., 1995). The modular structure of CREB is shown in Fig. 5. CREB is inactive in the dephosphorylated state and turns into an activator upon phosphorylation. The key enzyme leading to CREB activation is the cAMP-dependent protein kinase (PKA), but CREB serves also as a substrate for calcium/calmodulin-dependent protein kinase IV and the mitogen and stress-activated protein kinases (SAPK) MSK1 and 2 (Sun et al., 1994; Wiggin et al., 2002). CREB has been shown to be important in long-lasting changes in synaptic strength in diverse organisms including *Drosophila*, *Aplysia* and mouse (Frank and Greenberg, 1994; Silva et al., 1998). Several studies have revealed a role for the CREB family of activators in the control of cell survival and proliferation (Long et al., 2001; Ionescu et al., 2001). It has been demonstrated that CREB DNA-binding activity and phosphorylation are necessary for the nerve growth factor (NGF)-dependent survival of neurons (Riccio et al., 1999; Bedogni et al., 2003). In addition, several cell cycle genes such as *cyclin D1* and *cyclin A* are regulated by CREB via a functional CRE element (Desdouets et al., 1995; Lee et al., 1999; D'Amico et al., 2000). Additionally, alteration of CRE binding proteins with loss of CREB expression were observed in the human adrenocortical cancer cell line H295R. Similar alterations were found

in human malignant adrenocortical tumors (Rosenberg et al., 2002a, b). Also the involvement of CREB in the control of tumor metastasis was demonstrated in cases of melanoma cells (Jean and Bar-Eli, 2000, 2001). Several evidences strongly support a role of CREB as a neuroprotectant (Hu et al., 1999; Walton et al., 1999; Tanaka et al., 2000; Mabuchi et al., 2001). While the complete disruption of CREB is lethal in mice (Rudolph et al., 1998) and is therefore not likely to be compatible with human life, several pathological conditions exist in which CREB function may be disrupted subtly or incompletely. Some such conditions are genetic disorders in which CREB interactors are defective. A heterozygous mutation in *CBP*, for example, produces Rubenstein-Taybi Syndrome, which is characterized by multiple deficits including mental retardation (Petrij et al., 1995). In most cases, the mechanism by which the disruption of CREB function may produce disease is poorly understood. One notable exception is the group of polyglutamine repeat diseases, which includes Huntington's Disease, a devastating neurodegenerative disorder (Lonze and Ginty, 2002).

ATF2

Activating transcription factor 2 (ATF2), also known as cAMP response element binding protein-1 CRE-BP-1 (Nomura et al., 1993), is a member of the activating protein-1 (AP-1) family of bZIP proteins. ATF2 binds to the classical CRE motif 5`-TGACGTCA-3` (Thiel et al., 2005a). Furthermore, the related DNA target sequence 5`-TTGCGTAA-3` has been reported as high affinity consensus binding site for ATF2 homodimers (Benbrook and Jones, 1994). ATF2 is able to form homodimers and heterodimers through the leucine zipper and the preferred dimerization partner is c-Jun (Hai and Curran, 1991; Van Dam et al., 1993; Benbrook and Jones, 1994; Tsai et al., 1996). ATF2 is ubiquitously expressed with the highest level of expression being observed in the brain (Takeda et al., 1991). ATF2 contains at its N-terminus a phosphorylation-dependent transcriptional activation domain (Livingstone et al., 1995) and at its C-terminus a bZIP domain, responsible for DNA-binding and dimerization. In the absence of appropriate inducers, ATF2 remains transcriptionally inactive due to an inhibitory intramolecular interaction between the activation domain and the bZIP domain of ATF2 (Li and Green, 1996). The modular structure of ATF2 is shown in Fig. 19. The activity of the activation domain of ATF2 appears to be masked in the intact protein and is dependent upon two closely situated threonine residues, Thr 69 and Thr 71. These residues are phosphorylated *in vivo* and can be efficiently phosphorylated *in vitro* by the JNK/SAPK group of MAPK family (Livingstone et al., 1995). The replacement of these phosphorylation sites with alanine inhibited the transcriptional activity of ATF2. The phosphorylation of ATF2

N-terminus can also be mediated by ERK2 *in vitro* although in much less efficiently. It is doubtful, however, that ERK1/2 play any role in the regulation of ATF2 activity *in vivo* (Livingstone et al., 1995). ATF2 has been implicated in a wide variety of biological processes including skeletal and central nervous system (CNS) development (Reimold et al., 1996), proinflammatory cytokine stimulation (Gupta et al., 1995) and the cellular stress response (Clerk and Sugden, 1997; Gupta et al., 1995; Morooka et al., 1995; Wilhelm et al., 1995). Additionally, elevated ATF2 expression was found in Alzheimer's Disease cortex (Yamada et al., 1997b). These studies raise the possibility that activation of ATF2 protein may have a role in neuronal programmed cell death (Walton et al., 1998). The inactivation of ATF2 gene in transgenic mice revealed an absolute requirement of ATF2 for skeletal and central nervous system development. Moreover, induction of genes regulated by CRE sites such as E-selectin were impaired (Reimold at al., 1996). In addition, mice lacking ATF2 display features of a severe form of meconium aspiration syndrome (Maekawa at al., 1999), a neonatal disease that results in acute and chronic respiratory morbidity. These ATF2 deficient mice die shortly after birth.

c-Jun

The transcription factor c-Jun is a bZIP protein, which is a central component of the transcriptional complex AP-1. Like all AP-1 family members c-Jun recognizes either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements (TREs) (5`-TGAGCTCA-3`) or cAMP response elements (5`-TGACGTCA-3`) (Hirai and Yaniv, 1989; Sassone-Corsi et al., 1990; Ryseck and Bravo, 1991). Studies of *c-jun* expression indicate that it belongs to the immediate-early class of genes, which are rapidly transient induced in response to mitogenic stimuli in quiescent cells (Curran and Franza, 1988). Although many cells contain low levels of c-Jun protein, the transcription of the *c-jun* gene is greatly stimulated both by hormones (e.g., epidermal growth factor) and by compounds that stimulate the activity of protein kinase C (e.g., Phorbol esters) (Dixit et al., 1989; Lamph et al., 1988). c-Jun contains at its C-terminus a bZIP domain, responsible for DNA-binding and dimerization. The N-terminus of c-Jun contains a phosphorylation-dependent transcriptional activation domain (Pulverer et al., 1991; Smeal et al., 1991). The modular structure of c-Jun is shown in Fig. 37. One crucial property of c-Jun is the formation of functional heterodimers with multiple transcription factors, and these partners alter the affinity of c-Jun for DNA-binding sites, and, subsequently, determine the downstream target genes controlled by c-Jun (Angel and Karin, 1991; Hai and Curran, 1991). Furthermore, c-Jun and its heterodimer partners are targets for

phosphorylation by kinases linked to specific signaling pathways. c-Jun N-terminal kinases (JNK) phosphorylate the serine residues 63 and 73 within the N-terminus of c-Jun and this enables c-Jun to activate the mRNA polymerase complex resulting in transcription of effector genes (Derijard et al., 1994; Gupta et al., 1995). c-Jun plays a general role in the regulation of cell proliferation and apoptosis. It has been shown in fibroblasts isolated from E11.5 *c-jun*$^{-/-}$ and *c-jun*$^{+/-}$ embryos that the absence or diminished expression of c-Jun resulted in greatly reduced growth rates, and that this proliferation defect could not be compensated by addition of purified mitogens (Johnson et al., 1993; Schreiber et al., 1999). Evidences concerning the role of c-Jun and c-Jun phosphorylation in apoptosis was obtained in neuronal cells where transient overexpression of c-Jun induced apoptosis and expression of a dominant-negative *c-jun* mutant inhibited apoptosis *in vitro* (Estus et al., 1994; Ham et al., 1995, Behrens et al., 1999). *In vitro* experiments have demonstrated that expression of c-Jun can kill neonatal neurons (Schlingensiepen et al., 1993) but, in the adult nervous system, c-Jun might be involved in neuroprotection and regeneration (Aznar et al., 1995; Sommer et al., 1995). Furthermore, targeted disruption of *c-jun* is lethal (Hilberg et al., 1993; Johnson et al., 1993; Schorpp-Kistner et al., 1999), where lethality of *c-jun*$^{-/-}$ fetuses has been suggested to be due to defective liver development (Hilberg et al., 1993).

c-Fos

The transcription factor c-Fos is a nuclear phosphoprotein belonging to the AP-1 family of bZIP proteins. In most cell types *c-fos* mRNA and protein are present at low levels, however, they can be induced by a diverse array of agents in a variety of situations (Morgan and Curran, 1991). *c-fos* is not able to form homodimers and one way *c-fos* can mediate transcription is via heterodimerization with *jun* family members and binding TRE/AP-1 sites (Rauscher et al., 1988; McBride and Nemer, 1998). The c-Fos protein contains several transcriptionally active regions, including some autonomous transactivation domains (Sutherland et al., 1992; Wisdom and Verma, 1993; Deng and Karin, 1994; Jooss et al., 1994), a carboxy-terminal transrepression domain (Sassone-Corsi et al., 1988a; Gius et al., 1990; Ofir et al., 1990) and a region that interacts with the TATA box-binding protein (Metz et al., 1994). Like all other bZIP proteins, c-Fos contains on its C-terminus a bZIP domain, responsible for DNA-binding and dimerization (Landschulz et al., 1988). Fos protein has been implicated as playing a pivotal role during cell growth, differentiation and development (Muller and Wagner, 1984; Holt et al., 1986; Distel et al., 1987; Dony and Gruss, 1987; Nishikura and Murray, 1987; Ruther et al., 1987; Verma and Graham, 1987). It has been

shown that a *c-fos* null mutation leads to a number of tissue specific deficits of function, some of the most striking of them occurring in the bone and CNS (Johnson et al., 1992; Wang et al., 1992). c-Fos is persistently induced in brain of mice treated with kainic acid, a potent activator of glutamate receptors that causes apoptosis of hippocampal neurons (Smeyne et al., 1993).

CREB2/ATF4

cAMP response element binding protein 2, also called activating transcription factor 4 (ATF4), is a ubiquitously expressed 351-amino acid protein (Karpinski et al., 1992). CREB2/ATF4 has been described to repress transcription (Karpinski et al., 1992; Jungling et al., 1994) but new data show that CREB2/ATF4 is a strong transcriptional activator (Liang and Hai, 1997; He et al., 2001; Schoch et al., 2001; Luo et al., 2003, Thiel et al., 2005a). CREB2/ATF4 can form dimers with other transcription factors including those in the CCAAT/enhancer binding protein family (Vallejo et al., 1993). *CREB2* mRNA is expressed ubiquitously in human tumor cell lines and mouse organs suggesting that it is involved in regulating transcription in a wide variety of cell types (Karpinski et al., 1992). CREB2/ATF4 contains in the C-terminal end a leucine-zipper motif and an adjacent basic domain that have been shown to be involved in dimerization and DNA binding, respectively. It also contains on the N-terminus a consensus MAPK phosphorylation sites but lacking protein kinase A and protein kinase C phosphorylation sites (Karpinski et al., 1992). The modular structure of CREB2 is shown in Fig. 5. Mammalian *ATF4* has been implicated in various physiological processes including wound healing (Estes et al., 1995), eye development (Tanaka et al., 1998; Hettmann et al., 2000), stress responses (Fawcett et al., 1999), apoptosis (Kawai et al., 1998) and cancer (Mielnicki et al., 1996). It has been reported that targeted disruption of *ATF4* in mice causes perinatal lethality, dwarfism and severe osteoporosis, which are abnormalities caused by a failure of osteoblasts to fully differentiate and to properly function (Yang and Karsenty, 2004).

ATF5

Activating transcription factor 5, also known as ATF7 and ATFx, is a bZIP transcription factor that forms homodimers, which, at least *in vitro*, bind the CRE (Peters et al., 2001). ATF5 displays 58% amino acid identity to CREB2/ATF4 in the carboxy-terminal bZIP region (Hansen et al., 2002). Like other bZIP proteins, ATF5 contains C-terminal basic leucine zipper DNA-binding domain and N-terminal transcriptional domain. In the leucine zipper

domain, ATF5 has only three leucines instead of the five that are present in CREB2/ATF4 with the distal two leucines replaced by valines (Pati et al., 1999). It has been reported that the expression of *ATFx* is down-regulated in a variety of cells undergoing apoptosis following growth factors deprivation, indicating that ATFx has an anti-apoptotic function. The role of ATFx in cell survival appears to be purely anti-apoptotic, as overexpression of the gene did not affect the rate of cell growth or cell cycle progression (Persengiev et al., 2002). It has been shown that constitutive expression of ATF5 blocks neuronal differentiation of cultured embryonic neural progenitor cells and maintains them in a proliferating state. It has been also shown that loss of ATF5 function, or ATF5 expression, leads to neuronal differentiation of cultured neural progenitors (Angelastro et al., 2005).

C/EBPα

CCAAT/enhancer binding protein α is the prototype of the C/EBP family and belongs to the bZIP class of transcription factors (Johnson et al., 1987; Grayson et al., 1988; Landschulz et al., 1988). C/EBPα is expressed at high levels in the adipose tissue, liver, intestine, lung, adrenal gland, peripheral-blood mononuclear cells and placenta (Cao et al., 1991; Williams et al., 1991; Antonson and Xanthopoulos, 1995; Lekstrom-Himes and Xanthopoulos, 1998). The C/EBP proteins bind specifically to the dyad symmetrical sequence 5´-ATTGCGCAAT-3´ (Agre et al., 1989). However, it has been also reported that C/EBP proteins bind to ATF/CRE motifs (5´-TGACGTCA-3´) (Bakker and Parker, 1991). All members of the C/EBP family have a C-terminally located bZIP domain that is responsible for DNA binding and dimerization (Landschulz et al., 1988). The modular structure of C/EBPα is depicted in Fig. 60. C/EBPα activates transcription of several liver and fat cell specific genes (MacDougald and Lane, 1995; Christy et al., 1989; Friedman et al., 1989; Friedman and McKnight, 1990). It has been demonstrated that C/EBPα can induce growth arrest in adipocytes suggesting that it may play a role in regulating the balance between proliferation and differentiation (Freytag and Geddes, 1992; Umek et al., 1991). When overexpressed in cultured cells, C/EBPα acts as a strong inhibitor of cell proliferation (Diehl et al., 1996; Timchenko et al., 1996; Hendricks-Taylor and Darlington, 1995). It has been shown that C/EBPα is critical for the proper development of both the liver and the lung since animals deficient in C/EBPα display severe abnormalities in these organs and die within 10 h after birth (Flodby et al., 1996).

CHOP

The C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage protein 153 (GADD153), was first identified as a gene that was induced by DNA damage (Fornace et al., 1988; Ron and Habener, 1992). CHOP can form heterodimers with other members of the C/EBP family, regulating gene expression either as a dominant-negative factor preventing the binding of the C/EBPs to their canonical DNA targets (Ron and Habener, 1992) or as a transcriptional activator by directing CHOP-C/EBP heterodimers to other DNA sequences (Ubeda et al., 1996). Similar to the Fos proteins, CHOP does not form stable homodimers and thereby it depends on heterodimerization with other proteins to exert functions on the control of gene transcription (Ron and Habener, 1992). CHOP contains in its C-terminus a bZIP domain (Ramji and Foka, 2002), while the N-terminus of CHOP contains a transcriptional activation domain which is inducible by cellular stress (Ubeda et al., 1996). Several studies have reported a relationship between CHOP expression in cultured cells and various stress conditions such as the depletion of Ca^{2+} stores (Brewer et al., 1997), oxidative stress (Guyton at al., 1996) and glucose or amino acids deprivation (Carlson et al., 1993; Bruhat et al., 1997). The expression of CHOP appears to play a role in the control of the cell division cycle (Barone et al., 1994; Zhan et al., 1994). CHOP has also been implicated in the inhibition of adipocyte differentiation (Batchvarova et al., 1995) and seems to be involved in cell apoptosis induced by endoplasmic reticulum stress (Zinszner et al., 1998), alkylating agents (Wang and Ron, 1996), or by Fas and ceramides (Brenner et al., 1997).

A.3. Problems and aim

The bZIP transcription factor CREB is inactive in the dephosphorylated state and needs phosphorylation to be activated. To study CREB-mediated gene transcription, a signaling cascade leading to CREB phosphorylation and activation needs to be triggered. This can be accomplished either by adding cAMP analogues such as the cell permeable dibutyryl cAMP, or by adding forskolin, a direct activator of adenylyl cyclase. These experiments are problematic in that high levels of phosphodiesterase enzymes in the cells may immediately hydrolyze cAMP to 5`-AMP, resulting in that no cAMP-mediated gene transcription can be monitored (Klar et al., 2002). Additionally, high levels of cAMP may also activate the exchange proteins directly activated by cAMP/Rap1 (EPAC/Rap1) pathway, which is a PKA independent signaling pathway (Kawasaki at al., 1998; De Rooij et al., 1998). Thus, many

functions of cAMP previously attributed to PKA may be in fact the result of EPAC activation (Mai et al., 2002). Another way to study CREB-mediated gene transcription is to overexpress the catalytic subunit of PKA. This approach has the advantage that by excluding a parallel signaling cascade via the cAMP-induced EPAC, only the biological outcome of PKA activation is studied. However, the translocation of the catalytic subunit of PKA into the nucleus is not efficient and may rely solely on diffusion (Harootunian at al., 1993). Therefore, high amounts of expression vector in the range of 1-5 µg/plate are often used (Grewal et al., 2000; Streeper et al., 2000, 2001) in order to observe an effect on gene transcription. The problem is that these high amounts of expressed catalytic subunit are far away from the physiological concentrations within the cells. The best way to study CREB-regulated gene transcription is to uncouple the investigation of transcriptional targets of CREB from the signaling pathways in the cell leading to CREB phosphorylation and activation. This was accomplished in this work by using the constitutively active mutant of CREB termed C2/CREB. Constitutively active mutants of ATF2 (C2/ATF2), c-Jun (C2/c-Jun) and C/EBPα (C2/CEBPα) were also used to study gene transcription mediated by ATF2, c-Jun and C/EBPα, respectively.

The bZIP proteins dimerize to bind specific DNA sequences. Many conflicting results were published concerning the dimerization of bZIP proteins but the dimerization code is not yet completely understood. To analyze the specificity of dimerization between different bZIP domains and also to determine bZIP dimerization partners the dominant-negative bZIP mutants termed A-ZIP mutants were used (Fig. 4). The dominant-negative bZIP mutants A-ZIPs are reagents that inhibit DNA-binding of the wild-type bZIP proteins in a leucine zipper-dependent fashion. A-ZIP proteins are amphipathic molecules that contain an acidic region instead of the natural basic domain N-terminal to the leucine zipper domain. This acidic extension of the leucine zipper forms a heterodimeric coiled coil structure with the basic region of its target that is more stable than the bZIP dimer bound to DNA. The heterodimer complexes formed between an A-ZIP mutant and a wild-type bZIP protein are then defective for DNA-binding (Vinson et al., 2002).

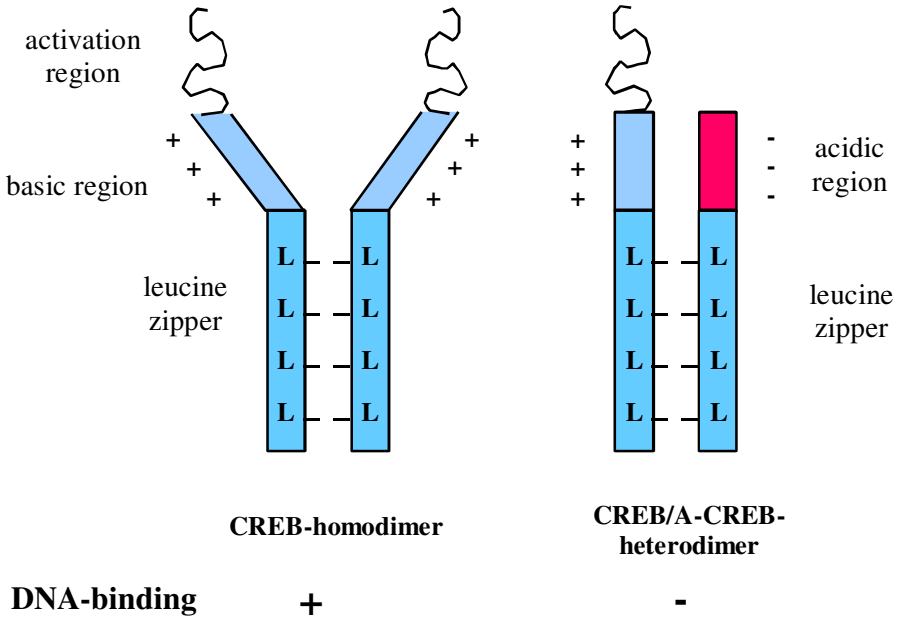


Fig.4. Schematic representation of the dominant-negative bZIP mutants termed A-ZIP mutants. CREB homodimer and CREB/A-CREB heterodimer are shown.

The aim of this work was to analyze and clarify genes regulated by different bZIP proteins by using either gain-of-function mutants (the constitutively active mutants, C2/bZIPs), or loss-of-function mutants (the dominant-negative mutants, A-ZIPs). Furthermore, gain-of-function experiments allowed the identification of target genes where the bZIP proteins are involved and loss-of-function experiments allowed better understanding of the dimerization code of different bZIP proteins.

B. Materials and Methods

B.1. Materials

B.1.1. Chemicals and Reagents

The chemicals used in this work were obtained from the following companies:

Acros organics (Geel, Belgium)
Biochrom (Berlin, Germany)
Fisher Scientific (Nidderau, Germany)
Fluka (Neu-Ulm, Germany)
Calbiochem (Bistro, GB)
Merck Biosciences (Schwalbach, Germany)
Pierce (Rockford, USA)
Roth (Karlsruhe, Germany)
Sigma (Taufkirchen, Germany)
Tocris (BIOTREND chemicals, Cologne, Germany)
VWR (Darmstadt, Germany)

The reagents were obtained from:

Axxora platform (Alexis Biochemicals, Gruenberg, Germany)
Biomol (Hamburg, Germany)
GIBCO BRL (Invitrogen Life Technologies, Karlsruhe, Germany)
MBI Fermentas (St. Leon-Rot, Germany)
PAA (Coelbe, Germany)
Promega (Mannheim, Germany)
Roche (Mannheim, Germany)
Stratagene Europe (Amsterdam, Holland)

The following substances and compounds used in this work were prepared as follows:

BAPTA-AM [1,2-*bis*(*o*-Aminophenoxy)ethane-N-N-N´-N´-tetraacetic Acid Tetra (acetoxymethyl) Ester]

Calbiochem # 196419

Stock Solution: 10 mM in DMSO

BAPTA-AM is a calcium chelator (Tsien, 1980, 1981).

Buserelin

Sigma # B-3303

Stock Solution: 1 mg/ml (806 µM) in distilled water

Amino acid sequence: Glu-His-Trp-Ser-Tyr-D-Ser-Leu-Arg-Pro-NHEt (Pinter et al., 1999).

Carbachol (Carbamylcholine chloride)

Sigma # C-4382

Stock solution: 100 mM in distilled water

Carbachol is an cholinergic receptor agonist (Yamada et al., 1997a; Pugh and Margiotta, 2000).

Chloroquine diphosphate salt

Sigma # C-6628

Stock solution: 10 mM in distilled water

Chloroquine was used to increase transfection efficiency (Luthman and Magnusson, 1983).

Complete

Roche # 11 697 498 001

Stock solution: 25x conc (one tablet Complete was dissolved in 2 ml redist water)

Complete is a Protease Inhibitor Cocktail Tablets, that is used for the inhibition of serine, cysteine and metalloproteases in bacterial, mammalian, yeast and plant cell extracts in large volumes (North, 1969).

G 418 sulfate

Calbiochem # 345810

Stock solution: 300 mg/ml in distilled water

JNK Inhibitor 1 (L-stereoisomer) [L-JNK1]

Alexis # 159-600-R100

Stock solution: 1 mM in PBS

Amino acid sequence: GRKKRRQRRR-PP-RPKRPTTLNLFPQVPRSQD-amide (Bonny et al., 2001).

KN-62 (1-[N,O-*bis*-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine)

Calbiochem # 422706

Stock solution: 5 mM in DMSO

KN-62 is known as a cell-permeable selective inhibitor of the calcium/calmodulin-dependent protein kinases (Tokumitsu et al., 1990).

Lipopolysaccharide (LPS) from E.coli

Sigma # L2630

Stock solution: 1 µg/µl in distilled water

LPS is the major component of the cell wall of Gram negative bacteria and is known to trigger a variety of inflammatory reactions in macrophages and other cells (Wright et al., 1990; Downey et al., 1998).

PD98059 (2´-amino-3´-methoxyflavon)

Calbiochem # 513

Stock solution: 75 mM in DMSO

PD98059 is an inhibitor of the MAP kinase kinase MEK that acts by inhibiting the activation of MAP kinase and subsequent the phosphorylation of MAP kinase substrates (Pang et al., 1995; Langlois et al., 1995).

SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole]

Alexis # 270-179-M001

Stock solution: 13.2 mM in DMSO

SB203580 is a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995; Saklatvala et al., 1996).

B.1.2. Filters and X-ray films

The nitrocellulose filters and membranes were obtained from either Schleicher & Schuell (Dassel, Germany) or VWR (Darmstadt, Germany) and the X-ray films were obtained from Fuji.

B.1.3. Vectors and Plasmids

B.1.3.1. Vectors and expression plasmids

Plasmids were kindly obtained from G. Thiel if nothing else is mentioned.

pM1

The pM1 expression plasmid was obtained from I. Sadowski (Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada). It encodes the DNA-binding domain of the transcription factor GAL4 (amino acids 1-147) of *Saccharomyces cerevisiae* (Sadowski et al., 1992).

pGAL4-c-Jun

The pGAL4-c-Jun expression plasmid was obtained from Michael Karin, University of California, San Diego (Groot et al., 2000). The GAL4 fusion protein encoded by this plasmid contains the transcriptional activation sequence from amino acids 1-246 of c-Jun fused to the DNA-binding domain of GAL4. The modular structure of GAL4-c-Jun is shown in Fig. 53A.

pGAL4-ATF2

The pGAL4-ATF2 expression plasmid was purchased from Stratagene (La Jolla, CA, USA). The GAL4 fusion protein encoded by this plasmid contains the transcriptional activation sequence from amino acids 1-96 of ATF2 fused to the DNA-binding domain of GAL4. The modular structure of GAL4-ATF2 is shown in Fig. 53A.

pGAL4-CREB2/ATF4

The GAL4 fusion protein encoded by this plasmid contains the transcriptional activation sequence from amino acids 6-181 of CREB2/ATF4 fused to the DNA-binding domain of

GAL4 (Schoch et al., 2001). The modular structure of GAL4-CREB2/ATF4 is shown in Fig. 53A.

pGAL4-CREB

The pGAL4-CREB expression plasmid was purchased from Stratagene (La Jolla, CA, USA). The fusion protein encodes the transcriptional activation sequence of CREB (amino acids 1-281) fused to the DNA-binding domain of GAL4. The modular structure of GAL4-CREB is shown in Fig. 16A.

pGAL4-ATF5

The pGAL4-ATF5 plasmid encodes the GAL4 fusion protein encompassing amino acids 14-204 of ATF5 fused to the DNA-binding domain of GAL4. The modular structure of pGAL4-ATF5 is shown in Fig. 53A.

pEBGN

The pEBGN expression vector encodes *Schistosoma japanicum* glutathione S-transferase GST under the control of elongation factor 1α gene promoter. In addition, pEBGN has a nuclear localization sequence (NLS) derived from the Simian Virus 40 large T antigen which was inserted to accomplish nuclear targeting of the GST fusion protein (Thiel and Cibelli, 1999).

pEBGN-CREB and pEBGN-KCREB

The pEBGN-CREB and pEBGN-KCREB plasmids encode the GST fusion proteins GST-CREB and GST-KCREB encompassing amino acids 235-326 of CREB or KCREB, respectively (Thiel et al., 2005a). The modular structure of GST-CREB and GST-KCREB is shown in Fig. 22A.

CREB133A and K-CREB

The CREB133A and K-CREB expression plasmids were a kind gift of Wilhart Knepel and Elke Oetjen, Department of Molecular Pharmacology, University of Göttingen, Germany. CREB133A contains a serine to alanine mutation at the position 133, while K-CREB is a CREB mutant containing a point mutation R236L within the basic DNA-binding domain, impairing DNA-binding (Thiel et al., 2005b).

pCMV5

The pCMV5 is an expression vector, where gene expression is controlled by the cytomegalovirus gene IE promoter/enhancer (NCBI accession # AF239249).

pCMV-FLAG-CREB

The pCMV-FLAG-CREB expression plasmid encodes amino acids 1-326 of CREB. In addition, a triple FLAG tag (sequence MDYKDHDGDYKDHDIDYKDDDDK) is present on the N-terminus for immunodetection of the protein (Thiel et al., 2005a). The modular structure of pCMV-FLAG-CREB is shown in Fig. 5.

pCMV-FLAG-CREB2

The pCMV-FLAG-CREB2 expression plasmid encodes amino acids 1-351 of CREB2/ATF4. In addition, a triple FLAG tag is present on the N-terminus (Thiel et al., 2005a). The modular structure of pCMV-FLAG-CREB2 is shown in Fig. 5.

pCMX-PL1-ATF2

The pCMX-PL1-ATF2 expression plasmid consists of the cDNA of the transcription factor ATF2 and is controlled by the cytomegalovirus gene IE promoter enhancer. This plasmid was a kind gift of Jon D. Shuman (NIAID, Rockville, USA) (Steinmüller and Thiel, 2003).

pCMV-FLAG-C2/CREB

The pCMV-FLAG-C2/CREB plasmid encodes a constitutively active C2/CREB chimera. The C2/CREB fusion protein encodes amino acids 1-187 of CREB2 (the transcriptional activation domain of CREB2) fused to amino acids 182-326 of CREB (the C-terminal region of CREB encompassing the bZIP domain). In addition, a triple FLAG tag is present on the N-terminus (Thiel et al., 2005a, b). The modular structure of pCMV-FLAG-C2/CREB is shown in Fig. 5.

pCMV-FLAG-C2/ATF2

The pCMV-FLAG-C2/ATF2 expression plasmid encodes a constitutively active ATF2 mutant. The encoded protein contains the transcriptional activation domain of CREB2 (amino acids 1 to 187) linked to the C-terminal region of ATF2 (amino acids 92-389), encompassing the bZIP domain of ATF2. In addition, a triple FLAG tag is present on the N-terminus (Steinmüller and Thiel, 2003). The modular structure of pCMV-FLAG-C2/ATF2 is shown in Fig. 19.

pCMV-FLAG-C2/c-Jun

The pCMV-FLAG-C2/c-Jun expression plasmid encodes a constitutively active C2/c-Jun chimera. The encoded protein contains the transcriptional activation domain of CREB2 (amino acids 1 to 187) linked to the C-terminal region of c-Jun (amino acids 188 to 331), encompassing the bZIP domain of c-Jun. In addition, a triple FLAG tag is present on the N-terminus. The modular structure of pCMV-FLAG-C2/c-Jun is shown in Fig. 37.

pCMV-FLAG-C/EBPα

The pCMV-FLAG-C/EBPα expression plasmid encodes amino acids 1-358 of C/EBPα. In addition, a triple FLAG tag is present on the N-terminus (Thiel et al., 2005a). The modular structure of pCMV-FLAG-C/EBPα is shown in Fig. 60.

pCMV-FLAG-C2/C/EBPα

The pCMV-FLAG-C2/C/EBPα expression plasmid encodes the chimeric transcription factor C2/C/EBPα consisting of the N-terminal transcriptional activation domain of CREB2 (amino acids 1 to 187) and the C-terminal bZIP domain of C/EBPα (amino acids 245 to 358). In addition, a triple FLAG tag is present on the N-terminus The modular structure of pCMV-FLAG-C2/C/EBPα is shown in Fig. 60.

pCMV-FLAG-ATF5

The pCMV-FLAG-ATF5 expression plasmid encodes amino acids 1-281 of ATF5. In addition, a triple FLAG tag is present on the N-terminus.

pCMV-CHOP

The pCMV-CHOP expression vector contains 170 amino acids of CHOP. Additionally, a triple FLAG tag is present on the N-terminus.

pCMV-FLAG-ATF2ΔN

The pCMV-FLAG-ATF2ΔN expression plasmid encodes a truncated form of ATF2, consisting of the C-terminus of the transcription factor ATF2 (Steinmüller and Thiel, 2003). The modular structure of pCMV-FLAG-ATF2ΔN is shown in Fig. 41B.

pCMV-FLAG-c-JunΔN

The pCMV-FLAG-c-JunΔN expression plasmid encodes a truncated form of c-Jun, consisting of the C-terminus of the transcription factor c-Jun (Steinmüller and Thiel, 2003). The modular structure of pCMV-FLAG-c-JunΔN is shown in Fig. 41B.

MEKK1Δ

The MEKK1Δ expression plasmid was obtained from Michael Karin, University of California, San Diego. It encodes a truncated form of MEK kinase 1 lacking the amino acids sequence from 1 to 351 (Minden et al., 1994).

pMKP-1

The pMKP-1 plasmid was obtained from N. G. Tonks, Cold Spring Harbour Laboratory, New York, USA (Sun et al., 1993; Kaufmann et al., 2001).

pcDNA3MKK6(E)

The pcDNA3MKK6(E) expression plasmid contains a mutated MAP kinase kinase 6 (MKK6)-cDNA inserted into the expression vector pcDNA3 (Invitrogen, San Diego, USA) with HA-Epitope Tag. This mutant was designed by a replacement of serine 151 and threonine 155 with glutamate. This plasmid was kindly obtained from J. Han, Department of Immunology, The Scripps Research Institute, La Jolla, USA (Han et al., 1996).

pcDNA3p38

The pcDNA3p38 plasmid that contains the p38-cDNA and a FLAG-epitope inserted into the pcDNA3 vector (Invitrogen, San Diego, USA) was a kind gift of J. Han, Department of Immunology, The Scripps Research Institute, La Jolla, USA (Jiang et al., 1996).

pCMVCα

The pCMVCα plasmid that encodes the catalytic subunit of PKA (amino acids 2-351) was a kind gift of Michael Uhler from the University of Michigan, Ann Arbor (Uhler and McKnight, 1987). The modular structure of pCMVCα is shown in Fig. 13.

NLSCα

The NLSCα expression plasmid has a triple FLAG-tag and a nuclear localization signal on the N-terminus, followed by amino acids 19 to 351 of the catalytic subunit of PKA (Thiel et al., 2005a, b). The modular structure of NLSCα is shown in Fig. 13.

pSV40LacZ

The pSV40LacZ plasmid encodes β-galactosidase under the control of the Simian Virus 40 promoter (Schoch et al., 2001).

pRSVβ

The pRSVβ plasmid encodes β-galactosidase under the control of the LTR (Long Terminal Repeat) of the Rous Sarcoma Virus derived from the *Escherichia coli* (Jüngling et al., 1994).

B.1.3.2. Reporter plasmids

pGTPCHIluc

The pGTPCHIluc reporter plasmid contains the luciferase open reading frame under control of the GTP cyclohydrolase I promoter sequences from –97 to –60 upstream of a minimal promoter encompassing a TATA box derived from the HIV long terminal repeat and an initiator element from the adenovirus major late promoter. The schematic representation of the modular structure of pGTPCHIluc is depicted in Fig. 6A.

pGTPCHICREmutluc

The pGTPCHICREmutluc reporter plasmid contains three point mutations within the CRE-like sequence of the GTP cyclohydrolase I promoter/luciferase reporter gene. The schematic representation of the modular structure of pGTPCHIluc is depicted in Fig. 8A.

pTNFα(-1311)luc

The TNFα promoter/luciferase reporter plasmid pTNFα(-1311)luc (Rhoades et al., 1992) was a kind gift of James S. Economou, UCLA, Los Angeles. The schematic representation of the modular structure of pTNFα(-1311)luc is depicted in Fig. 28.

pTNFα(-131)luc

The pTNFα(-131)luc reporter plasmid consists of a TNFα promoter/luciferase reporter gene containing one copy of the cAMP Response Element/Activating Protein 1 (CRE/AP1) element of the human TNFα gene. The schematic representation of the modular structure of pTNFα(-131)luc is depicted in Fig. 11B.

pTNFα(CRE/AP1)^{2}luc

The pTNFα(CRE/AP1)^{2}luc reporter plasmid consists of a TNFα promoter/luciferase reporter gene containing two copies of the CRE/AP1 element of the human TNFα gene. (Thiel et al., 2005b). The schematic representation of the modular structure of pTNFα(CRE/AP1)^{2}luc is depicted in Fig. 11A.

pASluc

The asparagine synthetase promoter/luciferase reporter gene contains nucleotides from –79 to –35 of the human asparagine synthetase gene. The schematic representation of the modular structure of pASluc is depicted in Fig. 46B.

2xC/EBPluc

The 2xC/EBPluc reporter plasmid has two C/EBP binding sites upstream of a minimal herpes simplex virus thymidine kinase promoter. It was a kind gift from Peter F. Johnson, NCI-Frederick Cancer Research and Development Center (Thiel et al., 2005a). The schematic representation of the modular structure of 2xC/EBPluc is depicted in Fig. 61A.

pSgIICRE^{4}luc

The pSgIICRE^{4}luc reporter plasmid contains four copies of the CRE derived from the secretogranin II gene upstream of the HIV-LTR TATA box and the adenovirus later promoter initiator element. (Thiel et al., 2005a). The schematic representation of the modular structure of pSgIICRE^{4}luc is depicted in Fig. 31.

pSgIIluc

The pSgIIluc reporter plasmid contains a secretogranin II promoter/luciferase reporter gene encompassing the sequence from –167 to +35 of the murine secretogranin II gene (Thiel et

al., 2005a). The schematic representation of the modular structure of pSgIIluc is depicted in Fig. 32.

pc-fosCRE^{4}luc

The pc-fosCRE^{4}luc reporter plasmid contains four copies of the CRE derived from the c-Fos gene upstream of the HIV LTR TATA box and the adenovirus later promoter initiator element (Thiel et al., 2005a). The schematic representation of the modular structure of pc-fosCRE^{4}luc is depicted in Fig. 35.

pUAS^{5}luc

The pUAS^{5}luc reporter plasmid contains five binding sites for the yeast transcription factor GAL4 in pHIVTATAluc (Thiel et al., 2000). The schematic representation of the modular structure of pUAS^{5}luc is depicted in Fig. 16C.

pEgr-1.1luc

The pEgr-1.1luc reporter plasmid consists of an Egr-1 promoter/luciferase reporter plasmid encompassing sequence from –237 to +235 of the human Egr-1 gene was inserted into pGL3-Basic (Promega) (Groot et al., 2000). The schematic representation of the modular structure of pEgr-1.1luc is depicted in Fig. 20B.

pTHluc

The pTHluc reporter plasmid consists of the tyrosine hydroxylase/luciferase reporter plasmid which expression is controlled by the tyrosine hydroxylase promoter sequences from -760 to +18 (Thiel et al., 2005a). The schematic representation of the modular structure of pTHluc is depicted in Fig. 36.

pG6PCRE1/CRE2luc

The pG6PCRE1/CRE2luc reporter plasmid contains the luciferase open reading frame under control of glucose-6-phosphatase promoter sequences from –166 to –122 encompassing both CRE-like sequences CRE1 and CRE2, upstream of a minimal promoter consisting of the TATA box of the human immunodeficiency virus and the adenovirus major late promoter initiator element (Thiel et al., 2005b). The schematic representation of the modular structure of pG6PCRE1/CRE2luc is depicted in Fig. 9B.

pG6PCRE1mut/CRE2luc and pG6PCRE1/CRE2mutluc

The pG6PCRE1mut/CRE2luc and pG6PCRE1/CRE2mutluc are glucose-6-phosphatase promoter/luciferase reporter genes carrying mutations inactivating either the CRE1 or the CRE2 sites (Thiel et al., 2005b). The schematic representation of the modular structure of pG6PCRE1mut/CRE2luc and pG6PCRE1/CRE2mutluc is depicted in Fig. 9B.

pG6PCRE1luc

The pG6PCRE1luc reporter plasmid contains glucose-6-phosphatase promoter sequences from –173 to –140 encompassing the CRE1 motif (Thiel et al., 2005b). The schematic representation of the modular structure of pG6PCRE1luc is depicted in Fig. 9C.

pG6PCRE2luc

The pG6PCRE2luc reporter plasmid contains glucose-6-phosphatase promoter sequences from –152 to –117 encompassing the CRE2 motif (Thiel et al., 2005b). The schematic representation of the modular structure of pG6PCRE2luc is depicted in Fig. 9C.

B.1.4. Cell Culture

The human hepatoma cell line HepG2 was purchased from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.

The mouse microglial cell line BV-2 was obtained from G. Cibelli, Department of Biomedical Science, University of Foggia, Italy.

The locus coeruleus-like cell line CATH.a was obtained from D. Chikaraishi, Duke University Medical Center, Durham, NC, USA.

The mouse gonadotrope cell line αT3M3 was obtained from G. Willars, Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, U.K.

The human embryonal kidney cell line 293T/17 was obtained from D. Baltimore, Rockefeller University, New York, USA.

HepG2, BV-2, αT3M3 and 293T/17 cells were maintained in Dulbecco´s modified Eagles medium (DMEM) supplemented with:
10% heat-inactivated fetal calf serum (FCS)
100 U/ml penicillin
100 µg/ml streptomycin
2 mM glutamine
Cells were incubated at 37°C in 5% CO_2.
For αT3M3 cells 300 µg/ml G418 were added to the medium.

CATH.a cells were cultured in RPMI medium supplemented with:
4% heat-inactivated fetal calf serum
8% heat-inactivated horse serum
100 U/ml penicillin
100 µg/ml streptomycin
Cells were incubated at 37°C in 5% CO_2.

PBS, used for all washing and harvesting steps, consists of:
170 mM NaCl
3.3 mM KCl
4 mM Na_2HPO_4
1.84 mM KH_2PO_4
pH was adjusted to 7.2.

B.2. Methods

B.2.1. Transformation of competent E.coli

1-2 µl of plasmid was added to a Falcon tube containing 60 µl CM buffer and 50 µl of competent E.coli. The mixture was incubated for 20 min on ice and then at room temperature for 10 min. 500 µl LB medium was added and the mixture was incubated at 37°C for 1 h with shacking. The mixture was then plated on LB agar supplemented with ampicillin plates and incubated overnight at 37°C.

Solutions

- CM buffer: 33 mM $CaCl_2$, 55 mM $MgCl_2$, 110 mM KCl
- LB medium: 10 g/l Trypton, 5 g/l yeast extract, 5 g/l NaCl

B.2.2. DNA preparation

A bacterial conlony was picked from plate with transformed competent cells and dissolved in 200 µl LB medium containing ampicillin. The mixture was incubated overnight at 37°C with shacking and then centrifuged for 30 min at 4000 rpm. The pellet was used to purify the plasmids according to the standard protocols of Qiagene company.

B.2.3. Transfection applying calcium phosphate method

The calcium phosphate method was used to transfect HepG2 and CATH.a cells. HepG2 cells were seeded in quadruplicate at a density of $8x10^5$ cells/60 mm plate. Cells were maintained overnight in 10% DMEM at 37°C in 5% CO_2.

CATH.a cells were seeded in quadruplicate at a density of $125x10^4$ cells/60 mm plate. Cells were maintained overnight in RPMI complete medium at 37°C in 5% CO_2.

For each calcium phosphate transfection a mix "calcium phosphate/DNA suspension" of two solutions was prepared as follows:

Solution 1: certain amount of the DNA was filled up to a final volume of 1 ml with H_2O mixed with 110 µl 2.5 M $CaCl_2$, which was slowly added to solution 2 and mixed by bubbling for 20 sec.

Solution 2: 1100 µl 2 x HBSS (274 mM NaCl, 25 mM KCl, 3.75 mM Na_2HPO_4, 27.75 mM glucose and 105 mM HEPES, pH 7.05-7.12, sterile filtration).

HepG2 cells: The medium in which the cells were maintained overnight was aspirated, 500 µl of the calcium phosphate/DNA suspension was added to the cells. After 20 min incubation at 37°C in 5% CO_2, 10% medium containing 10 µM chloroquine was added and the cells were again incubated at 37°C in 5% CO_2. Four to six hours later a glycerol shock was performed as follows:

Cells were washed twice with 1x PBS and incubated for two min with 15% glycerol (in 1x PBS) then washed two times with 1x PBS and again incubated with 10% DMEM overnight. Forty eight hours later cells were harvested and the β-galactosidase and luciferase activities were determined as described in the next chapter.

CATH.a cells: Cells were seeded and maintained overnight in complete RPMI medium at 37°C in 5% CO_2. The next day cells were transfected using the same way used to transfect HepG2. Three hours post-transfection a glycerol shock was performed, cells were incubated with RPMI complete medium overnight. Forty eight hours later cells were harvested and the β-galactosidase and luciferase activities were determined as described in the next chapter.

B.2.3.1. Determination of β-galactosidase and luciferase activities

The pellet obtained after transfection was lyzated using 50 µl 1x reporter lysis buffer (Promega, Heidelberg, # E3971), incubated for five min on ice and then centrifuged at 16000 x g at 4°C for 5 min, the supernatant was used for the following measurement:

1- Measurement of β-galactosidase activity:

30 µl cell extract were added to:

3 µl 100 x Mg

66 µl 1x ONPG (*O*-nitrophenyl-2-D-galactopyranoside)

201 µl 0.1 M Sodium phosphate (pH 7.5)

The reaction was incubated at 37°C maximum for two hours. When the color turned yellow the absorption was measured using a test wavelength of 405 nm.

The β-galactosidase activity was calculated as follow:

$$\frac{(380 \times OD_{405})}{\text{Reaction time [min]}}$$

Solutions:

- 100 x Mg: 0.1 M $MgCl_2$, 4.5 M β-Mercaptoethanol

- ONPG: 13 mM ONPG in 0.1 M sodium phosphate buffer, pH 7.5
- 0.1 M Sodium phosphate: 41 ml 0.2 M $Na_2HPO_4.2H_2O$, 9 ml 0.2 M $NaH_2PO_4.2H_2O$, 50 ml distelled water

2- Measurement of luciferase activity:

10 µl of cell extracts were mixed with 100 µl "Working Luciferase Assay Reagent" the luminescence was measured using the luminometer (Berthold Detection Systems). Luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities in order to obtain the relative luciferase activity.

Solutions:
- Working Luciferase Assay Reagent (WLAR):
20 mM Tricin (N-[Tris(hydroxymethyl)methyl]glycin) pH 7.8
1.07 mM $(MgCO_3)_4Mg(OH)_2$ 5 H_2O
2.67 mM $MgSO_4$ 7 H_2O
0.1 mM EDTA
33 mM DTT
270 µM Coenzyme A
530 µM ATP
470 µM Luciferin

B.2.4. Preparation of nuclear extracts

This preparation was performed according to Gerber et al., 1992 and also according to Andrews and Faller, 1991. BV-2 and αT3M3 cells were seeded at a density of $1x10^6$ cells/60 mm plate and $2.5x10^6$ cells/60 mm plate, respectively. Cells were maintained in 10% DMEM (standard medium) at 37°C in 5% CO_2. Twenty-four hours later cells were washed for two times with PBS and incubated with serum free medium for further twenty-four hours and then stimulated. Cells were then harvested with cold PBS and centrifuged at 16000 x g for 5 min and the pellet was redissolved in 100 µl lysis buffer containing:
10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH, pH 7.9
1.5 mM $MgCl_2$

10 mM KCl
0.2% NP40 (was added freshly)
0.5 mM DTT (was added freshly)
0.2 mM PMSF (was added freshly)
25xconc Complete Protease Inhibitor (was added freshly)
10 mM NaF (was added freshly)
1 mM Na_3VO_4 (was added freshly)

Pellet was incubated on ice for 10 min, vortexed, incubated for further 10 min and centrifuged for 3 min at 16000 x g at 4°C. After removing the supernatant, then the pellet was resuspended in 20 µl of nuclear extraction buffer consisting of:
20 mM HEPES-KOH, pH 7.9
25% glycerol
420 mM NaCl
1.5 mM $MgCl_2$
0.2 mM PMSF (was added freshly)
25xconc Complete Protease Inhibitor (was added freshly)

Pellet was incubated for 20 min on ice and centrifuged for 3 min at 16000 x g at 4°C to extract the nuclear proteins. Supernatant was collected in a new tube, some of the supernatant was used to measure the protein concentrations and the rest was stored at –20°C after adding SDS-Stop buffer and boiling for 5 min at 95°C.

SDS-Stop buffer (stock solution):
125 mM Tris pH 6.8
3 mM EDTA
20% (v/v) Glycerol
9% SDS
0.05% Bromophenol blue

SDS-Stop working buffer:
9 Vol. SDS-Stop buffer (stock solution)
1 Vol. β-Mercaptoethanol

B.2.5. Preparation of whole cell extracts

BV-2 cells were seeded at a density of $1x10^6$ cells/60 mm plate. Cells were maintained in 10% DMEM (standard medium) at 37°C in 5% CO_2. Twenty-four hours later cells were washed two times with PBS and incubated with serum free medium for further twenty-four hours and then stimulated. Twenty-four hours later cells were harvested with cold PBS and centrifuged at 16000 x g for 5 min and the pellet was redissolved in 50-100 µl lysis buffer containing:
10 mM Tris pH 7.5
400 mM NaCl
0.1 mM EDTA
0.5% Triton
0.02% NaN_3
0.2 mM PMSF (was added freshly)
0.5 mM DTT (was added freshly)
25xconc Complete Protease Inhibitor (was added freshly)

Pellet was strongly vortexed and incubated on ice for 20 min and then centrifuged for 10 min at 16000x g at 4°C. Supernatant was collected in a new tube, some of supernatant was used for protein estimation and the rest was stored at –20°C after adding SDS-Stop buffer and boiling for 5 min at 95°C.

B.2.6. Protein estimation

Protein concentrations were determined using BCA Protein Assay Reagent (Pierce, # 23225) by mixing:
5 ml reagent A [Bicinchoninic acid solution (BCA)]
100 µl reagent B ($CuSO_4$ 4%)
Bovine serum albumin was used as standard proteins. The reaction was incubated at 37°C for 30 min and the absorbance was measured using a test wavelength of 490 nm.

B.2.7. Western blot analysis

Preparing SDS-PAGE gel:

- Separating gel is prepared as follow (final volume 10 ml):

% polyacrylamide	**Buffer A**	**Buffer C**	**H_2O**
7.5%	2.5 ml	2.5 ml	5 ml
10%	2.5 ml	3.3 ml	4.2 ml

Polymerization: 200 µl APS 10%
20 µl TEMED

- **Stacking gel** is prepared as follow (final volume 5 ml):

1.25 ml buffer B

583 µl buffer C

3.17 ml H_2O

Polymerization: 200 µl APS 10%
20 µl TEMED

Buffer A (separating gel buffer, store at RT):

1.5 M Tris, pH 8.9

0.4% SDS

Buffer B (stacking gel buffer, store at RT):

0.5 M Tris, pH 6.8

0.4% SDS

Buffer C (store at 4°C):

Acrylamide/Bisacrylamide buffer (Rotiphorese Gel 30, Roth # 3029.1)

Running buffer (Laemmli buffer):

250 mM Tris

2.5 M Glycine

1% SDS

The proteins were first denaturated at 95°C for 5 min. Twenty micrograms of the proteins were separated on 7.5% or 10% gel SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (0,2 µm pore size). Membranes were blocked in 3%-5% non-fat dry milk in 1x TBS or 1x TBS containing 0.05% Tween 20 (1x TBST) for 30 min-1 h at room temperature or overnight at 4°C on an orbital shaker. Blots were washed three times with distilled water then incubated for 2.5 h at room temperature with the primary antibodies and then washed three times with distilled water. Secondary antibodies were incubated for 1-1.5 h at room temperature and then washed three times in H_2O. Blots were developed using ECL plus (Amersham, Freiburg, Germany).

Solutions for Immunoblotting:

- Blotting buffer (transfer buffer):
25 mM Tris
250 mM Glycine
20% Methanol

- 10x TBS:
10 mM Tris-HCl, pH 8.0
150 mM NaCl

Antibodies

Different primary antibodies were used; the source, species and dilution are shown in the table below. The secondary antibodies were obtained from:

- Goat anti-rabbit peroxidase-labeled antibody, Dianova, Hamburg, Germany # 111-035-003
- Goat anti-mouse antibody, Sigma, Taufkirchen, Germany # A-4416

Primary Antibody	Source	Species	Dilution	Secondary Antibody	Dilution
Anti-i-NOS/NOSII	Upstate, Biomol, Hamburg, Germany # 06-573	Mouse, bovine, sheep and guinea pig	1:1000 in 3% non-fat dry milk in TBS	Goat anti-rabbit peroxidase-labeled antibody	1:10000 in 3% non-fat dry milk in TBS
ATF2 (N-96)	Santa Cruz, Heidelberg, Germany # sc-6233	Human, mouse and rat	1:3000 in 3% non-fat dry milk in TBS	Goat anti-rabbit peroxidase-labeled antibody	1:10000 in 3% non-fat dry milk in TBS
p-ATF2 (F-1)	Santa Cruz, Heidelberg, Germany # sc-8398	Human, mouse and rat	1:1000 in 3% non-fat dry milk in TBS containing 0.05% Tween 20 (TBST)	Goat anti-mouse antibody	1:10000 in 3% non-fat dry milk in TBST
Anti-c-Jun	Upstate, Biomol, Hamburg, Germany # 06-225	Human and mouse	1:1000 in 3% non-fat dry milk in TBS	Goat anti-rabbit peroxidase-labeled antibody	1:10000 in 3% non-fat dry milk in TBS
Anti-phospho-c-Jun (Ser 73)	Upstate, Biomol, Hamburg, Germany # 06-659	Human and mouse	1:2500 in 5% non-fat dry milk in TBST	Goat anti-rabbit peroxidase-labeled antibody	1:10000 in 5% non-fat dry milk in TBST
c-FOS(4)	Santa Cruz, Heidelberg, Germany # sc-52	Human, mouse and rat	1:1000 in TBST	Goat anti-rabbit peroxidase-labeled antibody	1:10000 in TBST

B.2.8. Stripping and reprobing of membranes

This method was perfomed as described in ECL Plus booklet as follow:

1- Submerge the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.7) and incubate at 50°C for 30 min with occasional agitation.
2- Wash the membrane twice in 1xTBST at room temperature using large volumes of wash buffer.
3- Block the membrane in blocking solution (non-fat dry milk in TBS or TBST).
4- Perform immunodetection.

B.2.9. Lentiviral gene transfer

293T/17 cells were seeded at a density of $2.3x10^6$ cells/60 mm plate and were maintained in 10% DMEM (standard medium) at 37°C in 5% CO_2. Next day the viral particles were produced by performing transient transfection experiment using the calcium phosphate co-precipitation technique described earlier. Three plasmids were transfected into the cells: 6.6 µg of lentiviral transfer vector, 5 µg of packaging vector (pCMVΔR8.91) and 2.3 µg of plasmid encoding the vesicular stomatitis virus glycoprotein (pCMVG). Seventy-two hours post-transfection viral supernatants were collected, filtered through a 0.45 µm filter and used to infect BV-2 and/or αT3M3 cells in the presence of 8 µg/ml polybrene. The cells were maintained at 37°C in 5% CO_2. Forty-eight to seventy-two hours later the infected cells were harvested, part of the cells were used to prepare nuclear extracts in order to detect the presence of the construct by performing Western blot analysis and the rest of the cells was seeded at a density of $1x10^6$ cells/60 mm plate (for BV-2) and $2.5x10^6$ cells/60 mm plate (for αT3M3) and, following induction, nuclear extracts and/or whole cells extracts were prepared.

C. Results

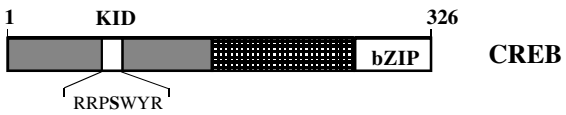
C.1. Regulation of gene expression by CREB

C.1.1. The constitutively active CREB mutant C2/CREB activates transcription of reporter genes containing CRE-like sequences

The bZIP transcription factor CREB is transcriptionally silent in the unphosphorylated state and this excludes a simple overexpression strategy to analyze the biological impact of the CRE or CRE-like sequences within different genes. Therefore, to study CREB-mediated gene transcription a constitutively active CREB mutant termed C2/CREB was used.

C.1.1.1. Modular structure of CREB, CREB2 and the constitutively active CREB mutant C2/CREB

The bZIP proteins CREB and CREB2 contain on their C-termini a bZIP domain responsible for DNA-binding and dimerization, while the N-termini contain activation domains. The activation domain of CREB2 is constitutively active and transferable to heterologous DNA-binding domains (Schoch et al., 2001), while the activation domain of CREB is controlled by phosphorylation. The bZIP domain of CREB, which is responsible for DNA-binding and dimerization, was expressed as a fusion protein with the constitutively active transcriptional activation domain of CREB2, generating the chimeric transcription factor C2/CREB. Additionally, the C2/CREB fusion protein contains an immunological tag used for detection of the protein. This mutant is constitutively active and does not require phosphorylation for activation.



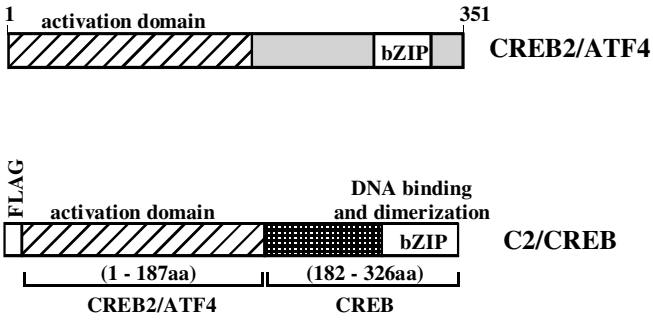


Fig. 5. Modular structure of CREB, CREB2 and C2/CREB.
Schematic representation of the bZIP proteins CREB, CREB2 and the constitutively active mutant C2/CREB.

C.1.1.2. C2/CREB mutant transactivates a reporter gene under the control of the proximal regulatory region of the human GTP cyclohydrolase I gene

GTP cyclohydrolase I is the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin, it catalyzes the formation of dihydroneopterin triphosphate from GTP. It has been shown that signaling molecules that elevate the intracellular concentration of cAMP may also be involved in the regulation of GTP cyclohydrolase I expression, because cell-permeable analogues of cAMP and the adenylate cyclase activator forskolin activate GTP cyclohydrolase I gene transcription in PC12 and mesangial cells (Plüss et al., 1996, 1997; Hirayama et al., 2001). Additionally, the sequence motif 5`-TGACGCGA-3`, that resembles the cAMP response element (CRE), has been identified in the core promoter of the human GTP cyclohydrolase I gene (Kapatos et al., 2000). The constitutively active CREB mutant C2/CREB was used to test whether the GTP cyclohydrolase I gene is transactivated by CREB via the CRE-like sequence. In addition, a reporter plasmid containing the proximal region of the human GTP cyclohydrolase I gene upstream of a minimal promoter was also used (Fig. 6A). Transient transfection experiments of the human hepatoma cell line HepG2 were performed to study the regulation of the GTP cyclohydrolase I promoter/luciferase reporter gene by C2/CREB. GTP cyclohydrolase I was purified from rat liver (Hatakeyama et al., 1989) and it was cloned from rat and human hepatocytes (Hatakeyama et al., 1991; Togari et al., 1992). The result shows that transcription of the GTP cyclohydrolase I reporter gene was strongly activated by the constitutively active CREB mutant C2/CREB and the induction was on the order of 77-fold (Fig. 6B).

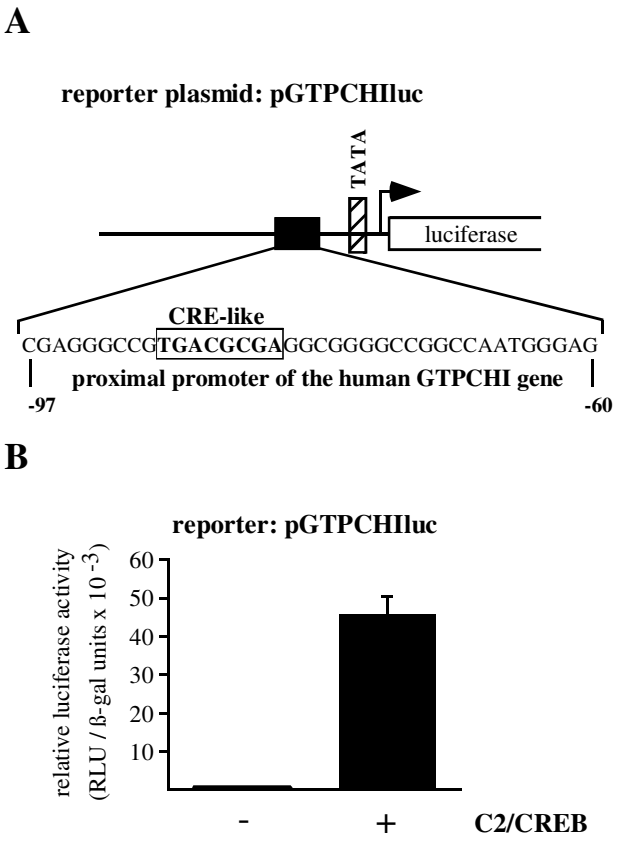


Fig. 6. C2/CREB activates the transcription of a GTP cyclohydrolase I promoter/luciferase reporter gene.
(A) Schematic representation of the reporter plasmid GTPCHIluc. Position and sequence of the CRE-like motif are shown. (B) The reporter plasmid pGTPCHIluc (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pRSVβ, encoding β-galactosidase under the control of the Rous sarcoma virus long-terminal repeat, to correct for variations in transfection efficiencies (2 µg/plate). In addition, either an empty expression vector pCMV5 (25 ng/plate) or an expression vector encoding C2/CREB (pCMV-FLAG-C2/CREB) (25 ng/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the luciferase activities were normalized for transfection efficiency by dividing luciferase light units by β-galactosidase activities. Data are mean ±SD.

C.1.1.2.1. C2/CREB-induced transcription of the GTP cyclohydrolase I reporter gene is impaired by dominant-negative CREB mutant (A-CREB)

The DNA-binding and dimerization specificity of C2/CREB and CREB are identical, since they share an identical bZIP domain. To check whether other bZIP proteins are able to interfere with C2/CREB-induced transcriptional activation of the GTP cyclohydrolase I promoter, the dominant-negative mutants A-ZIPs were used. The result shows that only A-CREB was able to inhibit C2/CREB-induced transcription of the GTP cyclohydrolase I reporter gene (Fig. 7). Expression of A-ATF2, a dominant-negative of ATF2, did not change

C2/CREB-induced reporter gene transcription, indicating that ATF2 does not heterodimerize with CREB. A-ATF4, a dominant-negative of CREB2/ATF4, A-Fos, a dominant-negative of c-Jun, and A-C/EBP, a dominant-negative of C/EBP, even enhanced transcription of the reporter gene, maybe by sequestering inhibitory cofactors away from the transcription units. These data indicate that only A-CREB interferes with C2/CREB-induced transactivation of the GTP cyclohydrolase I/luciferase reporter gene and that the bZIP proteins CREB2/ATF4, c-Jun, ATF2 and C/EBP are unable to heterodimerize with CREB.

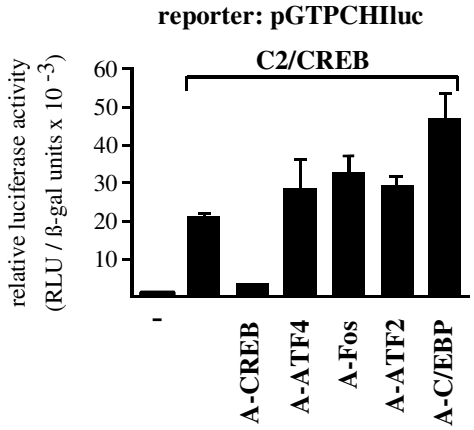


Fig. 7. Dominant-negative mutant of CREB impairs C2/CREB-induced transcriptional activation of the GTP cyclohydrolase I reporter gene.
HepG2 cells were transfected with 1 µg/plate of the reporter plasmid pGTPCHIluc, 2 µg/plate of the internal standard plasmid pRSVβ, 25 ng/plate of either an empty expression vector "pCMV5" or an expression vector encoding C2/CREB and 250 ng/plate of expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined by measuring β-galactosidase and luciferase activities of the transfected cells. Data are mean ± SD.

C.1.1.2.2. Mutations of the CRE-like sequence of the GTP cyclohydrolase I promoter impair transactivation by CREB

From the data shown above, it was presumed that the CRE-like sequence is important for CREB-mediated transactivation of the GTP cyclohydrolase I/luciferase reporter gene. To confirm this observation, GTP cyclohydrolase I reporter gene that contains three point mutations within the CRE-like sequence was generated (Fig. 8A). *In vitro* DNA/protein binding experiments with annealed oligonucleotides carrying these point mutations showed decreased but not eliminated binding of nuclear proteins by these mutations. Furthermore, these mutated oligonucleotides retained the ability to compete with the wild-type

oligonucleotide that contains the intact CRE-like sequence (Kapatos et al., 2000). Transfection experiments employing both the intact and the CRE-like mutated reporter genes show that the CRE-like sequence is of major importance for the constitutively active CREB mutant to transactivate the GTP cyclohydrolase I promoter/luciferase reporter gene (Fig. 8B).

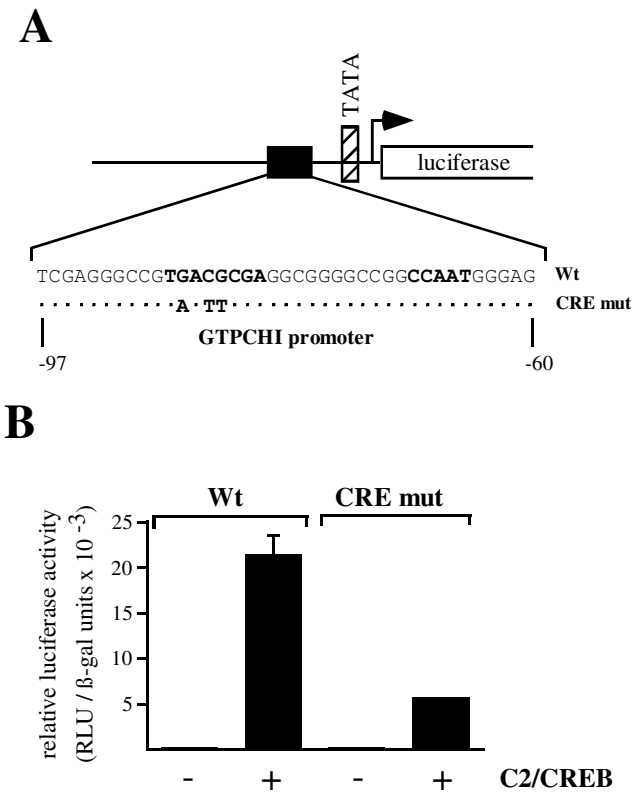


Fig. 8. Point mutations within the CRE-like sequence of the GTP cyclohydrolase I promoter impair transactivation mediated by C2/CREB.
(A) Schematic representation of the GTP cyclohydrolase I reporter plasmids containing an intact or a mutated CRE-like sequence. (B) 1 µg/plate of the wild-type or the mutated GTP cyclohydrolase I reporter plasmid was transfected into HepG2 cells, together with the standard plasmid RSVβ (2 µg/plate). In addition, either an empty expression vector pCMV5 (25 ng/plate) or an expression vector encoding C2/CREB (25 ng/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.1.1.3. C2/CREB transactivates the transcription of glucose-6-phosphatase promoter

Glucose-6-phosphatase is a multicomponent system located in the endoplasmic reticulum that catalyzes the terminal step in glycogenolysis in the liver and gluconeogenesis in the liver and kidney (Foster et al., 1997; Mithieux, 1997; Van de Werve et al., 2000). The proximal region of the human glucose-6-phosphatase promoter contains two CRE-like sequences that

resemble the canonical CRE sequence 5´-TGACGTCA-3´ (Lin et al., 1997; Schmoll et al., 1999). The distal CRE-like site (CRE1) and the proximal CRE-like site (CRE2) differ on two or three positions, respectively, in comparison to the canonical CRE. To study the biological function of these elements reporter plasmids that contain both CRE-like elements or only one of them were used. All the reporter genes contained a TATA box derived from the HIV long terminal repeat, an initiator element from the adenovirus major late promoter and the luciferase open reading frame (Fig. 9).

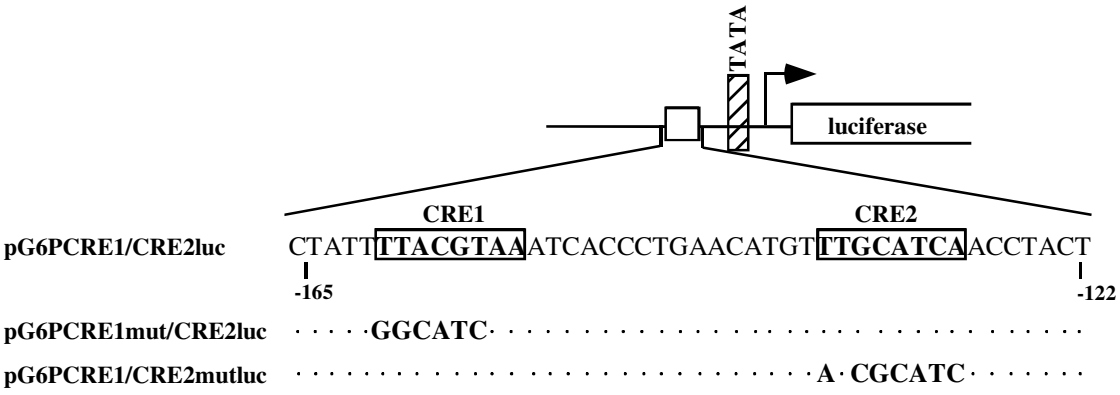
A

Human glucose-6-phosphatase promoter

TGTGTGCCTGTTTTTCTATT**TTACGTAA**ATCACCCTGAACATGT**TTGCATCA**ACCTACTGGTGATGCACCT

-180 **CRE1** **CRE2** **-110**

B



C

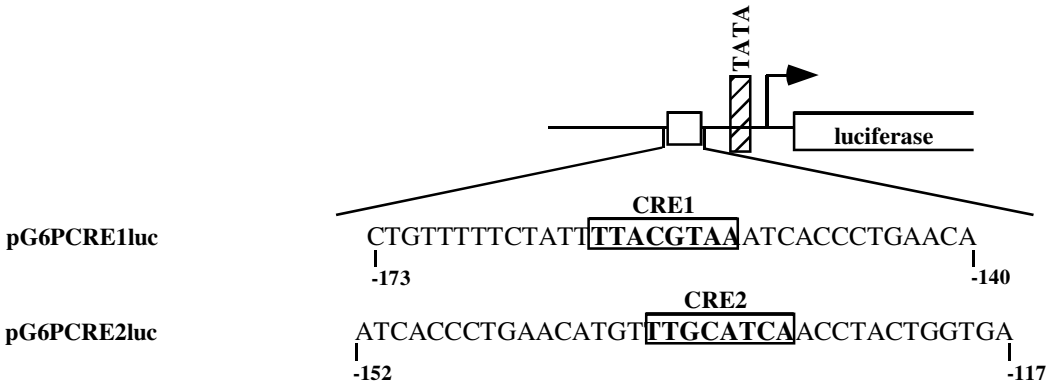


Fig. 9. The human glucose-6-phosphatase gene promoter.
(A) Sequence of a portion of the human glucose-6-phosphatase gene promoter including the CRE-like sequences CRE1 and CRE2. (B) The reporter plasmid pG6PCRE1/CRE2luc contains a minimal promoter consisting of the TATA box of the human immunodeficiency virus, the adenovirus major late promoter initiator element, the luciferase open reading frame and glucose-6-phosphatase promoter sequences encompassing both CRE-like sequences CRE1 and CRE2. The reporter plasmids pG6PCRE1mut/CRE2luc and pG6PCRE1/CRE2mutluc carry mutations in either the CRE1 or the CRE2, respectively, to inactivate these sites. (C) Reporter plasmids pG6PCRE1luc and pG6PCRE2luc contain glucose-6-phosphatase promoter sequences encompassing either the CRE1 or the CRE2.

The human hepatoma cell line HepG2 was chosen to study the regulation of the glucose-6-phosphatase promoter/luciferase reporter genes by C2/CREB since it has been reported that activation of the cAMP signaling pathway stimulates glucose-6-phosphatase gene transcription in these cells (Li and Van de Werve, 2000; Hornbuckle et al., 2001; Streeper et al., 2001). Transient transfection experiments of HepG2 cells were performed and the data show that transcription of the pG6PCRE1/CRE2luc reporter gene, that contains both CRE-like sequences in its regulatory region, was strongly induced following expression of C2/CREB (Fig. 10A, upper panel). Mutation of CRE1 or CRE2 did not abolish the transactivation potential of C2/CREB (Fig. 10A, bottom panel), indicating that both CRE-like sequences function independently. To confirm this observation reporter plasmids that contain only one of the CRE-like sequences in their regulatory regions were tested and the data show that both reporter genes pG6PCRE1luc and pG6PCRE2luc were transactivated by C2/CREB (Fig. 10B). This leads to the conclusion that both CRE1 and CRE2 motifs of the glucose-6-phosphatase gene promoter function as *bona fide* CREs.

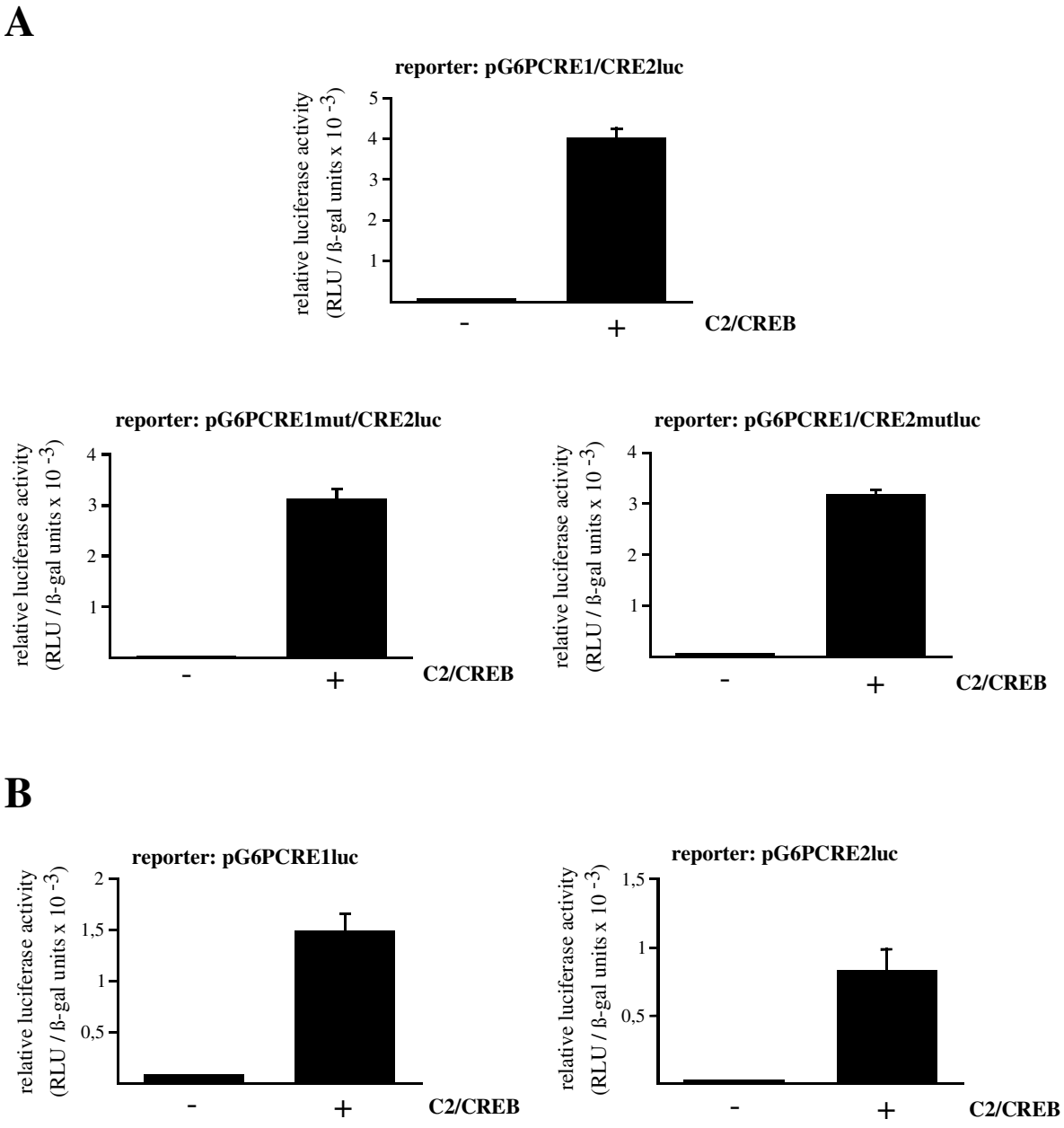


Fig. 10. Biological activity of C2/CREB.
One of the reporter plasmids pG6PCRE1/CRE2luc, pG6PCRE1mut/CRE2luc, pG6PCRE1/CRE2mutluc (A), pG6PCRE1luc or pG6PCRE2luc (B) (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pRSVβ (2 µg/plate) and either the "empty" expression vector pCMV5 or an expression vector encoding C2/CREB (20 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.1.1.4. C2/CREB activates the transcription of the tumor necrosis factor α (TNFα) reporter gene

It has been reported that the TNFα gene belongs to the target genes of CREB (Mayr and Montminy, 2001). To test whether C2/CREB is able to induce the transcription of the TNFα promoter, the reporter plasmids pTNFα(CRE/AP1)^{2}luc and pTNFα(-131)luc were used. The reporter plasmid pTNFα(CRE/AP1)^{2}luc contains two copies of the CRE/AP1-like element derived from TNFα gene promoter. It also contains a TATA box derived from the HIV long terminal repeat, an initiator element from the adenovirus major late promoter and the luciferase open reading frame (Fig. 11A). The reporter plasmid pTNFα(-131)luc contains only one copy of the CRE/AP1-like element derived from TNFα gene promoter. It also contains an initiator element from the adenovirus major late promoter and the luciferase open reading frame (Fig. 11B).

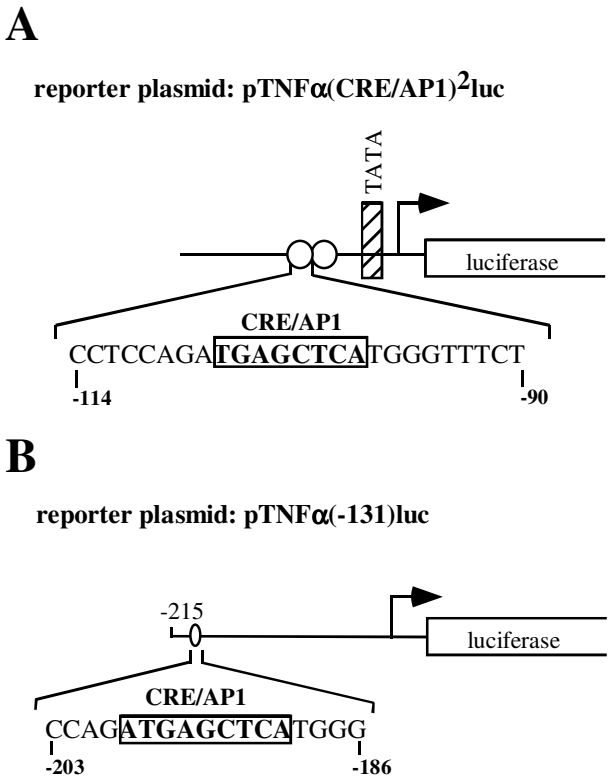


Fig. 11. The reporter plasmids pTNFα(CRE/AP1)^{2}luc and pTNFα(-131)luc.
(A) Schematic representation of the reporter plasmid pTNFα(CRE/AP1)^{2}luc. It contains two copies of the composite CRE/AP1 sequence derived from the human TNFα gene, upstream of a minimal promoter. The sequence of the CRE-like motif within the promoter is depicted. (B) Schematic representation of the reporter plasmid pTNFα(-131)luc. It contains one copy of the composite CRE/AP1 sequence derived from the human TNFα gene, upstream of a minimal promoter. The sequence of the CRE-like motif within the promoter is depicted.

To check whether overexpression of C2/CREB influences the transcriptional activity of the TNFα reporter genes, transient transfection experiments of HepG2 cells were performed. The results show that C2/CREB transactivated both the pTNFα(CRE/AP1)^{2}luc (Fig. 12A) and pTNFα(-131)luc (Fig. 12B).

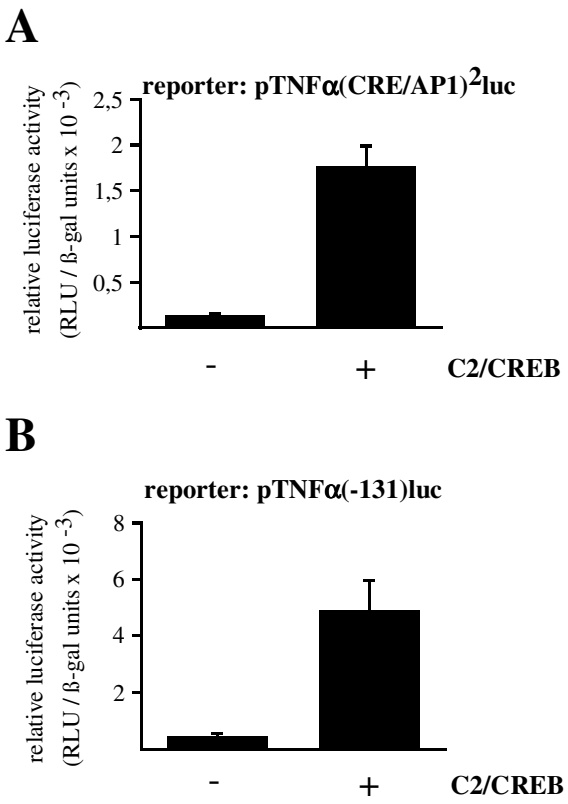


Fig. 12. C2/CREB activates the transcription of TNFα reporter genes.
One of the reporter plasmids pTNFα(CRE/AP1)^{2}luc (A) or pTNFα(-131)luc (B) (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pRSVβ (2 µg/plate) and either the "empty" expression vector pCMV5 or an expression vector encoding C2/CREB [20 ng plasmid/plate (A) and 25 ng plasmid/plate (B)]. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.1.2. Expression of a nuclear targeted mutant of the catalytic subunit of cAMP-dependent protein kinase (NLSCα)

C.1.2.1. Modular structure of the catalytic subunit of cAMP-dependent protein kinase (Cα) and the modified kinase NLSCα

The modular structure of the catalytic subunit of cAMP-dependent protein kinase (Cα) and

the modified kinase NLSCα are shown in Fig. 13. The protein is myristylated on the N-terminus, but myristylation is not essential for enzyme activation (Clegg et al., 1989). The mutant NLSCα should be sorted to the nuclear compartment, due to the presence of the nuclear localization signal (NLS).

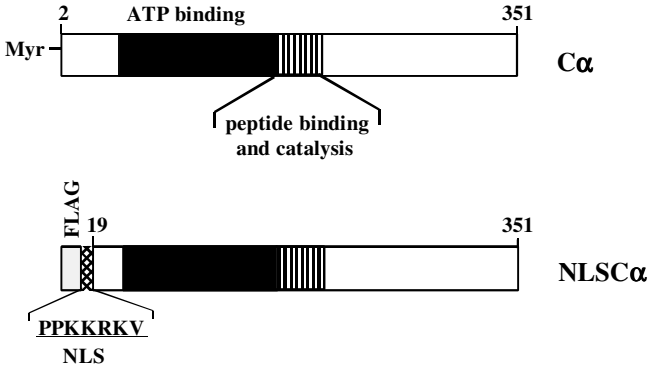


Fig. 13. Modular structure of the catalytic subunit of cAMP-dependent protein kinase (Cα) and the modified kinase NLSCα.
Schematic representation of Cα and NLSCα. The wild-type enzyme is myristylated as indicated (Myr). The location of the nuclear localization signal (sequence PPKKRKV) and the triple FLAG epitope in the NLSCα are shown.

C.1.2.2. Activation of GTP cyclohydrolase I promoter/luciferase reporter gene transcription following expression of the nuclear targeted mutant of the catalytic subunit of cAMP-dependent protein kinase

To check whether the transcription of the GTP cyclohydrolase promoter/luciferase reporter gene is increased following overexpression of NLSCα, transfection experiments of HepG2 cells were performed. As expected, the data show that transfection of a nanomolar concentration of an expression vector encoding the wild-type form of CREB did not change the basal transcription of the reporter gene, because only the concentration of inactive and unphosphorylated CREB was increased. However, transfection of an NLSCα expression vector stimulated reporter gene transcription in the order of 9-fold (Fig. 14). In contrast, overexpression of the wild-type form of the catalytic subunit (Cα) did not activate the transcription of the reporter gene. These results confirm the previous observations, obtained by overexpression experiments of C2/CREB, that the cAMP/PKA signaling pathway enhances GTP cyclohydrolase I gene expression via CREB.

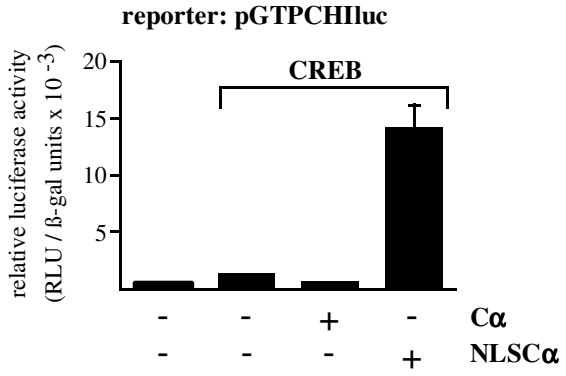


Fig. 14. Expression of the catalytic subunit of cAMP-dependent protein kinase in the nucleus stimulates GTP cyclohydrolase I promoter activity.
The reporter plasmid pGTPCHIluc (1 µg/plate) was transfected into HepG2 cells together with pRSVβ internal standard plasmid (2 µg/plate) and either an "empty" expression vector or an expression vector encoding wild-type CREB (25 ng/plate). In addition, an expression vector encoding either Cα or NLSCα (100 ng/plate) was transfected. Cell extracts were prepared forty-eight hours later and β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.1.2.2.1. NLSCα-mediated upregulation of the GTP cyclohydrolase I promoter/luciferase reporter gene is impaired by the dominant-negative CREB mutant (A-CREB)

Competition experiment using the dominant-negative mutants A-ZIPs was performed and the result shows that expression of wild-type CREB did not stimulate reporter gene transcription, whereas expression of NLSCα strongly enhanced transcription of the reporter gene. The competition assay showed that only A-CREB impaired CREB/NLSCα-mediated transcription (Fig. 15). These data indicate that only A-CREB interferes with NLSCα-induced transactivation of the GTP cyclohydrolase I/luciferase reporter gene and confirm the previous observation that the bZIP proteins CREB2/ATF4, c-Jun, ATF2 and C/EBP are unable to heterodimerize with CREB.

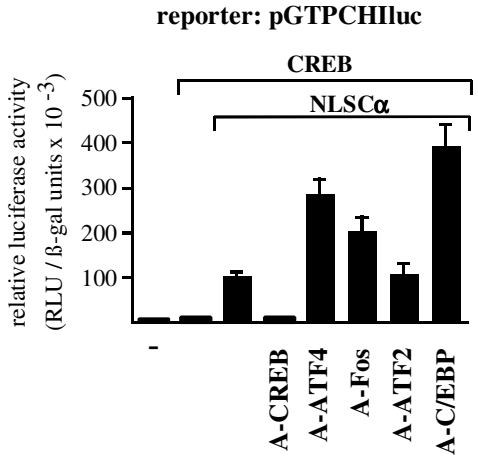


Fig. 15. Dominant-negative mutant of CREB impairs NLSCα-induced transcriptional activation of the GTP cyclohydrolase I reporter gene.
HepG2 cells were transfected with the reporter plasmid pGTPCHIluc (1 µg/plate), the internal standard plasmid pRSVβ (2 µg/plate), an expression vector encoding wild-type CREB (25 ng/plate), an expression vector encoding NLSCα (100 ng/plate) and expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP (250 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.1.2.3. Expression of a nuclear targeted mutant of the catalytic subunit of cAMP-dependent protein kinase strongly stimulated transcription of the glucose-6-phosphatase promoter/luciferase reporter genes

To test the biological activity of NLSCα, in comparison to the wild-type form of the catalytic subunit (Cα), the activity of CREB was measured using a fusion protein consisting of the GAL4 DNA-binding domain fused to the kinase inducible activation domain of CREB. Transcriptional activation was monitored by co-transfection of the reporter plasmid pUAS^{5}luc that contains five copies of the GAL4 binding site termed "upstream activating sequence" (UAS) upstream of a luciferase reporter gene. Since mammalian cells do not express transcription factors that bind to the UAS, this system directly measures the effect of NLSCα or Cα on the transcriptional activation potential of CREB. The results show that the nuclear targeted mutant of the catalytic subunit of PKA (NLSCα) was highly potent in stimulating the transcriptional activation potential of CREB. The activation was on the order of 60-fold. In contrast, no transcriptional activation was observed following overexpression of the wild-type form of the catalytic subunit (Fig. 16).

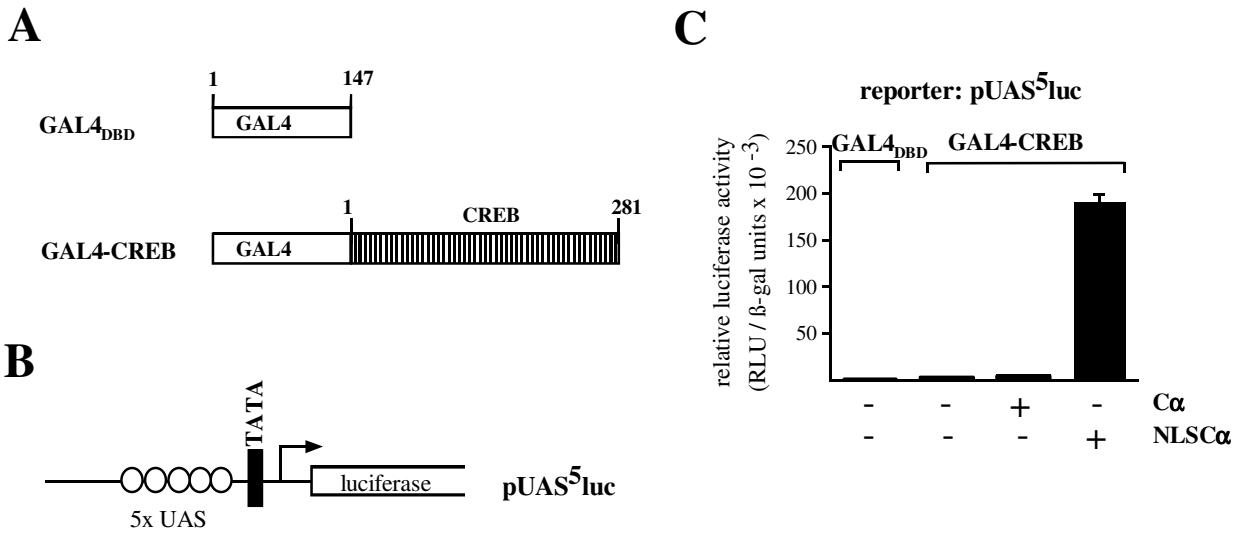
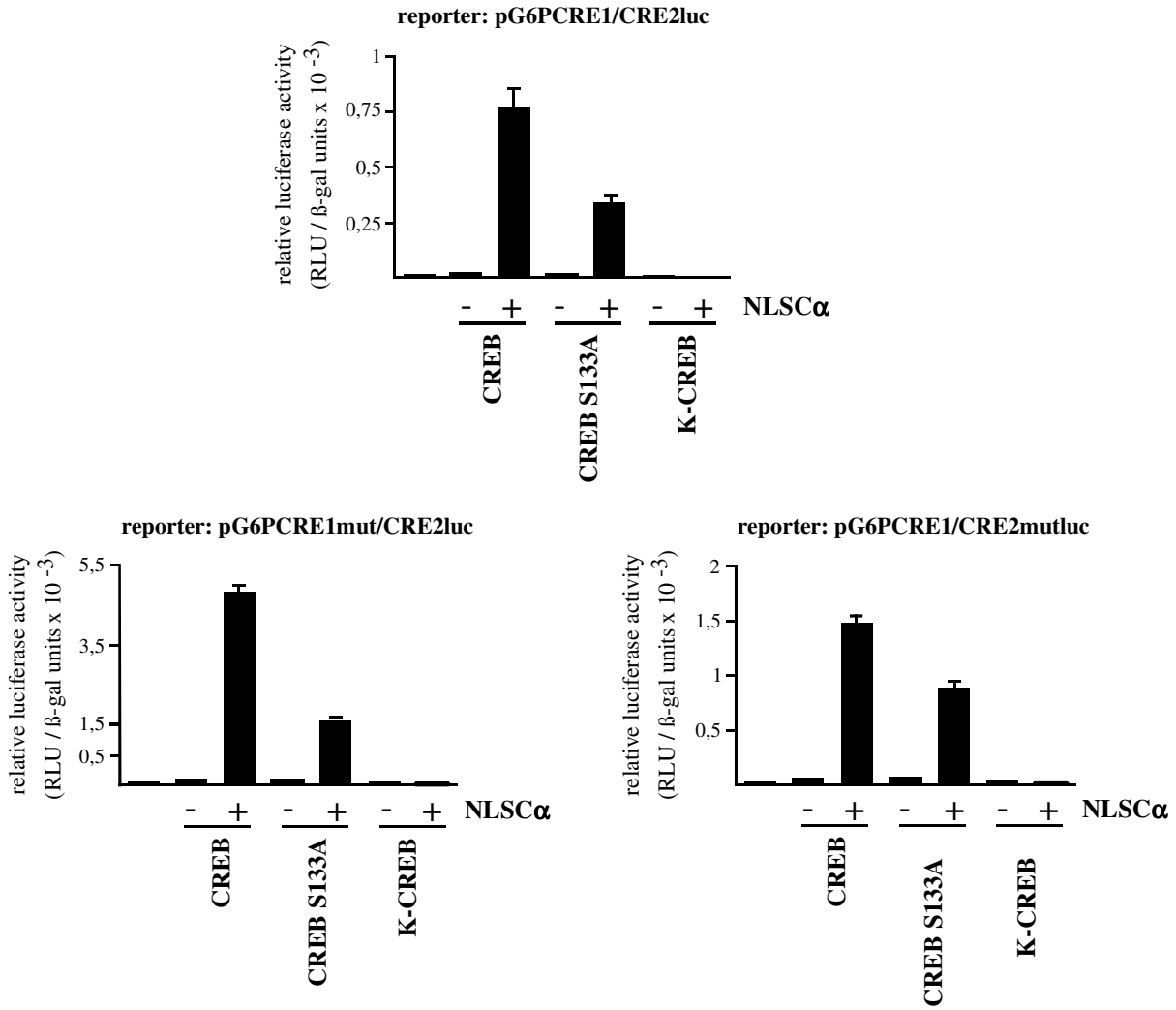


Fig. 16. Biological activity of the nuclear targeted mutant of the catalytic subunit of cAMP-dependent protein kinase.
(A) Modular structure of the GAL4-CREB fusion protein. This protein consists of the DNA-binding domain of GAL4 (amino acids 1-147) and the activation domain of CREB (amino acids 1-281). (B) Schematic representation of the reporter plasmid pUAS^{5}luc that contains a transcription unit encompassing the luciferase open reading frame and a minimal promoter that consists of five copies of the upstream activating sequence (UAS), a TATA box derived from the HIV long terminal repeat and the initiator element from the adenovirus major late promoter. (C) The reporter plasmid pUAS^{5}luc (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pRSVβ (2 µg/plate) and either an expression vector encoding the DNA-binding domain of GAL4 (plasmid pM1) or the GAL4-CREB fusion protein (1 µg/plate). In addition, cells were transfected with an expression vector encoding either Cα or NLSCα (100 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activity was determined. Data are mean ±SD.

Next, the biological activity of NLSCα in controlling the transcription of the glucose-6-phosphatase promoter/luciferase reporter genes was tested. In addition, wild-type CREB, CREBS133A, a CREB mutant containing a serine to alanine mutation at position 133, and K-CREB, a CREB mutant containing the point mutation R286L within the basic DNA-binding domain, impairing DNA-binding (Walton et al., 1992) were overexpressed. The results show that transfection of a nanomolar concentration of expression vectors encoding the wild-type form of CREB, CREBS133A or K-CREB did not significantly change the basal transcriptional activity of the reporter genes. However, transfection of an NLSCα expression vector strongly stimulated transcription of the glucose-6-phosphatase promoter/luciferase reporter genes (Fig. 17). These results confirm the previous observations obtained via overexpression of C2/CREB that both CRE motifs of the glucose-6-phosphatase gene function as independent genetic elements responsive to couple elevated cAMP and PKA levels with enhanced glucose-6-phosphatase gene transcription. Surprisingly, reporter genes

are still activated following coexpression of NLSCα with CREBS133A, lacking the major PKA phosphorylation site. Compared to the coexpression experiments of NLSCα with the wild-type form of CREB, the reduced activation of reporter gene transcription in coexpression experiments of NLSCα with CREBS133A indicates that NLSCα-catalyzed phosphorylation of serine residue 133 of CREB is important for reporter gene transcription. The fact that CREBS133A is still able to transactivate the reporter genes following expression of NLSCα suggests that NLSCα triggers further phosphorylation reactions leading to enhanced transcription via the CRE-like sequences within the glucose-6-phosphatase gene. In contrast, expression of K-CREB is transcriptionally inactive in the presence or absence of NLSCα indicating that DNA-binding is of major importance for CREB and CREBS133A to transactivate the reporter genes.



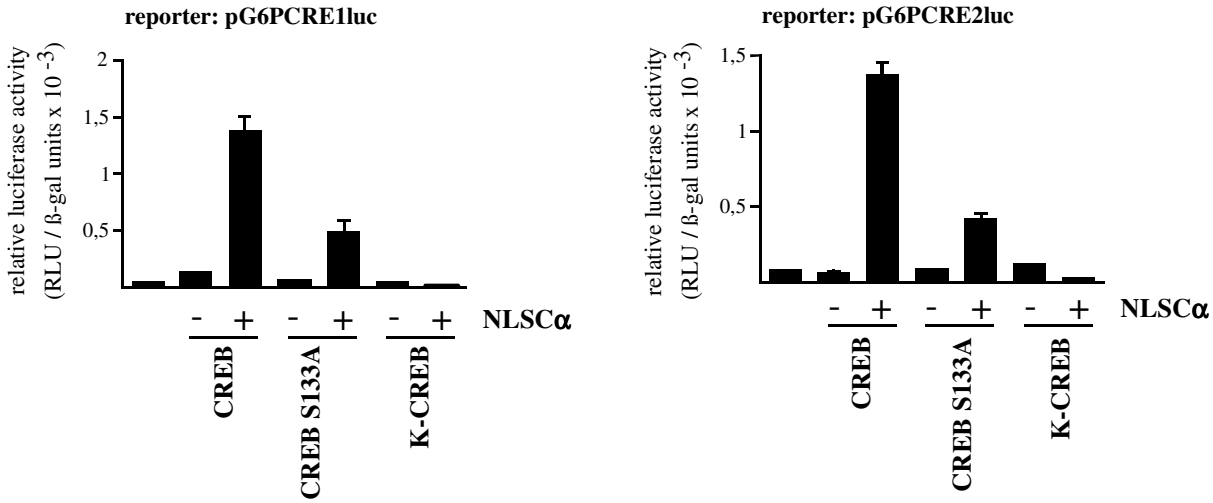


Fig. 17. Transcriptional activity of CREB and CREB mutants in the presence of a nuclear targeted mutant of the catalytic subunit of cAMP-dependent protein kinase.
One of the reporter plasmids pG6PCRE1/CRE2luc, pG6PCRE1mut/CRE2luc pG6PCRE1/CRE2mutluc, pG6PCRE1luc or pG6PCRE2luc (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pRSVβ (2 µg/plate) and the "empty" expression vector pCMV5 or an expression vector encoding either CREB, CREBS133A or K-CREB (20 ng plasmid/plate). In addition, an expression vector encoding NLSCα (100 ng/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. The mean +/- SD is depicted.

C.1.2.4. Expression of NLSCα strongly stimulated transcription of the TNFα reporter gene containing two copies of the CRE/AP1 site

The biological activity of NLSCα in controlling the transcription of the TNFα reporter gene (pTNFα(CRE/AP1)^{2}luc) was tested. In addition, wild-type CREB, CREBS133A and K-CREB were overexpressed. Transfection of expression vectors encoding the wild-type form of CREB, CREBS133A or K-CREB did not change the basal transcriptional activity of the reporter gene. However, transfection of an NLSCα expression vector strongly stimulated transcription of the TNFα promoter/luciferase reporter gene (Fig. 18). These results confirm the previous observations, obtained by overexpression experiments of C2/CREB, that the cAMP/PKA signaling pathway enhances TNFα gene expression via CREB.

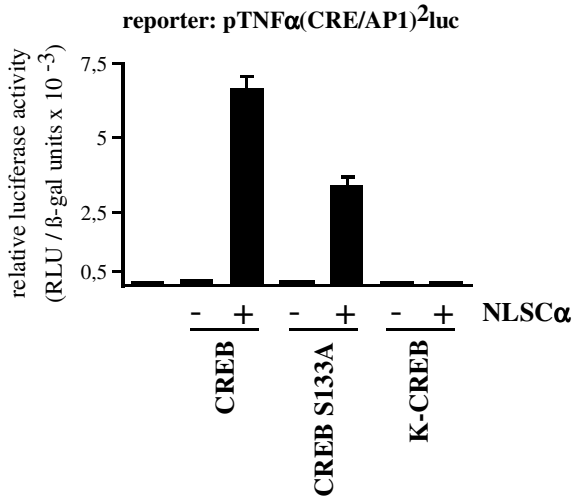


Fig. 18. Expression of the catalytic subunit of cAMP-dependent protein kinase in the nucleus stimulates TNFα promoter activity.
The reporter plasmid pTNFα(CRE/AP1)^{2}luc (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pRSVβ (2 µg/plate) and the "empty" expression vector pCMV5 or an expression vector encoding either CREB, CREBS133A or K-CREB (20 ng/plate). In addition, an expression vector encoding NLSCα (100 ng/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. The mean +/- SD is depicted.

C.2. Regulation of gene expression by ATF2

C.2.1. Biological activity of a constitutively active ATF2 mutant C2/ATF2 towards CRE-containing reporter genes

The bZIP transcription factor ATF2 binds to the classical cAMP responsive element (CRE) 5´-TGACGTCA-3´ (Maekawa et al., 1989) and also with high affinity to the related DNA target sequence 5´-TTACGTAA-3´ (Benbrook and Jones, 1994). ATF2 contains a phosphorylation-regulated activation domain, therefore, it is inactive in the dephosphorylated state. Nonetheless ATF2 turns into a biologically active transcriptional activator upon phosphorylation. To test the effect of ATF2 on CRE-containing reporter genes, a constitutively active ATF2 mutant C2/ATF2 was used.

C.2.2. Modular structure of ATF2 and the constitutively active ATF2 mutant C2/ATF2

The protein ATF2 contains on its C-terminus a bZIP domain that is responsible for DNA-binding and dimerization. The N-terminus contains an activation domain which is controlled by phosphorylation (Livingstone et al., 1995). The bZIP domain of ATF2, which is responsible of DNA-binding and dimerization, was expressed as a fusion protein with the constitutively active transcriptional activation domain of CREB2, generating the chimeric transcription factor C2/ATF2. The C2/ATF2 fusion protein contains additionally an immunological tag used for protein detection.

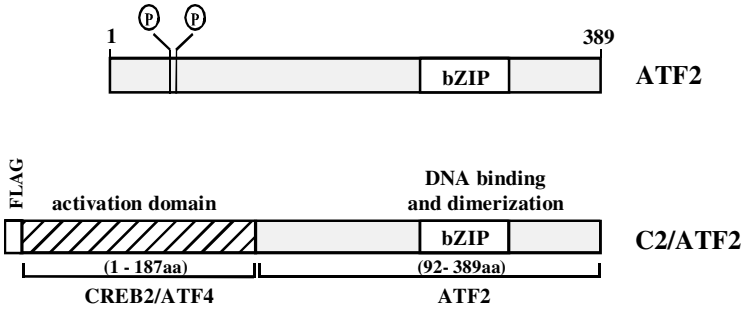


Fig. 19. Schematic representation of ATF2 and C2/ATF2.
Modular structure of the bZIP proteins ATF2 and the constitutively active mutant C2/ATF2.

C.2.3. C2/ATF2 activates the transcription of the GTP cyclohydrolase I promoter/luciferase reporter gene

The constitutively active mutant C2/ATF2 has been shown to transactivate luciferase reporter genes under control of the c-Jun, TNFα, Fas ligand or CHOP promoter, whereas the unphosphorylated wild-type ATF2 was inactive (Steinmüller and Thiel, 2003; Averous et al., 2004). *In vitro* DNA/protein binding experiments suggested that ATF2 binds to the GTP cyclohydrolase core promoter (Hirayama et al., 2001). To test the biological activity of ATF2 on the GTP cyclohydrolase I promoter/luciferase reporter gene, expression experiments using the mutant C2/ATF2 were performed. As a control, the effect of ATF2 and C2/ATF2 on the Egr-1 promoter activity was tested. The results show that C2/ATF2 transactivated both reporter genes (Fig. 20A, B). The transcriptional activation of the GTP cyclohydrolase I and Egr-1 promoter was on the order of 10-fold and 2.8-fold, respectively. In contrast, the wild-type ATF2 was inactive.

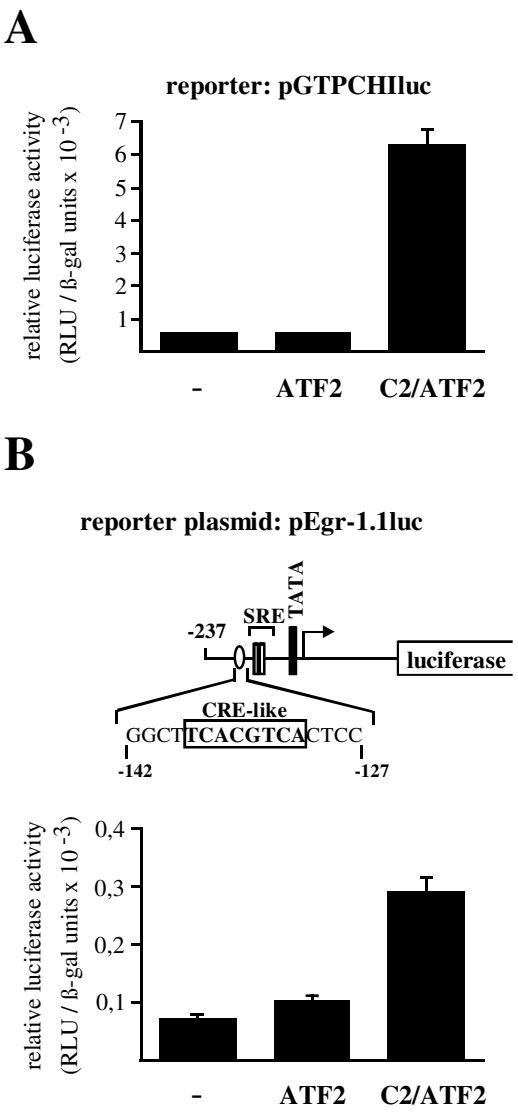


Fig. 20. The constitutively active ATF2 mutant C2/ATF2 activates the transcription of GTP cyclohydrolase I promoter/luciferase reporter gene.
(A) The reporter plasmid pGTPCHIluc was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding ATF2 or C2/ATF2 (500 ng/plate). Forty-eight hours post-transfection lysates were prepared and the relative luciferase activities were determined. The mean ±SD is depicted. (B) Schematic representation of the Egr-1 promoter/luciferase reporter plasmid (pEgr-1.1luc). The sequence of CRE-like motif within this promoter is depicted. The reporter plasmid pEgr-1.1luc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the expression vector pCMV5 or expression vectors encoding ATF2 or C2/ATF2 (500 ng/plate). Forty-eight hours post-transfection lysates were prepared and the relative luciferase activities were determined. The mean ±SD is depicted.

C.2.3.1. C2/ATF2-stimulated GTP cyclohydrolase I reporter gene was impaired by dominant-negative ATF2 mutant A-ATF2, and by A-Fos, a dominant-negative of c-Jun

Competition experiments using the A-ZIP expression vectors were performed. The results show that C2/ATF2-induced GTP cyclohydrolase I promoter/luciferase reporter was impaired by A-ATF2 and A-Fos, a dominant-negative of c-Jun. Neither A-CREB, nor A-ATF4 nor A-C/EBP were able to block the transcriptional activity of C2/ATF2 (Fig. 21). These results, together with the competition assay performed after expression of C2/CREB (depicted in Fig. 7), indicate that constitutively active mutants of CREB and ATF2 are independently able to transactivate the GTP cyclohydrolase I gene, although these experiments argue against a heterodimerization of CREB and ATF2.

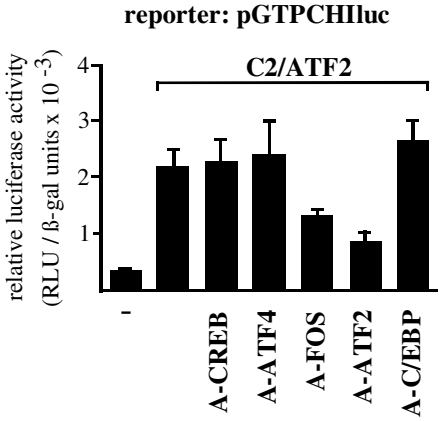


Fig. 21. The dominant-negative mutants A-ATF2 and A-Fos impair C2/ATF2-induced transcriptional activation of the GTP cyclohydrolase I reporter gene.
HepG2 cells were transfected with the reporter plasmid pGTPCHIluc (1 µg/plate), the internal standard plasmid pRSVβ (2 µg/plate), an expression vector encoding C2/ATF2 (500 ng/plate) and expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP (50 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.2.3.2. CREB and ATF2 independently transactivate the GTP cyclohydrolase I reporter gene transcription

To check whether CREB and ATF2 can heterodimerize, the effect of the dominant-negative CREB mutants GST-CREB and GST-KCREB on C2/ATF2-mediated GTP cyclohydrolase I promoter/luciferase reporter gene transcription was tested. GST-CREB has an intact bZIP domain derived from CREB and is therefore able to bind to the cognate sites of CREB, making these sites unavailable to bind to any wild-type bZIP protein. In addition, GST-CREB may also form inactive heterodimers with the wild-type CREB. In contrast, GST-KCREB is

unable to bind to DNA, due to a point mutation R286L in the basic region (Walton et al., 1992) (Fig. 22A). As depicted in Fig. 22B, GST-CREB impaired C2/ATF2-stimulated transcription of the reporter gene. GST-KCREB did not show any negative effect on C2/ATF2-mediated transactivation, because of its inability to bind to the CRE. Thus, constitutively active mutants of CREB and ATF2 are independently able to transactivate the GTP cyclohydrolase gene, without forming heterodimers, suggesting that ATF2 and CREB compete for the same DNA-binding site, the CRE.

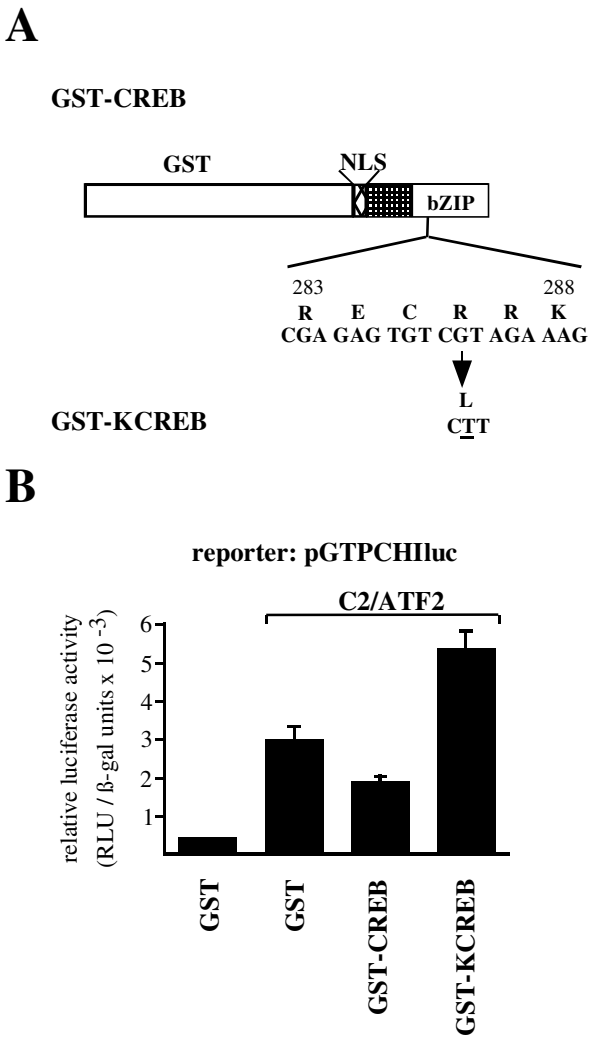


Fig. 22. ATF2 and CREB compete for the same DNA-binding site.
(A) Schematic representation of the modular structure of the CREB mutants GST-CREB and GST-KCREB. These mutants consist of amino-terminal glutathione-S-transferase, a nuclear localization signal derived from the SV40 large T antigen, and the bZIP domains of CREB or KCREB on the C-termini. (B) HepG2 cells were transfected with the pGTPCHIluc reporter plasmid (1 µg/plate), the internal standard plasmid pRSVβ (2 µg/plate), an expression vector encoding C2/ATF2 (500 ng/plate) and expression vectors encoding either GST-CREB or GST-KCREB (250 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.2.3.3. Stimulation of GTP cyclohydrolase I promoter/luciferase reporter gene transcription by p38 MAP kinase and a constitutively active MAP kinase kinase 6

ATF2 is activated via phosphorylation of the threonine residues 69 and 71 situated within its activation domain. This phosphorylation is catalyzed by the protein kinases p38 MAP kinase (p38 MAPK) or c-Jun N-terminal protein kinase (JNK). In order to activate p38 protein kinase, a constitutively active mutant of p38 MAPKK, MKK6 [MKK6(E)] was used. p38 MAPK is phosphorylated by MKK6 on the residues Ser^{207} and Thr^{211}. These residues have been exchanged to glutamic acid residues, generating a constitutively active protein kinase mutant termed MKK6(E) (Jiang et al., 1996). To study the transcriptional activation of ATF2, the activity of the GAL4-ATF2 fusion protein depicted in Fig. 23A was measured. The transcriptional activation was monitored by co-transfection of the reporter plasmid $pUAS^5luc$ described earlier. Transfection efficiency was monitered by co-transfecting pSV40lacZ due to an observed effect of p38 and MKK6(E) upon Rous sarcoma virus long terminal repeat controlled β-galactosidase expression. The data show that the transcriptional activation potential of the GAL4-ATF2 fusion protein was elevated following expression of MKK6(E) on the order of 2.5-fold (Fig. 23B). As shown in Fig. 23C, transfection of a nanomolar concentration of an expression vector encoding wild-type p38 MAPK did not change reporter gene transcription since only the concentration of an inactive and unphosphorylated p38 protein kinase was increased. However, coexpression of the p38 protein kinase together with the constitutively active MKK6 mutant MKK6(E) strongly increased the ATF2 transactivation potential. The protein consisting of the GAL4 DNA-binding domain ($GAL4_{DBD}$) did not stimulate transcription of the luciferase reporter gene under any condition.

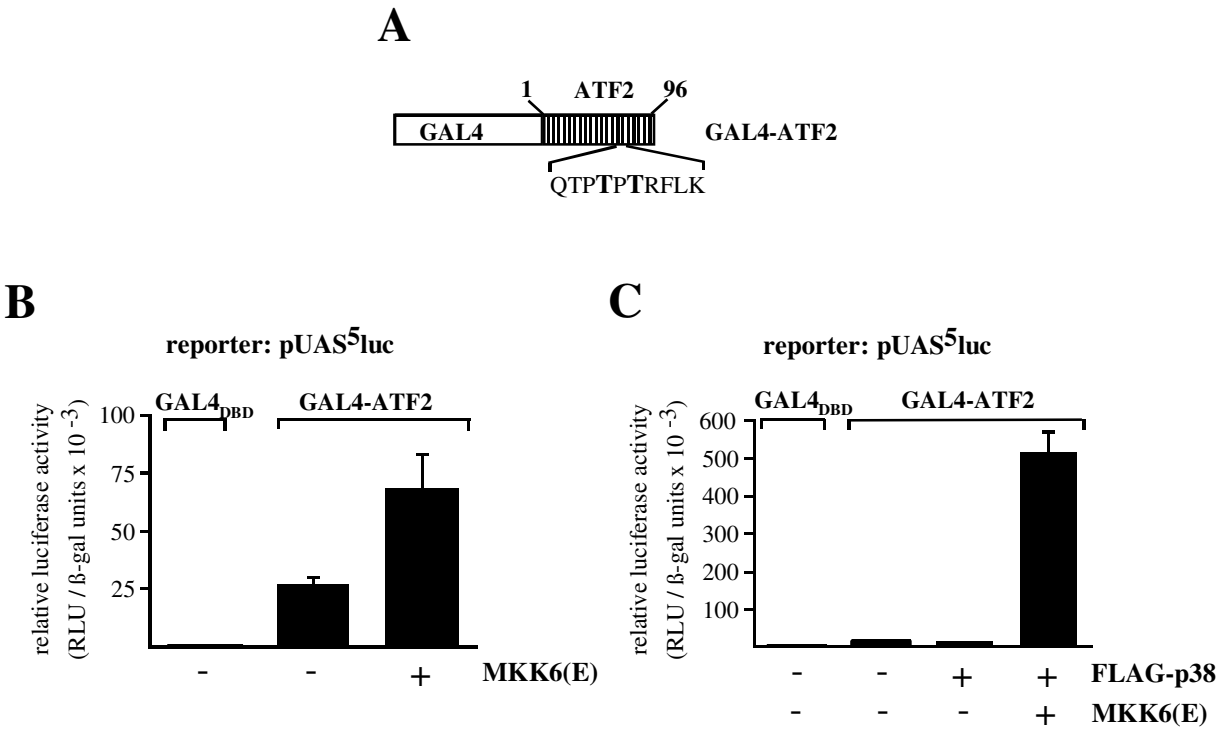


Fig. 23. Transcriptional activation of ATF2.
(A) Modular structure of GAL4 fusion protein GAL4-ATF2. The protein contains the DNA-binding domain of the yeast transcription factor GAL4 (amino acids 1-147) and the phosphorylation-dependent activation domain of ATF2 (amino acids 1-96). The sequence encompassing the phosphorylation sites is depicted. (B, C) The reporter plasmid pUAS^{5}luc (1 µg/plate) and the expression plasmid encoding GAL4-ATF2 (1 µg/plate) were transfected into HepG2 cells together with either only expression vector encoding MKK6(E) (25 ng/plate) (B) or with expression vectors encoding MKK6(E) (25 ng/plate) and p38 MAPK (50 ng/plate) (C). As a control, plasmid pM1 encoding only the DNA-binding domain of GAL4 ($GAL4_{DBD}$) was used (1 µg/plate). As a reference plasmid pSV40lacZ (3 µg/plate) was transfected. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

Additionally, the impact of activating the p38 MAPK signaling pathway on GTP cyclohydrolase I promoter/luciferase reporter gene transcription was tested. The results show that MKK6(E) alone was not able to induce reporter gene transcription (Fig. 24A), while expression of MKK6(E) in the presence of p38 MAPK increased GTP cyclohydrolase I promoter/luciferase gene transcription on the order of 9.6-fold (Fig. 24B). These data indicate that the activation of p38 protein kinase signaling pathway activated GTP cyclohydrolase I gene transcription.

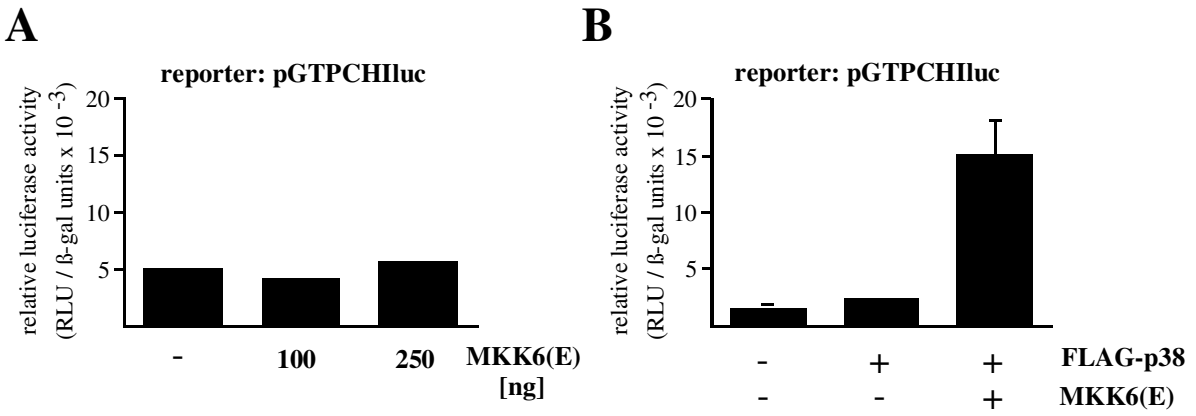


Fig. 24. Activation of GTP cyclohydrolase I promoter by p38 MAPK and a constitutively active mutant of MAP kinase kinase 6.
HepG2 cells were transfected with the pGTPCHIluc reporter plasmid (1 µg/plate), the reference plasmid pSV40lacZ (3 µg/plate) and only expression vector encoding MKK6(E) (100, 250 ng/plate) (A) or expression vectors encoding MKK6(E) (25 ng/plate) and p38 MAPK (50 ng/plate) (B). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.2.3.4. p38/MKK6(E)-stimulated GTP cyclohydrolase I promoter/luciferase reporter gene was impaired by dominant-negative ATF2 mutant A-ATF2, and by A-Fos, a dominant-negative of c-Jun

Competition experiment using the A-ZIP mutants was performed. The results revealed that p38/MKK6(E)-stimulated GTP cyclohydrolase I promoter/luciferase reporter was inhibited by A-ATF2 and A-Fos, while A-CREB, A-ATF4 and A-C/EBP had no effect (Fig. 25). This competition experiment confirms that the GTP cyclohydrolase I gene transcription is regulated by ATF2. It also confirms that CREB, CREB2/ATF4 and C/EBP do not heterodimerize with ATF2.

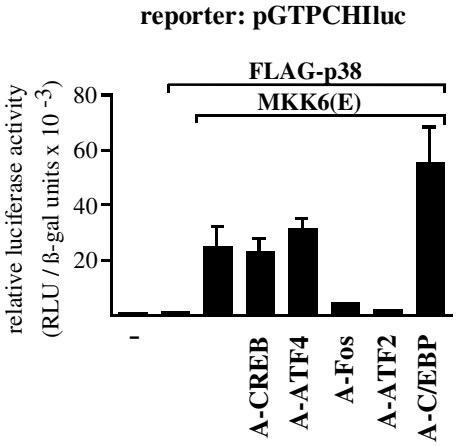


Fig. 25. p38/MKK6(E)-stimulated GTP cyclohydrolase I reporter gene was impaired by the dominant-negative mutants A-ATF2 and A-Fos.
The reporter plasmid pGTPCHIluc (1 µg/plate) was transfected into HepG2 cells together with the reference plasmid pSV40lacZ (3 µg/plate), expression vectors encoding either MKK6(E) (25 ng/plate) or p38 MAPK (50 ng/plate) and expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP (50 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.2.3.5. Mutations of the CRE-like sequence of the GTP cyclohydrolase I promoter impair transactivation by ATF2

To test whether the CRE-like sequence is essential for ATF2-mediated transactivation of the GTP cyclohydrolase I promoter/luciferase reporter gene, transfection experiments employing reporter genes carrying the intact or the mutated CRE-like site, together with the expression vector encoding C2/ATF2, were performed. The results show that the CRE-like sequence is of major importance for the constitutively active ATF2 mutant to transactivate the GTP cyclohydrolase I reporter gene (Fig. 26).

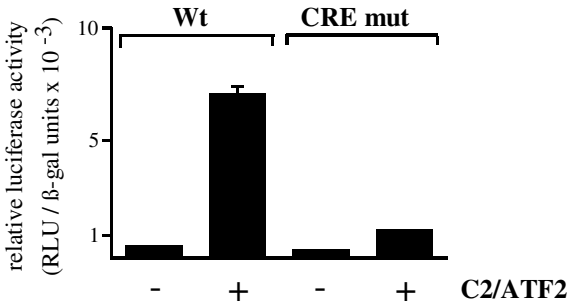


Fig. 26. Point mutations within the CRE-like sequence of the GTP cyclohydrolase I promoter impair transactivation mediated by C2/ATF2.
1 µg/plate of the wild-type or the mutated GTP cyclohydrolase I reporter plasmid, together with 500 ng/plate of C2/ATF2 and 2 µg/plate of RSVβ were transfected into HepG2 cells. Forty-eight hours post-transfection lysates were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.2.4. C2/ATF2 transactivates a reporter gene containing one copy of the CRE/AP1 motif derived from the TNFα promoter

The biological activity of ATF2 on the TNFα promoter/luciferase reporter gene was tested. Transient transfection experiments of HepG2 cells using the wild-type ATF2 and the constitutively active mutant C2/ATF2 show that C2/ATF2 transactivated the TNFα reporter gene that contains one copy of CRE/AP1 motif. This induction was on the order of 5.8-fold, in contrast, the wild-type ATF2 was inactive (Fig. 27).

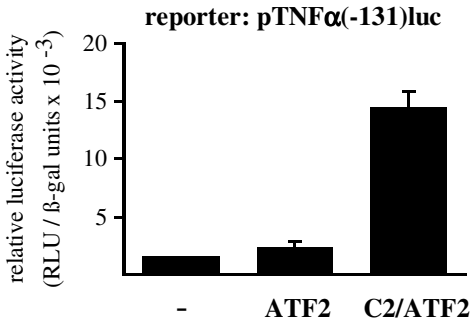


Fig. 27. C2/ATF2 activates the transcription of a TNFα reporter gene.
The reporter plasmid pTNFα(-131)luc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the expression vector pCMV5 or expression vectors encoding ATF2 or C2/ATF2 (500 ng/plate). Forty-eight hours post-transfection lysates were prepared and the relative luciferase activities were determined. The mean ±SD is depicted.

C.2.4.1. Induction of TNFα promoter/luciferase reporter gene expression by p38 MAPK and a constitutively active mutant of MAPKK 6

Members of the MAPKs have been identified to be responsible for TNFα gene expression in lymphocytes and non-lymphocyte tissues. It has been shown that in T lymphocytes the MAPKs, ERK, JNK and p38 MAPK cooperate in regulating the TNFα gene expression (Hoffmeyer et al., 1999). The role of p38 MAPK signaling pathway in the regulation of TNFα gene transcription was analyzed by overexpressiong a constitutively active mutant of MAPKK6 [MKK6(E)]. Two reporter plasmids were used, first, a reporter plasmid that contains -1311 nucleotides of the regulatory region of TNFα gene (Fig. 28) and second, a reporter gene that contains two copies of the CRE/AP1 site of the TNFα promoter.

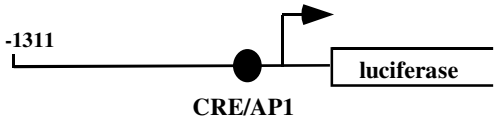


Fig. 28. The TNFα promoter/luciferase reporter gene (pTNFα(-1311)luc).
The reporter plasmid pTNFα(-1311)luc contains the luciferase reporter gene and the regulatory region derived from the human TNFα gene.

Transient transfection experiments employing the constitutively active mutant MKK6(E) were performed and the results show that expression of MKK6(E) was not sufficient to increase the transcription of the TNFα promoter/luciferase reporter gene containing -1311 nucleotides of the regulatory region of TNFα gene, while expression of MKK6E in the presence of p38 MAPK elevated promoter/luciferase reporter gene transcription (Fig. 29A). Similarly, MKK6(E) enhanced the transcription of the TNFα reporter gene controlled by the CRE/AP1 motif only in the presence of p38 MAPK (Fig. 29B).

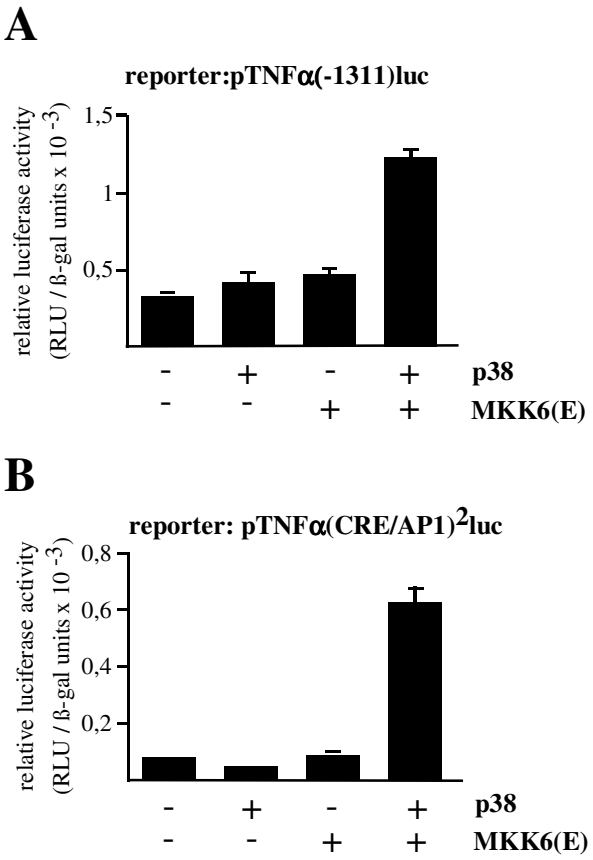


Fig. 29. Activation of TNFα promoter/luciferase reporter genes by p38 MAPK and a constitutively active MAPKK 6.
HepG2 cells were transfected with one of the reporter plasmids pTNFα(-1311)luc (A) or pTNFα(CRE/AP1)^{2}luc (B) (1 µg/plate), the reference plasmid pRSVβ (2 µg/plate) and one of the expression vectors p38 MAPK (50 ng/plate) and/or MKK6(E) (25 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.2.4.2. Stimulation of TNFα promoter gene by p38/MKK6(E) was impaired by MKP-1

The MAPK phosphatase-1 (MKP-1) is known to inactivate p38 MAPK in the nucleus via dephosphorylation (Raingeaud et al., 1995; Liu et al., 1995). To dephosphorylate p38 in the nucleus, MKP-1 was overexpressed. The results show that MKP-1 blocked the transactivation of both TNFα reporter genes mediated by p38/MKK6(E) (Fig. 30A, B). The results obtained by using the p38/MKK6(E) and p38/MKK6(E)/MKP-1 lead to the conclusion that p38 MAPK signaling pathway regulates TNFα gene expression in HepG2 cells. These date together with the data obtained by using C2/ATF2 suggest that TNFα gene transcription is regulated by ATF2.

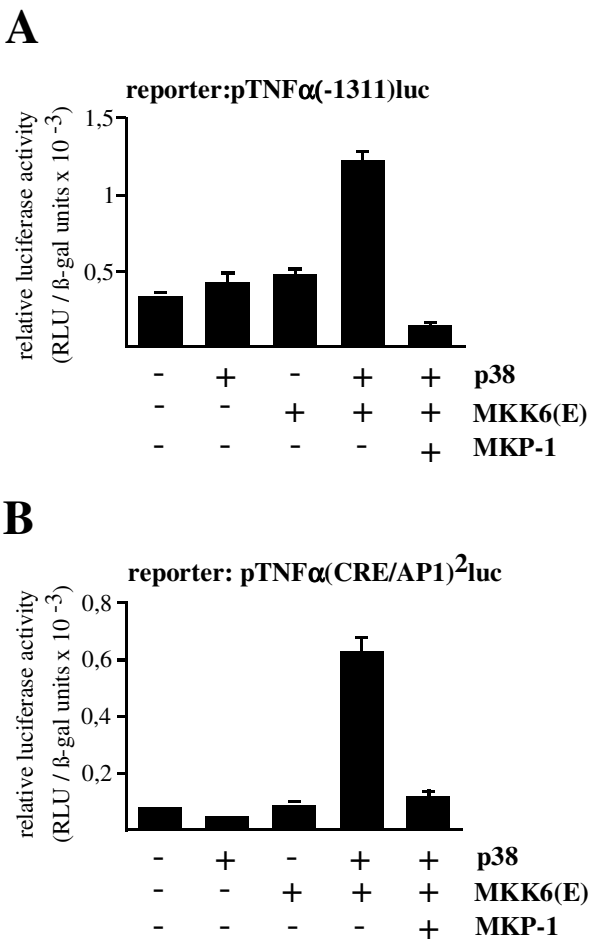


Fig. 30. The stimulating effect of p38/MKK6(E) expression upon TNFα reporter genes was attenuated by expressing MKP-1.
HepG2 cells were transfected with one of the reporter plasmids pTNFα(-1311)luc (A) or pTNFα(CRE/AP1)^{2}luc (B) (1 µg/plate), the internal standard plasmid pRSVβ (2 µg/plate) and one of the expression vectors p38 MAPK (50 ng/plate) and/or MKK6(E) (25 ng/plate). An expression vector encoding MKP-1 (1 µg/plate) was also transfected. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.2.5. C2/ATF2 transactivates a reporter gene containing four copies of the CRE derived from the secretogranin II gene

Secretogranin II, a member of the granin family, is an acidic secretory protein found in secretory granules of most peptidergic endocrine cells and neurons. It has been reported that secretogranin II plays a role in the sorting process to secretory granules (Scammell, 1993; Rosa and Gerdes, 1994; Huttner and Natori, 1995). The genes encoding the granin proteins contain CRE motifs in their promoter region. To test the biological activity of ATF2 towards a reporter gene containing four copies of the CRE derived from the secretogranin II gene in

noradrenergic locus coeruleus-like CATH.a cells, expression experiments employing C2/ATF2 were performed. The results show that C2/ATF2 transactivated the secretogranin II promoter/luciferase reporter gene (plasmid pSgIICRE^{4}luc). The transcriptional activation was approximately on the order of 6-fold (Fig. 31).

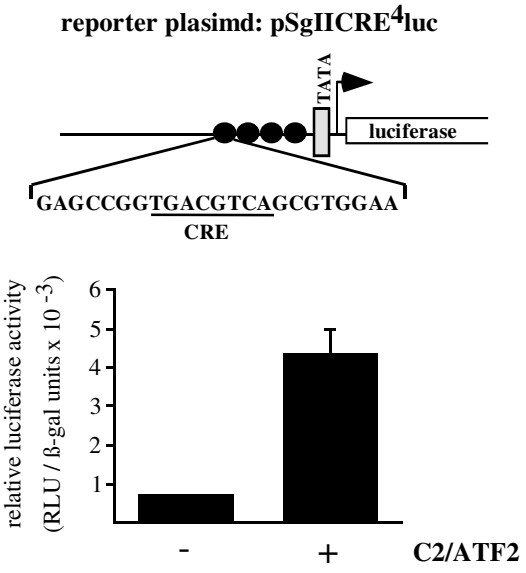


Fig. 31. Biological activity of C2/ATF2 towards a reporter gene containing four copies of the CRE derived from the secretogranin II gene.
Modular structure of the reporter plasmid pSgIICRE^{4}luc that contains a minimal promoter consisting of the human immunodeficiency virus TATA box, the adenovirus major late promoter initiator element, the luciferase open reading frame and four copies of CRE derived from the secretogranin II regulatory region. CATH.a cells were transfected with the reporter plasmid pSgIICRE^{4}luc (1 µg/plate), the internal reference plasmid pRSVβ (2 µg/plate) and either the 'empty' expression vector pCMV5 or an expression vector encoding C2/ATF2 (100 ng/plate). Lysates were prepared forty-eight hours post-transfection and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted.

To confirm that the CRE within the secretogranin II gene serves as a target for C2/ATF2, a secretogranin II reporter gene, containing one copy of the canonical CRE was tested. The results show that expression of either C2/ATF2 or C2/CREB transactivated the secretogranin II reporter gene (Fig. 32), indicating that both ATF2 and CREB target the secretogranin II gene promoter.

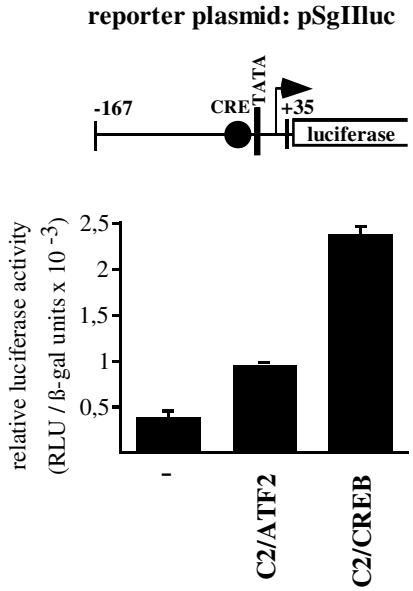


Fig. 32. Biological activity of C2/ATF2 towards a reporter gene containing one copy of the CRE derived from the secretogranin II gene.
Modular structure of the reporter plasmid pSgIIluc that contains secretogranin II promoter sequences from -167 to +35. The position of the CRE is depicted. CATH.a cells were transfected with the reporter plasmid pSgIIluc (1 µg/plate) together with the internal reference plasmid pRSVβ (2 µg/plate) and either the 'empty' expression vector pCMV5 or an expression vector encoding either C2/ATF2 or C2/CREB (100 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and β-galactosidase and luciferase activities were measured. Data are mean ±SD.

C.2.5.1. C2/ATF2 and C2/CREB compete for DNA-binding

As shown in Fig. 22, CREB and ATF2 do not heterodimerize to activate the GTP cyclohydrolase I reporter gene but they rather compete for the same DNA-binding site. To confirm that CREB and ATF2 do not heterodimerize, secretogranin II reporter gene transcription was activated by expression of C2/ATF2 and competed with the various dominant-negative bZIP mutants. As expected, the results show that A-ATF2 and A-Fos inhibited C2/ATF2-stimulated transcription, whereas A-CREB, A-ATF4 and A-C/EBP had no effect (Fig. 33).

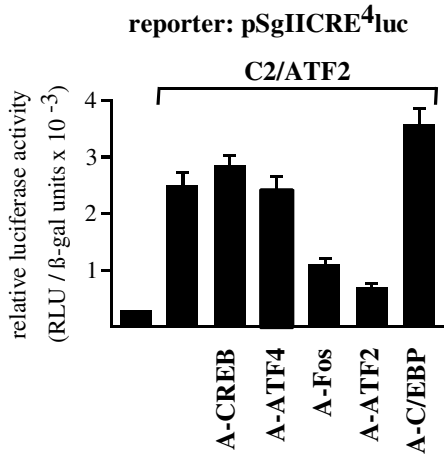


Fig. 33. A-ATF2 and A-Fos impaired C2/ATF2-induced secretogranin II reporter gene transcription.
CATH.a cells were transfected with the pSgIICRE^{4}luc reporter plasmid (1 µg/plate), an expression vector encoding C2/ATF2 (100 ng/plate) and the reference plasmid pRSVβ (2 µg/plate). In addition, expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP (100 ng/plate) were transfected. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined.

Additionally, the fusion proteins GST-CREB and GST-KCREB were used as competitors. The results show that GST-CREB impaired C2/ATF2-induced transcriptional activity, whereas GST-KCREB, which can not bind to DNA, had no inhibiting activity (Fig. 34). Taken together, these data indicate that both ATF2 and CREB compete for the same DNA-binding site, the CRE.

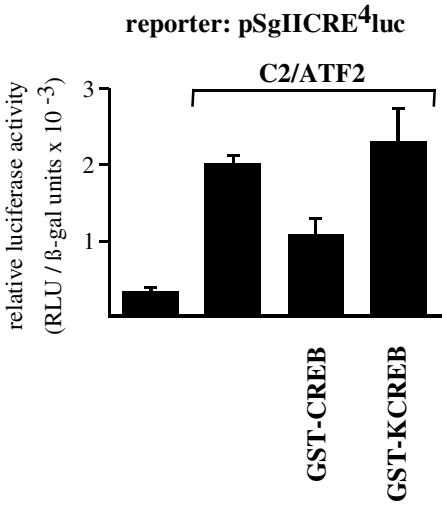


Fig. 34. CREB and ATF2 compete for binding to the CRE.
CATH.a cells were transfected with the pSgIICRE^{4}luc reporter plasmid (1 µg/plate), an expression vector encoding C2/ATF2 (100 ng/plate) and the internal standard plasmid pRSVβ (2 µg/plate). In addition, expression vectors encoding GST-CREB or GST-KCREB (100 ng/plate) were transfected. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined.

C.2.6. C2/ATF2 marginally activated the transcription of pc-fosCRE^{4}luc reporter gene

The c-Fos promoter has been shown to contain a consensus CRE (Sassone-Corsi et al., 1988b). The biological activity of ATF2 towards a reporter gene that contains four copies of the CRE derived from the c-Fos gene in noradrenergic locus coeruleus-like CATH.a cells was tested. Expression experiments using the mutant C2/ATF2 were performed and the results show that the pc-fosCRE^{4}luc reporter gene was only marginally activated by C2/ATF2. The induction was approximately on the order of 1.4-fold (Fig. 35).

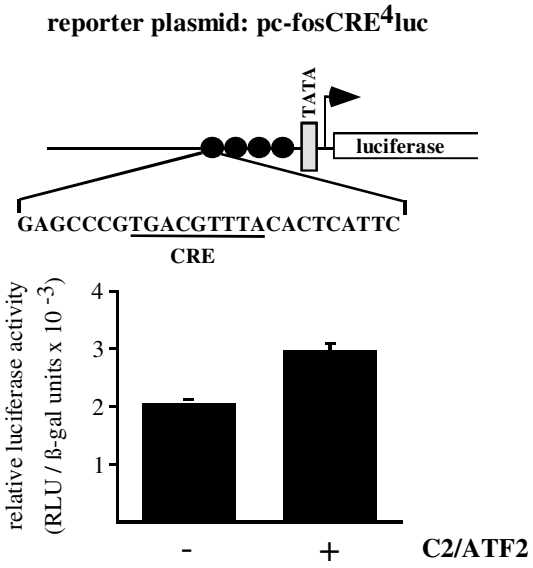


Fig. 35. Biological activity of constitutively active ATF2 mutant towards a reporter gene containing four copies of the CRE derived from the c-Fos gene.
Modular structure of the reporter plasmid pcfosCRE^{4}luc that contains a minimal promoter consisting of the human immunodeficiency virus TATA box, the adenovirus major late promoter initiator element, the luciferase open reading frame and four copies of CRE derived from the c-Fos regulatory region. CATH.a cells were transfected with the reporter plasmid pc-fosCRE^{4}luc (1 µg/plate), the internal reference plasmid pRSVβ (2 µg/plate) and either the 'empty' expression vector pCMV5 or an expression vector encoding C2/ATF2 (100 ng/plate). Lysates were prepared forty-eight hours post-transfection and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted.

C.2.7. C2/ATF2 is unable to transactivate the tyrosine hydroxylase promoter/luciferase reporter gene

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of catecholamines. Tyrosine hydroxylase promoter has been shown to contain a CRE sequence (D'Mello et al., 1989; Huang et al., 1991). The biological activity of ATF2 towards the tyrosine hydroxylase promoter/luciferase reporter gene in noradrenergic locus coeruleus-like CATH.a cells was tested by performing expression experiments using the constitutively active ATF2 mutant C2/ATF2. The results show that the tyrosine hydroxylase promoter/luciferase reporter gene was not transactivated following the expression of C2/ATF2 (Fig. 36). These data together with the data obtained by pSgIICRE^{4}luc, pSgIIluc and pc-fosCRE^{4}luc indicate that C2/ATF2 can select between different CRE sequences.

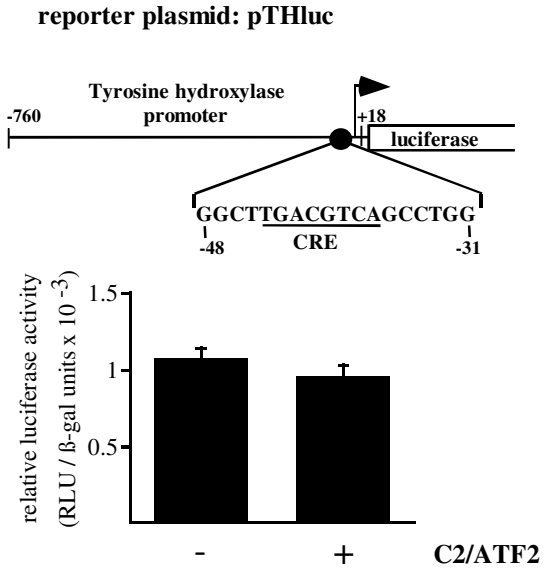


Fig. 36. Biological activity of C2/ATF2 towards the tyrosine hydroxylase reporter gene.
Modular structure of the tyrosine hydroxylase promoter/luciferase reporter gene that contains tyrosine hydroxylase promoter sequences from -760 to +18. The position and sequence of the CRE are shown. CATH.a cells were transfected with the reporter plasmid pTHluc (1 µg/plate), the internal reference plasmid pRSVβ (2 µg/plate) and either the 'empty' expression vector pCMV5 or an expression vector encoding C2/ATF2 (100 ng/plate). Lysates were prepared forty-eight hours post-transfection and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted.

C.3. Regulation of gene expression by c-Jun

C.3.1. Biological activity of a constitutively active c-Jun mutant C2/c-Jun towards CRE-containing reporter genes

The transcription factor ATF2 preferentially forms heterodimers through the leucine zipper with c-Jun, and ATF2/c-Jun heterodimers have been described to regulate c-Jun and TNFα gene transcription (Van Dam et al., 1993; Tsai et al., 1996). The transcription factor c-Jun contains a phosphorylation-regulated activation domain, therefore, it is inactive in the dephosphorylated state. Nonetheless c-Jun turns into a biologically active transcriptional activator upon phosphorylation, this phosphorylation occurs on the serine residues 63 and 73 present within the activation domain of c-Jun and is catalyzed by c-Jun N-terminal kinase. To test the effect of c-Jun on CRE-containing reporter genes, a constitutively active c-Jun mutant C2/c-Jun was used.

C.3.2. Modular structure of c-Jun and the constitutively active c-Jun mutant C2/c-Jun

The transcription factor c-Jun contains on its C-terminus a bZIP domain, which is responsible for DNA binding and dimerization, while its N-terminus contains an activation domain which is controlled by phosphorylation. The bZIP domain of c-Jun was expressed as a fusion protein with the constitutively active transcriptional activation domain of CREB2, generating the chimeric transcription factor C2/c-Jun. In addition the C2/c-Jun fusion protein contains an immunological tag used for detection of the protein. This construct is constitutively active and does not need phosphorylation to be activated.

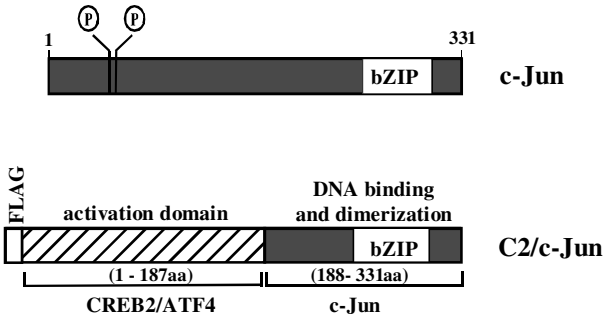


Fig. 37. Schematic representation of c-Jun and C2/c-Jun
Modular structure of the bZIP proteins c-Jun and the constitutively active mutant C2/c-Jun.

C.3.3. C2/c-Jun activates the transcription of the GTP cyclohydrolase I promoter/luciferase reporter gene

The biological activity of the constitutively active c-Jun mutant C2/c-Jun towards the GTP cyclohydrolase I reporter gene was measured. As a control, the impact of c-Jun and C2/c-Jun on the Egr-1 promoter activity was tested. The results show that C2/c-Jun activated both reporter genes (Fig. 38). The activation of the GTP cyclohydrolase I and Egr-1 promoter was on the order of 2.4-fold and 2.3-fold, respectively. In contrast, the wild-type c-Jun was inactive.

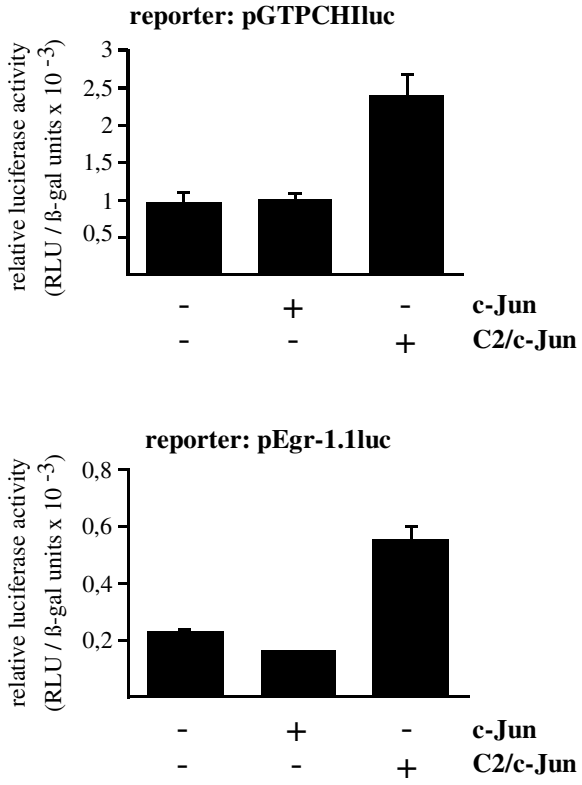


Fig. 38. The constitutively active c-Jun mutant C2/c-Jun activates the transcription of GTP cyclohydrolase I promoter/luciferase reporter gene.
One of the reporter plasmids pGTPCHIluc or pEgr-1.1luc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding c-Jun or C2/c-Jun (500 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. The mean ±SD is depicted.

C.3.3.1. Do CREB and c-Jun heterodimerize in order to activate the GTP cyclohydrolase I reporter gene transcription?

To check whether CREB and c-Jun are able to heterodimerize, the effect of the dominant-negative CREB mutants GST-CREB and GST-KCREB upon C2/c-Jun-mediated GTP cyclohydrolase I promoter gene activity was tested. The results show that GST-CREB impaired C2/c-Jun-stimulted transcription of the reporter gene, while GST-KCREB did not show any inhibiting effect, since it is unable to bind to the CRE (Fig. 39). Thus, constitutively active mutants of CREB and c-Jun are independently able to transactivate the GTP cyclohydrolase I reporter gene, without forming heterodimers, suggesting that c-Jun and CREB compete for the same DNA-binding site, the CRE.

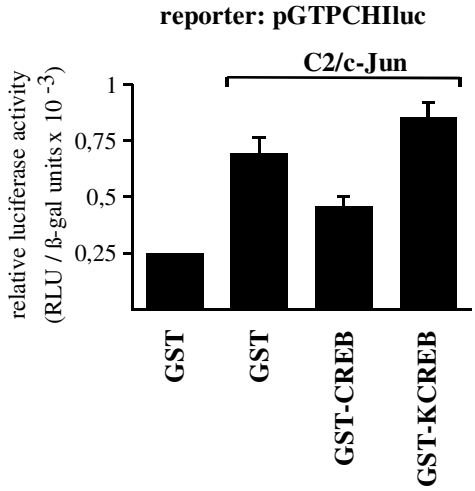


Fig. 39. c-Jun and CREB compete for the same DNA-binding site.
HepG2 cells were transfected with the pGTPCHIluc reporter plasmid (1 µg/plate), the internal standard plasmid pRSVβ (2 µg/plate), an expression vector encoding C2/c-Jun (500 ng/plate) and expression vectors encoding either GST-CREB or GST-KCREB (250 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.3.3.2. Expression of the constitutively active MAP3 kinase MEKK1Δ stimulates GTP cyclohydrolase I promoter/luciferase reporter gene

c-Jun is phosphorylated by the c-Jun N-terminal kinase (JNK). The activity of JNK is controlled by the MAP3 kinase mitogen-activated/extracellular signal responsive kinase kinase (MEK) kinase 1. An expression vector encoding a truncated form of MEK kinase 1 (MEKK1Δ) was used to stimulate JNK activity. It has been shown that MEKK1Δ strongly activated the transcriptional activity of a fusion protein consisting of the GAL4 DNA-binding domain and the c-Jun transactivation domain (Lin et al., 1995). To test the role of MEKK1Δ on the GTP cyclohydrolase I promoter/luciferase reporter gene transcription, transient transfection experiments were performed. The data show that overexpression of MEKK1Δ induced the transcription of the GTP cyclohydrolase I promoter/luciferase reporter gene on the order of 4-fold (Fig. 40). Transfection efficiency was monitered by co-transfecting pSV40lacZ due to an observed effect of MEKK1Δ upon Rous sarcoma virus long terminal repeat controlled β-galactosidase expression.

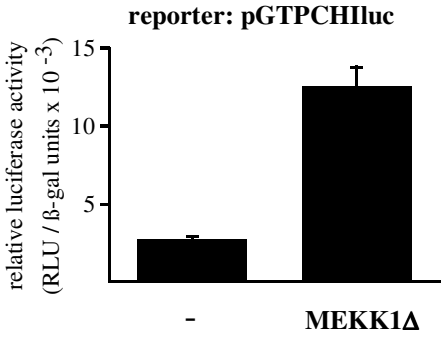


Fig. 40. Expression of a constitutively active mutant of MEKK1 increased GTP cyclohydrolase I promoter activity.
HepG2 cells were transfected with the pGTPCHIluc reporter plasmid (1 µg/plate) together with the internal standard plasmid pSV40lacZ (3 µg/plate) and either an empty expression vector "pCMV5" or an expression vector encoding MEKK1Δ (2 ng plasimd/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.3.3.3. c-Jun and ATF2 mediate the MEKK1Δ-induced upregulation of the GTP cyclohydrolase I reporter gene

To check whether c-Jun and/or ATF2 mediate the MEKK1Δ-induced upregulation of the GTP cyclohydrolase I promoter/luciferase reporter gene, competition assay using the A-ZIP expression vectors was performed. The results show that both A-ATF2 and A-Fos were able to block the transcriptional activation of the GTP cyclohydrolase I promoter gene mediated by MEKK1Δ (Fig. 41A). Additionally, another competition experiments involving dominant-negative ATF2 and c-Jun mutants ATF2ΔN and c-JunΔN were performed. These mutants contain the intact bZIP domains of ATF2 or c-Jun, respectively, and are therefore able to bind to the cognate sites of ATF2 and c-Jun, making these sites unavailable for wild-type bZIP proteins. The results show that both mutants ATF2ΔN and c-JunΔN blocked the transcriptional activation of the GTP cyclohydrolase I gene promoter mediated by MEKK1Δ (Fig. 41B). Taken together, these data indicate that either c-Jun or ATF2 or a heterodimer of c-Jun and ATF2 mediate the MEKK1Δ-induced upregulation of the GTP cyclohydrolase I promoter/luciferase reporter gene transcription.

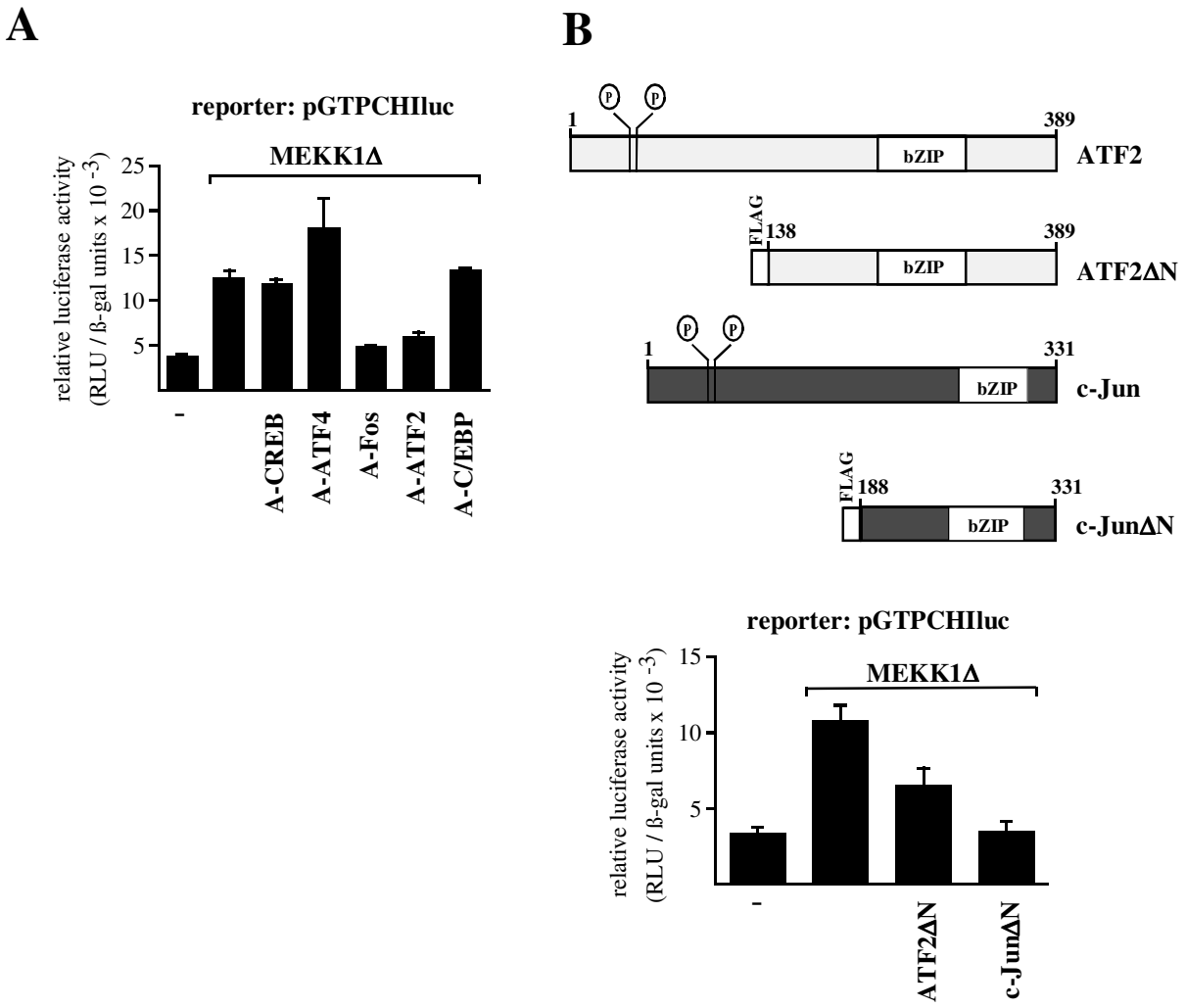


Fig. 41. MEKK1Δ-induced GTP cyclohydrolase I promoter activity was impaired by dominant-negative mutants of ATF2 and c-Jun.
(A) The reporter plasmid pGTPCHIluc (1 µg/plate) was transfected into HepG2 cells together with the internal standard plasmid pSV40lacZ (3 µg/plate), an expression vector encoding MEKK1Δ (2 ng plasmid/plate) and expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP (50 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD. (B) Schematic representation of the modular structure of ATF2 and c-Jun and the dominant-negative mutants ATF2ΔN and c-JunΔN. HepG2 cells were transfected with the pGTPCHIluc reporter plasmid (1 µg/plate) together with the standard plasmid pSV40lacZ (3 µg/plate), an expression vector encoding MEKK1Δ (2 ng/plate) and expression vectors encoding ATF2ΔN and c-JunΔN (250 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.3.3.4. Mutations of the CRE-like sequence of the GTP cyclohydrolase I promoter impair the activation by c-Jun

GTP cyclohydrolase I reporter genes carrying the intact or the mutated CRE-like site were used to analyze the importance of the CRE-like sequence for c-Jun-mediated transactivation. The results reveal that the CRE-like sequence is of major importance for the constitutively active c-Jun mutant to transactivate the GTP cyclohydrolase I promoter/luciferase reporter gene (Fig. 42).

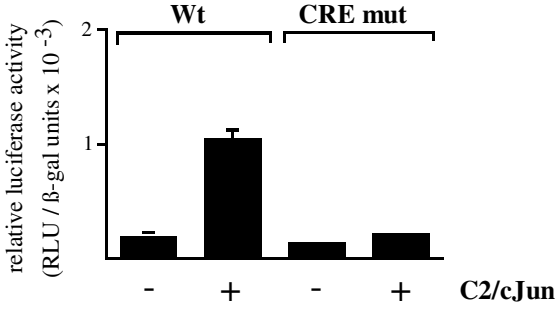


Fig. 42. Point mutations within the CRE-like sequence of the GTP cyclohydrolase I promoter impair transactivation mediated by C2/c-Jun.
1 µg/plate of the wild-type or the mutated GTP cyclohydrolase I reporter plasmid was transfected into HepG2 cells together with 500 ng/plate of C2/c-Jun and 2 µg/plate of the internal standard plasmid pRSVβ. Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.3.4. C2/c-Jun activates the transcription of the TNFα reporter gene containing one copy of the CRE/AP1 binding site

The biological activity of c-Jun on the TNFα promoter gene was tested. Transient transfection experiments of HepG2 cells using the constitutively active mutant C2/c-Jun were performed and the results show that C2/c-Jun transactivated the TNFα reporter gene (plasmid pTNFα(-131)luc). The transcriptional activation was approximately on the order of 20-fold. In contrast, the wild-type ATF2 was inactive (Fig. 43).

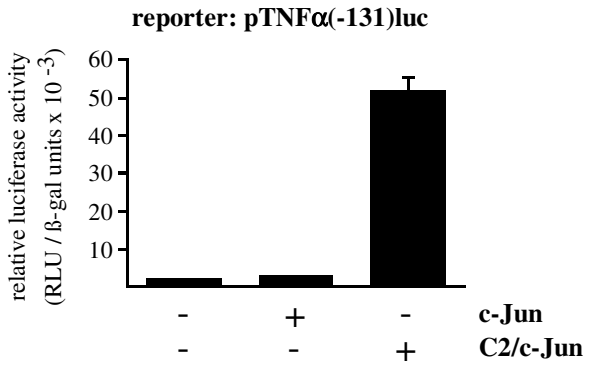


Fig. 43. C2/c-Jun activates the transcription of TNFα promoter/luciferase reporter gene.
The reporter plasmid pTNFα(-131)luc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding c-Jun or C2/c-Jun (500 ng/plate). Forty-eight hours post-transfection lysates were prepared and the relative luciferase activities were determined. The mean ±SD is depicted.

C.3.5. Expression of the constitutively active MAP3 kinase mutant MEKK1Δ activates TNFα reporter gene containing two copies of CRE/AP1 binding site

To test whether the JNK pathway regulates TNFα gene expression in HepG2 cells, an expression vector encoding a truncated form of MEK kinase 1 (MEKK1Δ) was used to stimulate JNK activity. To analyze the role of MEKK1Δ on the human TNFα gene promoter activity, a reporter gene containing two copies of the CRE/AP1 sequence upstream of a minimal promoter was used. Transient transfection experiments of HepG2 cells show that transfection of 2 ng of the expression plasmid MEKK1Δ was sufficient to activate the transcription of the luciferase reporter gene controlled by the CRE/AP1 element (Fig. 44).

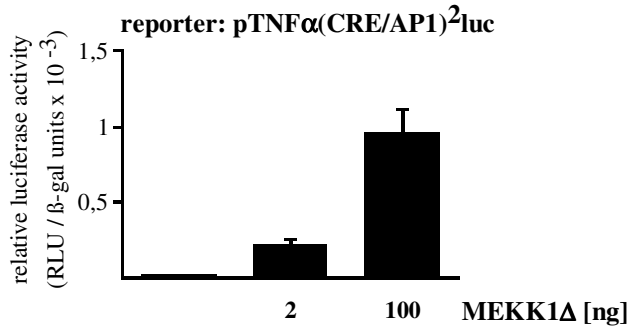


Fig.44. Expression of MEKK1Δ increased the transcription of TNFα reporter gene containing two copies of CRE/AP1 binding site.
HepG2 cells were transfected with the pTNFα(CRE/AP1)^{2}luc reporter plasmid (1 µg/plate), together with the internal standard plasmid pRSVβ (2 µg/plate) and an expression vector encoding MEKK1Δ (2 or 100 ng plasmid/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.3.5.1. MKP-1 inhibits MEKK1Δ-induced TNFα promoter/luciferase reporter gene containing two copies of CRE/AP1 binding site

MKP-1 is known to inactivate the protein kinase JNK in the nucleus (Sun et al., 1994; Liu et al., 1995; Gupta et al., 1996) via dephosphorylation, therefore the MKP-1 was overexpressed to dephosphorylate JNK in the nucleus. The results show that MKP-1 blocked MEKK1Δ-induced upregulation of TNFα promoter/luciferase reporter gene transcription via the CRE/AP1 site (Fig. 45). The results obtained by using the MEKK1Δ and MEKK1Δ/MKP-1 lead to the conclusion that JNK regulates TNFα gene expression in HepG2 cells. These data also suggest that the transcription of the TNFα gene is regulated by c-Jun and ATF2.

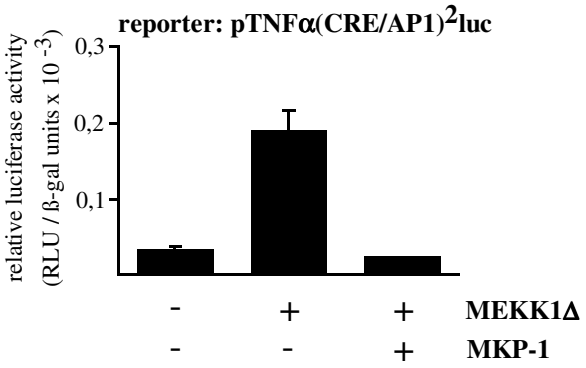


Fig. 45. The stimulating effect of MEKK1Δ expression upon TNFα reporter gene was attenuated by expressing MKP-1.
HepG2 cells were transfected with the pTNFα(CRE/AP1)^{2}luc reporter plasmid (1 µg/plate), together with the internal standard plasmid pRSVβ (2 µg/plate), either an empty expression vector "pCMV5" or an expression vector encoding MEKK1Δ (2 ng/plate) and an expression vector encoding MKP-1 (1 µg/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.4. Regulation of gene expression by CREB2/ATF4

C.4.1. CREB2/ATF4 is unable to transactivate a GTP cyclohydrolase I promoter/luciferase reporter gene

It has been reported that CREB2/ATF4 transactivates the GTP cyclohydrolase I promoter in PC12 cells (Kapatos et al., 2000). An expression vector encoding CREB2 was used to test the biological effect of CREB2 on GTP cyclohydrolase I promoter activity. The results show that overexpression of CREB2 did not enhance the GTP cyclohydrolase I promoter activity, while C2/CREB strongly enhanced the transcription of the reporter gene (Fig. 46A). As a positive control, a reporter gene containing the nutrient-sensing response unit of the asparagine synthetase promoter upstream of a minimal promoter and the luciferase open reading frame (plasmid pASluc) was used. The results show that overexpression of CREB2/ATF4 activates transcription of asparagine synthetase promoter via the nutrient-sensing response unit (Fig. 46B).

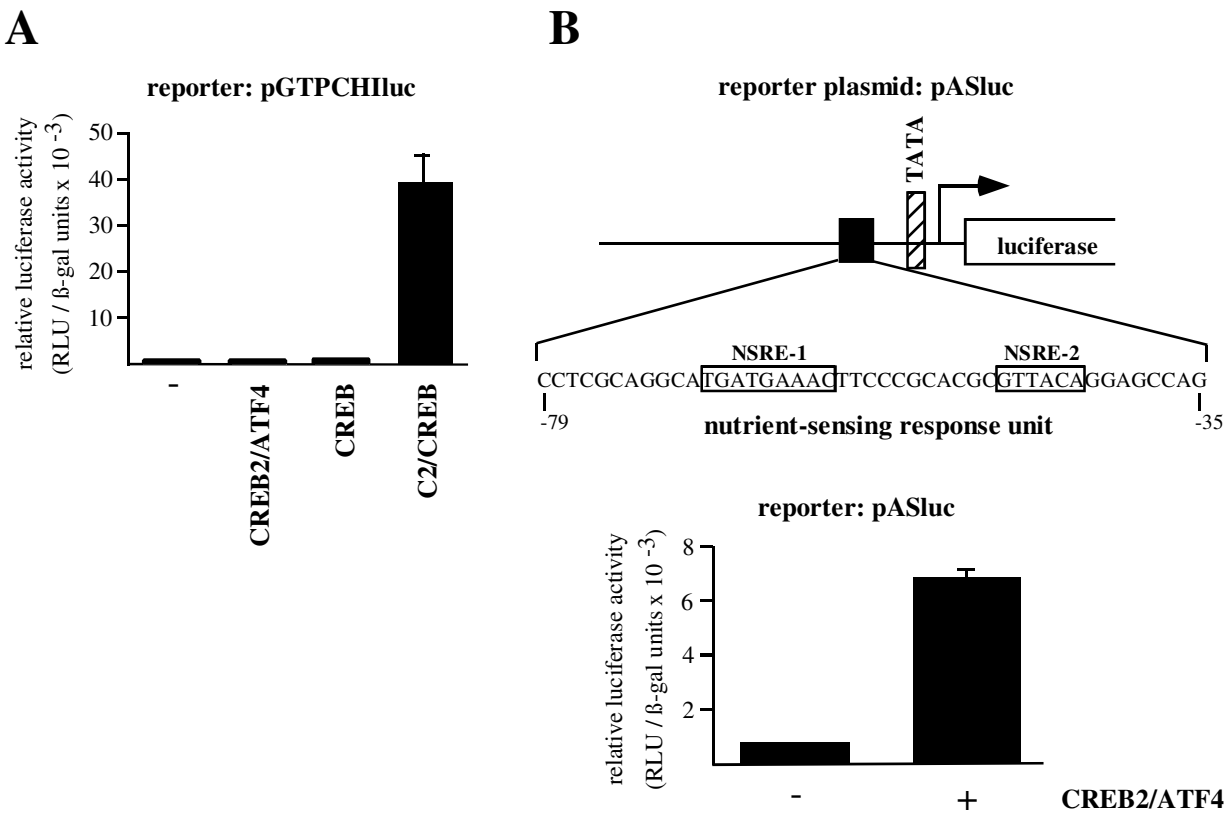


Fig. 46. CREB2/ATF4 does not upregulate the GTP cyclohydrolase I promoter activity.
(A) The reporter plasmid pGTPCHIluc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the expression vector pCMV5 or expression vectors encoding CREB2/ATF4 or CREB (25 ng/plate). For comparison, an expression vector encoding C2/CREB was transfected (25 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD. (B) Schematic representation of the reporter plasmid pASluc. The reporter plasmid pASluc contains the nutrient-sensing response unit upstream of a minimal promoter and the luciferase open reading frame. The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vector encoding CREB2/ATF4 (25 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

Likewise, CREB2/ATF4 did not activate a reporter gene under control of the Egr-1 promoter (plasmid pEgr-1.1luc). The Egr-1 promoter contains a CRE-like sequence within its proximal region. As shown in Fig. 47, C2/CREB strongly transactivated Egr-1 promoter/luciferase reporter gene. As a conclusion, CREB2 is unable to transactivate the genes encoding GTP cyclohydrolase I or Egr-1 via the CRE-like sequences in their proximal promoter region.

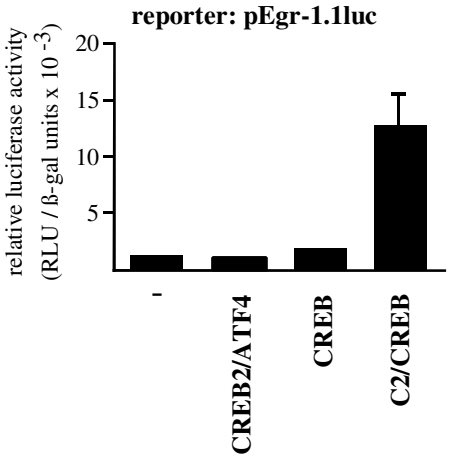


Fig. 47. CREB2/ATF4 does not upregulate the Egr-1 promoter activity.
The reporter plasmid pEgr-1.1luc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding CREB2/ATF4 or CREB (25 ng/plate). For comparison, an expression vector encoding C2/CREB was transfected (25 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.4.2. CREB2/ATF4 is unable to transactivate a TNFα reporter gene that contains one copy of CRE/AP1 site

The biological effect of CREB2 on the TNFα promoter activity was tested and the results show that overexpression of CREB2 does not activate the TNFα reporter gene via the CRE-like sequence in its proximal promoter region (Fig. 48). For comparison C2/CREB was also transfected.

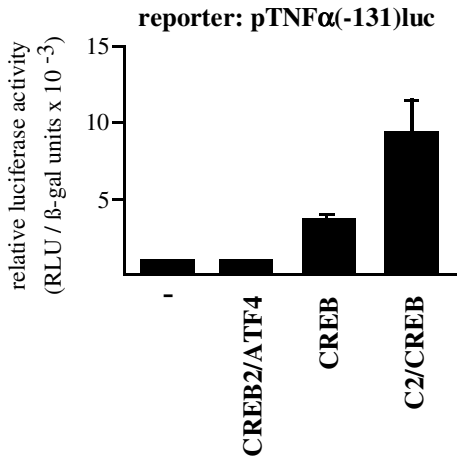


Fig. 48. CREB2/ATF4 does not upregulate the TNFα reporter gene transcription.
The reporter plasmid pTNFα(-131)luc (1 µg/plate) was transfected into HepG2 cells, together with the internal standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding CREB2/ATF4 or CREB (25 ng/plate). For comparison, an expression vector encoding C2/CREB was transfected (25 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.5. Regulation of gene expression by ATF5 and CHOP

C.5.1. Deprivation of human hepatoma cells of amino acids triggers a strong upregulation of the asparagine synthetase gene transcription

In mammals, plasma concentration of amino acids are affected by nutritional or pathological conditions. Recently, evidence has accumulated that amino acid availability regulates the expression of several genes involved in the regulation of a number of cellular functions or amino acid metabolism (Jousse et al., 2000). It has been shown that the transcription of the gene encoding asparagine synthetase is regulated by nutrient availability. To study the effect of amino acids deprivation on asparagine synthetase gene transcription HepG2 cells were cultured for 16 hours either in medium containing amino acids (DMEM) or in amino acid-free Krebs-Ringer-bicarbonate buffer (KRB). The results show that maintaining the cells in medium containing amino acids (incubation in DMEM) no asparagine synthetase mRNA was detectable, while maintaining the cells in medium lacking amino acids (KRB) strongly induced the biosynthesis of asparagine synthetase (Fig. 49).

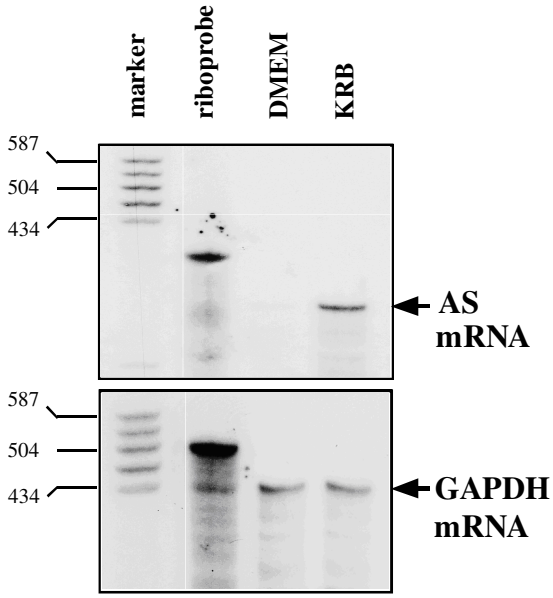


Fig. 49. Activation of asparagine synthetase gene transcription by amino acid deprivation. HepG2 human hepatoma cells were cultured in either DMEM or Krebs-Ringer-bicarbonat (KRB) medium lacking all amino acids for 16 hours. Cytoplasmic RNA was isolated and analyzed by RNase protection mapping using cRNAs specific for asparagine synthetase (AS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). An aliquot of the undigested riboprobes is shown. Size marker *Msp*I–cut PUC19 are shown in lane "marker". This experiment was performed by G.Thiel.

C.5.2. Amino acid deprivation induces the asparagine synthetase promoter/luciferase reporter gene transcription

It has been shown that the activation of asparagine synthetase gene transcription by amino acids deprivation is accomplished by a genetic element termed the nutrient-sensing response unit. The nutrient-sensing response unit consists of two elements termed NSRE-1 and NSRE-2, encompassing the sequences 5´-TGATGAAAC-3´ and 5´-GTTACA-3´, respectively (Kilberg and Barbosa-Tessmann, 2002). A reporter gene (pASluc) that contains both elements was used. To analyze the induction of asparagine synthetase reporter gene transcription by amino acid deprivation, transient transfection experiments of the human hepatoma cell line HepG2 were performed. The results show that amino acid deprivation induced asparagine synthetase promoter/luciferase reporter gene transcription (Fig. 50). The induction was on the order of 14-fold.

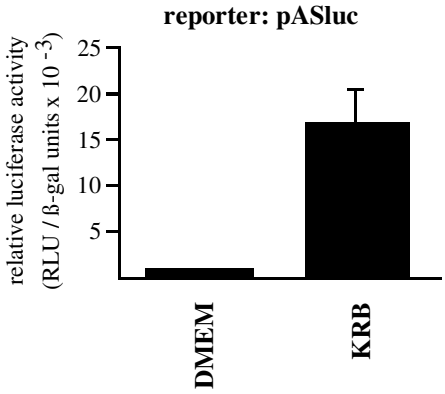


Fig. 50. Asparagine synthetase gene transcription is induced by amino acid deprivation.

The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 hepatoma cells together with the internal standard plasmid pRSVβ (2 µg/plate). Cells were cultured in medium containing amino acids (DMEM) or in amino acid-free Krebs-Ringer-bicarbonate (KRB) medium. Cells were harvested 16 hours later, cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.5.3. Amino acid deprivation-induced asparagine synthetase gene transcription was reduced by dominant-negative CREB2/ATF4 (A-ATF4) and C/EBP (A-C/EBP) mutants

It has been reported that the bZIP transcription factors ATF3, ATF4, and C/EBPβ mediate the signaling cascade from amino acid deprivation to enhanced asparagine synthetase gene transcription. Additionally, the bZIP protein ATF2 has been shown to be involved in the leucine deprivation-induced upregulation of CHOP gene transcription (Averous et al., 2004). To test the effect of the bZIP proteins CREB, ATF2, CREB2/ATF4, c-Jun and C/EBP on amino acid-deprivation induced asparagine synthetase gene transcription, the dominant-negative mutants A-ZIPs were used. The results show that A-ATF4 and A-C/EBP reduced the upregulation of reporter gene transcription, indicating that these bZIP proteins are important to stimulate gene transcription via the nutrient-sensing response unit. In contrast, dominant-negative mutants of CREB, ATF2 and c-Jun did not significantly impair reporter gene transcription (Fig. 51).

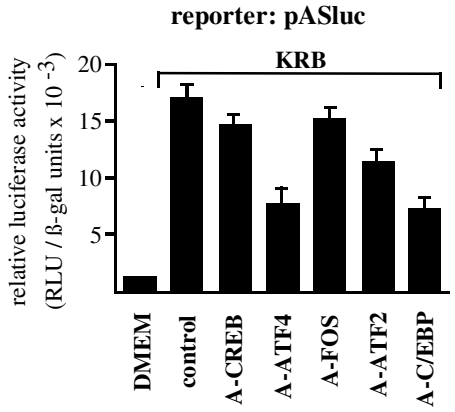


Fig. 51. Stimulation of asparagine synthetase gene transcription by amino acid deprivation involves the bZIP transcription factors CREB2/ATF4 and C/EBP.
The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 hepatoma cells together with the internal standard plasmid pRSVβ (2 µg/plate) and expression vectors encoding the dominant-negative mutants A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP. Cells were cultured in KRB medium for 16 hours. The control cells were maintained in DMEM. Cell extracts were prepared and the relative luciferase activities were determined. Data are mean ± SD.

C.5.4. Comparison of the primary structures of the bZIP transcription factors CREB2/ATF4 and ATF5

It has been shown that CREB2/ATF4 activates gene transcription of reporter genes containing the nutrient-sensing response unit (Siu et al., 2002). The primary structures of the bZIP transcription factors CREB2/ATF4 and ATF5 were compared. The comparison show that ATF5 is closely related to CREB2/ATF4 particularly in the bZIP domain. ATF5 contains a characteristic leucine-valine zipper, consisting of three leucine and three valine residues. An arginine-lysine rich basic region is N-terminal from this leucine-valine zipper that is required for sequence-specific binding to DNA (Fig. 52).

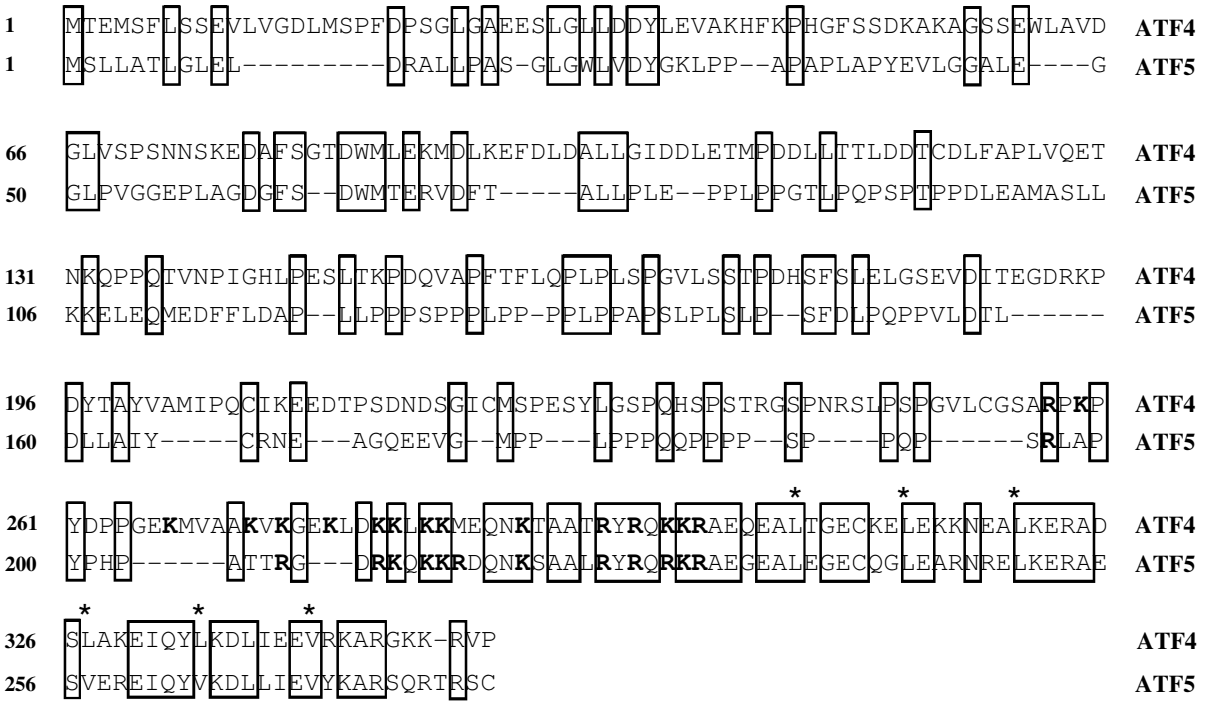


Fig. 52. Comparison of the amino acid sequences of the bZIP transcription factors CREB2/ATF4 and ATF5.
The basic residues of the "basic domain" are shown in bold. The leucine-valine zipper residues are indicated by stars above the amino acids.

C.5.5. Comparison of the transcriptional activation domains of CREB2/ATF4, ATF5, CREB, c-Jun and ATF2

CREB2/ATF4 contains a constitutively active transcriptional activation domain, while the bZIP proteins CREB, c-Jun and ATF2 have phosphorylation-inducible transcriptional activation domains. The transcriptional activation domains of CREB2/ATF4, ATF5, CREB, c-Jun and ATF2 were compared by expressing these activation domains as fusion proteins with the DNA-binding domain derived from the yeast transcription factor GAL4. Transcriptional activation was monitored by co-transfection of the reporter plasmid pUAS^{5}luc containing five copies of the GAL4 binding site upstream of a luciferase reporter gene. The data show that ATF5 and CREB2/ATF4 contain constitutively active trasncriptional activation domains. In contrast, transcription mediated by the fusion proteins GAL4-CREB, GAL4-c-Jun and GAL4-ATF2 is very low in the absence of stimulation.

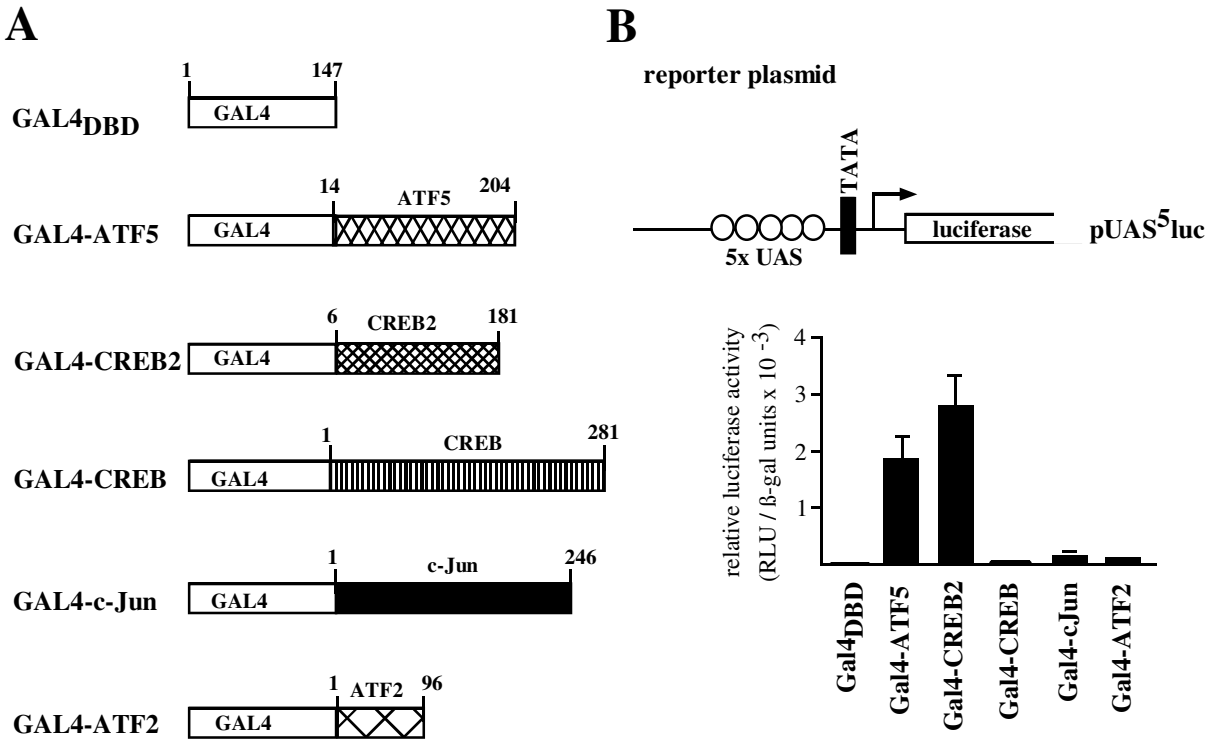


Fig. 53. Comparison of the transcriptional activation domains of CREB2/ATF4, ATF5, CREB, c-Jun and ATF2.
(A) Modular structure of the GAL4 fusion proteins GAL4-ATF5, GAL4-CREB2, GAL4-CREB, GAL4-c-Jun and GAL4-ATF2. (B) The reporter plasmid pUAS^{5}luc (1 µg/plate), the internal standard plasmid pRSVβ (2 µg/plate) and one of the expression vectors encoding GAL4-ATF5, GAL4-CREB2/ATF4, GAL4-CREB, GAL4-c-Jun or GAL4-ATF2 (1 µg/plate) were transfected into HepG2 hepatoma cells. As a control, plasmid pM1 encoding only the DNA-binding domain of GAL4 ($GAL4_{DBD}$) was transfected (1 µg/plate). Cells were cultured in DMEM medium containing 10% fetal bovine serum for forty-eight hours. Cell extracts were prepared and the relative luciferase activities were determined. Data are mean ± SD.

C.5.6. The biological activity of ATF5

C.5.6.1. ATF5 transactivates the asparagine synthetase promoter/luciferase reporter gene

The biological activity of ATF5 towards asparagine synthetase gene transcription was tested by performing transient transfection experiments using an expression vector encoding ATF5. As shown in Fig. 54, ATF5 transactivated a reporter gene containing the nutrient-sensing response unit of the asparagine synthetase gene in its regulatory region.

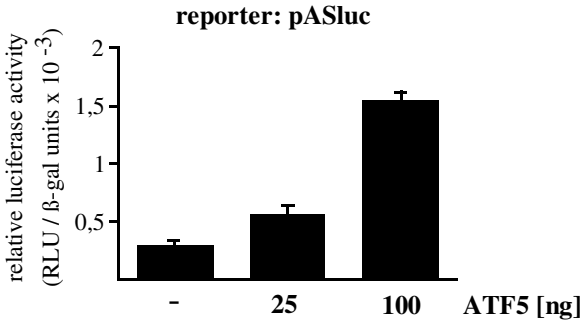


Fig. 54. ATF5 transactivates a reporter gene containing the nutrient-sensing response unit of the asparagine synthetase gene.
The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 hepatoma cells together with the internal standard plasmid pRSVβ (2 µg/plate) and either an empty expression vector "pCMV5" or an expression vector encoding ATF5 (25 or 100 ng plasmid/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.5.6.2. ATF5 can heterodimerize with CREB2/ATF4 and C/EBP proteins

A recent analysis of the "dimerization code" of CREB2/ATF4 and CREB revealed that both proteins do not form heterodimers. In contrast, a dominant-negative C/EBP protein impaired transcriptional activation induced by CREB2/ATF4, as expected, because C/EBP is a well-characterized dimerization partner of CREB2/ATF4 (Newman and Keating, 2003). To investigate the "dimerization code" of ATF5, the dominant-negative mutants A-ZIPs were used. The results show that dominant-negative mutants of CREB2/ATF4 and C/EBP impaired transcriptional activation of the reporter gene mediated by ATF5, indicating that ATF5 can heterodimerize with CREB2/ATF4 and C/EBP proteins. Expression of A-CREB, A-Fos or A-ATF2 did not change ATF5-induced reporter gene transcription, indicating that CREB, c-Jun and ATF2 do not heterodimerize with ATF5.

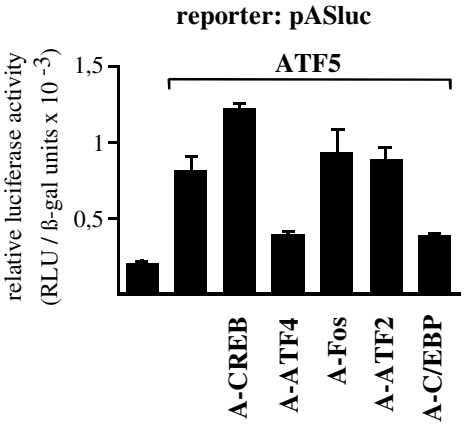


Fig. 55. The dominant-negative mutants A-ATF4 and A-C/EBP block ATF5-induced transcription of the asparagine synthetase gene.
The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 hepatoma cells together with the internal standard plasmid pRSVβ (2 µg/plate), an expression vector encoding ATF5 (25 ng/plate) and one of the expression vectors encoding either A-CREB, A-ATF4, A-Fos, A-ATF2 or A-C/EBP (50 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.5.6.3. ATF5 does not activate CRE-containing reporter genes

It has been reported that ATF5 homodimers can bind *in vitro* to the cAMP response element (CRE) (Peters et al., 2001). In the study reported here, it was shown that CREB2/ATF4 did not transactivate CRE-containing reporter genes, indicating that CREB2/ATF4 targets distinct genetic elements. Due to the similarities between ATF5 and CREB2/ATF4, it was proposed that ATF5 does not transactivate CRE-containing reporter genes. To clarify this presumption two reporter genes containing either the distal (CRE1) or the proximal CRE-like site (CRE2) derived from the glucose-6-phosphatase gene (plasmids pG6PCRE1luc and pG6PCRE2luc) were used. Additionally, a reporter gene containing four copies of the CRE derived from the c-Fos gene upstream of a minimal promoter (plasmid pc-fosCRE^{4}luc) was tested. The results show that ATF5 was not able to transactivate CRE-containing reporter genes. As a positive control, the constitutively active mutant C2/CREB was used and the data show that C2/CREB strongly induced the transcription of all three reporter genes (Fig. 56).

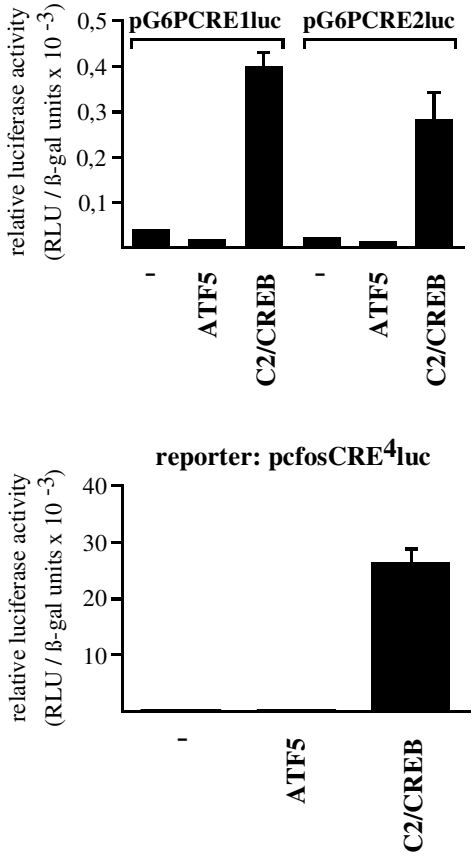


Fig. 56. ATF5 has no effect on CRE-containing reporter genes.
One of the reporter plasmids pG6PCRE1luc, pG6PCRE2luc or pc-fosCRE^{4}luc (1 µg/plate) was transfected into HepG2 cells, together with plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding either ATF5 or C2/CREB (25 ng/plate). Cell extracts were prepared forty-eight hours later and the relative luciferase activities of these extracts were determined. Data are mean ± SD.

C.5.7. The role of the C/EBP homologous transcription factor CHOP on amino acid deprivation-induced upregulation of asparagine synthetase gene transcription

As shown earlier the transcriptional activation mediated by the nutrient-sensing response unit of the asparagine synthetase gene involves C/EBP proteins or dimerization partners of C/EBP such as CREB2/ATF4 and ATF5. The role of the C/EBP homologous transcription factor CHOP on amino acid deprivation-induced upregulation of asparagine synthetase gene transcription was studied. Likewise, the role of CHOP upon CREB2/ATF4 or ATF5-induced upregulation of pASluc reporter gene transcription was tested.

C.5.7.1. CHOP inhibited the upregulation of reporter gene transcription, induced by amino acid deprivation

It has been reported that the biosynthesis of CHOP is strongly stimulated by nutrient deprivation (Bruhat et al., 1997; Averous et al., 2004). The role of CHOP on amino acid deprivation-induced asparagine synthetase gene transcription was tested and the results show that CHOP efficiently blocked the upregulation of asparagine synthetase reporter gene transcription, induced by amino acid deprivation in HepG2 cells.

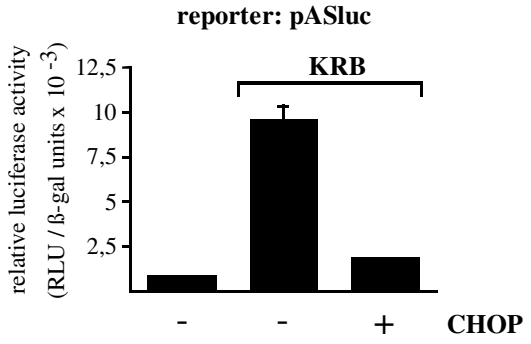


Fig. 57. CHOP functions as a shut-off-device of nutrient deprivation-induced gene transcription. The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 hepatoma cells together with the internal standard plasmid pRSVβ (2 µg/plate) and either the "empty" expression vector pCMV5 or an expression vector encoding CHOP (25 ng/plate). Cells were cultured in KRB medium for 16 hours. The control cells were transfected with an "empty" expression vector and cultured in DMEM medium. Cell extracts were prepared and the relative luciferase activities were determined. Data are mean ± SD.

C.5.7.2. The expression of CHOP blocked the activation of asparagine synthetase promoter/luciferase reporter gene by either CREB2/ATF4 or ATF5

The role of CHOP on CREB2/ATF4- or ATF5-induced upregulation of asparagine synthetase gene transcription was tested and the data show that the overexpression of CHOP abrogated the transactivation of the pASluc reporter gene mediated by CREB2/ATF4 or ATF5 (Fig. 58).

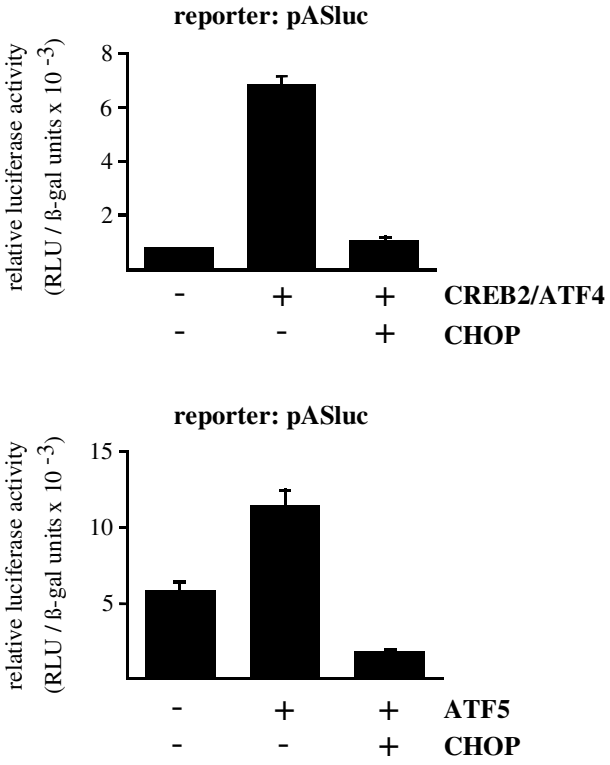


Fig. 58. CHOP inhibited the activation of the pASluc reporter gene by either CREB2/ATF4 or ATF5.
The reporter plasmid pASluc (1 µg/plate) was transfected into HepG2 hepatoma cells together with the internal standard plasmid pRSVβ (2 µg/plate) and expression vectors encoding CREB2/ATF4 or ATF5 (25 ng/plate). In addition, cells were transfected with either an "empty" expression vector or an expression vector encoding CHOP (25 ng/plate). Cells were cultured in DMEM medium. Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ± SD.

The expression of CHOP has been linked to cell cycle arrest and programmed cell death (Wang et al., 1998). To clarify that the induction of cell death is not responsible for the impairment of asparagine synthetase gene transcription following CHOP overexpression, the glucose-6-phosphatase promoter/luciferase reporter genes were induced by C2/CREB in the presence or absence of CHOP. The results show that CHOP did not interfere with the ability of C2/CREB to transactivate the reporter genes pG6PCRE1luc and pG6PCRE2luc (Fig. 59).

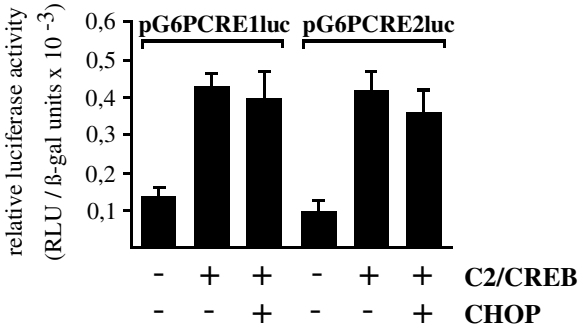


Fig. 59. CHOP does not interfere with the ability of C2/CREB to transactivate the reporter genes pG6PCRE1luc and pG6PCRE2luc.
One of the reporter plasmids pG6PCRE1luc or pG6PCRE2luc (1 µg/plate) was transfected into HepG2 cells, together with the standard plasmid pRSVβ (2 µg/plate), the empty expression vector pCMV5 or expression vectors encoding C2/CREB or CHOP (25 ng/plate). Cell extracts were prepared forty-eight hours later and the relative luciferase activities were determined. Data are means ± SD.

C.6. Regulation of gene expression by C/EBPα

C.6.1. Biological activity of C/EBPα and a constitutively active C/EBPα mutant C2/C/EBPα towards specific reporter genes

The proteins of the C/EBP family of bZIP proteins bind specifically to the dyad symmetric sequence 5´-ATTGCGCAAT-3´ (Agre et al., 1989), a motif quite distinct from the canonical CRE sequence 5´-TGACGTCA-3´. However, C/EBP proteins have also been reported to bind to ATF/CRE motifs (Bakker and Parker, 1991). To study C/EBP-mediated gene transcription expression vectors encoding either C/EBPα or the constitutively active mutant C2/C/EBPα were used.

C.6.2. Modular structure of C/EBPα and the constitutively active C/EBPα mutant C2/C/EBPα

The bZIP transcription factor C/EBPα contains in its C-terminus a bZIP domain, responsible for DNA-binding and dimerization, while the N-terminus contains an activation domain. The constitutively active C/EBPα mutant, C2/C/EBPα, consists of the transcriptional activation domain of CREB2 and the bZIP domain of C/EBPα.

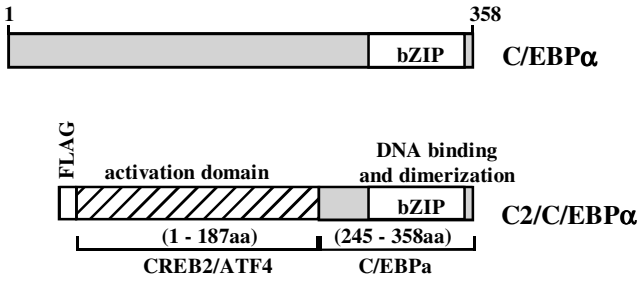
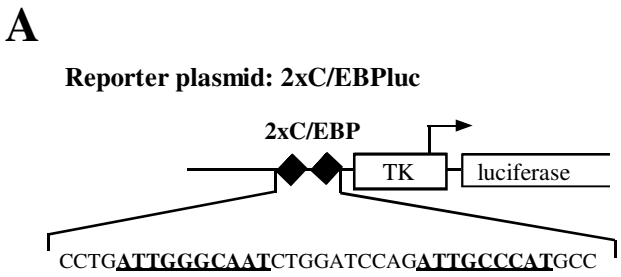


Fig. 60. Schematic representation of C/EBPα and C2/C/EBPα.
Modular structure of the bZIP proteins C/EBPα and the constitutively active mutant C2/C/EBPα.

C.6.3. C/EBPα and C2/C/EBPα strongly activate the C/EBP-specific reporter gene 2xC/EBPluc

The transcriptional activity of C/EBPα and C2/C/EBPα was compared by using the C/EBP-specific reporter gene 2xC/EBPluc (Fig. 61A). The data show that both C/EBPα and C2/C/EBPα proteins strongly transactivate the reporter gene on the order of 800- and 1200-fold, respectively (Fig. 61B). Transfection efficiency was monitored by co-transfecting pSV40lacZ due to an observed effect of C/EBPα and C2/C/EBPα upon Rous sarcoma virus long terminal repeat controlled β-galactosidase expression.



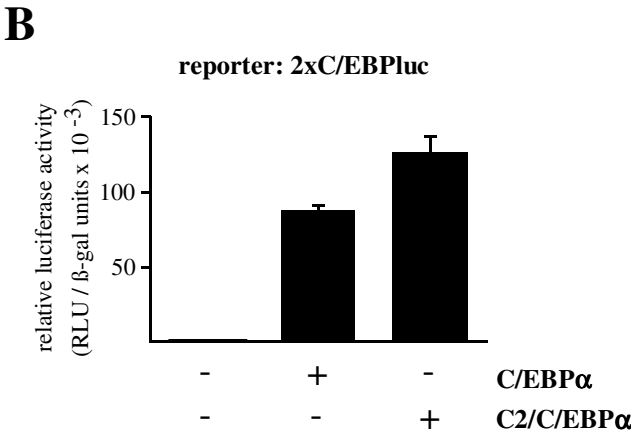


Fig. 61. C/EBP-specific reporter gene is strikingly induced by C/EBPα and C2/C/EBPα.
(A) Schematic representation of the reporter plasmid 2xC/EBPluc. The transcription unit includes two C/EBP binding sites upstream of a minimal thymidine kinase promoter. (B) The reporter plasmid 2xC/EBPluc (1 µg/plate) was transfected into HepG2 cells together with the internal reference plasmid pSV40lacZ (3 µg/plate) and either the "empty" expression vector pCMV5 or an expression vector encoding either C/EBPα or C2/C/EBPα (100 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the β-galactosidase and luciferase activities were determined. Data are mean ±SD.

C.6.4. C/EBPα proteins only marginally influence GTP cyclohydrolase I promoter activity

The influence of C/EBPα proteins on the GTP cyclohydrolase I promoter activity was tested. The results show that the GTP cyclohydrolase I promoter/luciferase reporter gene was only marginally transactivated by C/EBPα (1.8-fold). Overexpression of C2/C/EBPα increased GTP cyclohydrolase I reporter gene transcription on the order of 3.6-fold (Fig. 62). These results lead to the conclusion that the C/EBPα proteins play only a marginal role in the regulation of GTP cyclohydrolase I gene transcription.

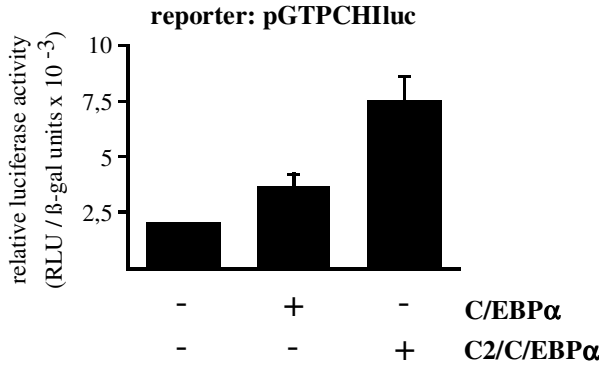


Fig. 62. C/EBPα and C2/C/EBPα marginally transactivated the GTP cyclohydrolase I promoter/luciferase reporter gene.
The reporter plasmid pGTPCHIluc (1 µg/plate) was transfected into HepG2 cells together with the internal reference plasmid pSV40lacZ (3 µg/plate) and either the "empty" expression vector pCMV5 or an expression vector encoding either C/EBPα or C2/C/EBPα (100 ng/plate). Forty-eight hours post-transfection cell extracts were prepared and the relative luciferase activities were determined. Data are mean ±SD.

C.6.5. Expression of C/EBPα slightly induced the transcription of the secretogranin II reporter gene that contains four copies of the CRE sequence

The effect of C/EBPα protein on a reporter gene that contains four copies of the CRE derived from the secretogranin II gene in noradrenergic locus coeruleus-like cell line CATH.a cells was tested. The results show that overexpression of C/EBPα slightly transactivated the secretogranin II reporter gene containing four copies of the CRE sequence (plasmid pSgIICRE^{4}luc). The induction was on the order of 1.5-fold (Fig.63).

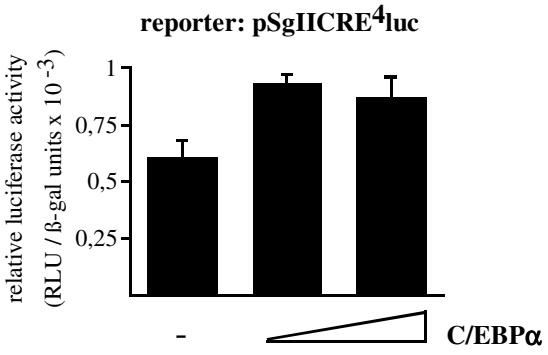


Fig. 63. C/EBPα marginally transactivated the secretogranin II promoter/luciferase reporter gene.
The reporter plasmid pSgIICRE^{4}luc (1 µg/plate) was transfected into CATH.a cells together with the pRSVβ internal reference plasmid (2 µg/plate) and either the 'empty' expression vector pCMV5 or an expression vector encoding C/EBPα (25 and 100 ng/plate). Lysates were prepared forty-eight hours post-transfection and β-galactosidase and luciferase activities were measured. The mean ± SD is depicted.

C.7. Test systems: activation of signaling cascades leading to the activation of target genes

Two test systems were studied in which the expression of genes embedded in the chromatin was analyzed and the involvement of different bZIP transcription factors was also investigated. The first gene studied is the inducible nitric oxide synthase (iNOS) gene in BV-2 murine microglial cells, while the second test system investigated the regulation of c-Jun and c-Fos in αT3M3 gonadotrope cells.

C.7.1. Expression of inducible nitric oxide synthase (iNOS) in microglial cells

iNOS generates nitric oxide from the amino acid arginine. Upon inflammatory conditions, iNOS is expressed by activated microglia (Perry et al., 1993; Kreutzberg, 1996; Merrill and Benveniste, 1996). Microglia are resident macrophages with wide distribution in nervous tissues (Barron, 1995). The regulation of iNOS expression by bZIP proteins in BV-2 microglial cells was analyzed.

C.7.1.1. LPS stimulates the expression of iNOS in BV-2 microglial cells

The lipopolysaccharide (LPS) is a component of the outer membrane of Gram negative bacteria. LPS has been shown to induce iNOS expression in BV-2 cells (Watters et al., 2002). To confirm these data the BV-2 cells were stimulated with different concentrations of LPS and then Western blot analysis was performed. The results show that LPS was able to induce iNOS expression in these cells in a dose dependent manner (Fig. 64).

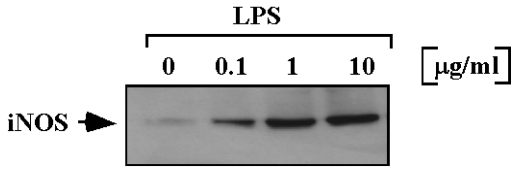


Fig. 64. LPS induces iNOS biosynthesis in BV-2 cells.
The BV-2 cells were serum starved for twenty-four hours then incubated with LPS (0.1, 1, 10 µg/ml) for further twenty-four hours. Whole cell extracts were prepared and then analyzed by Western blotting using iNOS specific antibody.

C.7.1.1.1. Activation of JNK is required for LPS-induced iNOS expression in BV-2 microglial cells

It has been shown that the MAPK signaling pathway is involved in LPS-mediated iNOS induction in primary glial cells (Da Silva et al., 1997; Bhat et al., 1998, 1999). To test whether JNK is required for LPS-induced iNOS expression in BV-2 microglial cells, a JNK peptide inhibitor termed L-JNK1 was used. This product is a potent inhibitor which is specific for JNK because it inhibits the interaction between JNK and its substrate, resulting in a JNK knockout phenotype (Bonny et al., 2001), in contrast to the chemical inhibitors that directly affect the kinase activity. The results show that L-JNK1 inhibited LPS-induced iNOS expression in BV-2 cells (Fig. 65).

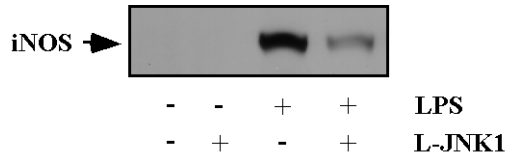


Fig. 65. L-JNK1 inhibits LPS-induced iNOS expression in BV-2 cells.
The BV-2 cells were serum starved for twenty-four hours. The cells were pretreated with the JNK inhibitor "L-JNK1" (1 µM) for 6 hours and then stimulated with LPS (0.1 µg/ml) for twenty-four hours. Whole cell extracts were prepared and then analyzed by Western blotting using iNOS specific antibody.

C.7.1.1.2. Activation of p38 MAPK is also required for LPS-induced iNOS expression in BV-2 microglial cells

A prominent role for p38-MAPK-mediated signaling in the transcriptional control of iNOS gene expression in primary glial cells (Bhat et al., 1998, 1999) and cultured rat glial cells (Bhat et al., 2002) has been suggested. To analyze the role of p38 MAPK upon LPS-induced iNOS expression in BV-2 cells, the p38 inhibitor SB203580 was used. The results show that SB203580 inhibited LPS-induced iNOS expression in a dose dependent manner (Fig. 66).

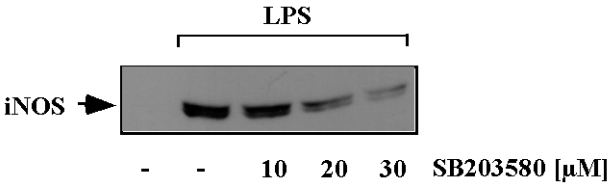


Fig. 66. SB203580 inhibited LPS-induced iNOS expression in BV-2 cells.
The BV-2 cells were serum starved for twenty-four hours. The cells were pretreated with the p38 inhibitor SB203580 (10, 20 and 30 µM) for 30 min then stimulated with LPS (0.1 µg/ml) for twenty-four hours. Whole cell extracts were prepared and then analyzed by Western blotting using an antibody against iNOS.

The MKP-1 is known to inactivate the phosphorylated forms of JNK and p38 in the nucleus. To study the role of MKP-1 in LPS-induced iNOS expression, the BV-2 cells were infected with lentiviruses expressing MKP-1. Lentiviral gene transfer was performed, the cells were then induced and subjected to Western blot analysis. The results show that MKP-1 completely blocked the induction of iNOS expression upon LPS stimulation (Fig. 67). This leads to the

conclusion that activation of JNK and p38 MAPK signaling pathways is required for LPS-induced iNOS gene expression in microglial cells.

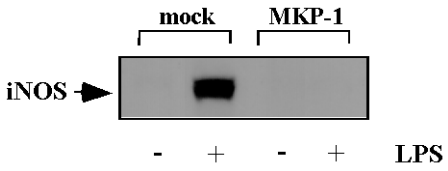


Fig. 67. MKP-1 overexpression impairs LPS-induced iNOS biosynthesis.
Lentiviral gene transfer of MKP-1. Viruses were pseudotyped with the vesicular stomatitis virus and used to infect BV-2 cells. As a control mock-infected cells were analyzed. The cells were serum starved for twenty-four hours then incubated with LPS (0.1 µg/ml) for further twenty-four hours. Whole cell extracts were prepared and then analyzed by Western blotting using an antibody against iNOS. The infection was performed by G. Thiel.

C.7.1.1.3. ATF2 is part of the signaling pathway involved in LPS-induced iNOS expression in microglial cells

p38 and JNK are the kinases that phosphorylate and activate the transcription factor ATF2. To analyze whether ATF2 is involved in LPS-induced iNOS expression, BV-2 cells were infected with lentiviruses expressing the dominant-negative mutant of ATF2 termed A-ATF2. The results show that A-ATF2 impaired LPS-induced iNOS gene expression. As a negative control, the dominant-negative CREB mutant A-CREB was used and the results indicate that CREB do not play any role in LPS-stimulated iNOS gene expression (Fig. 68).

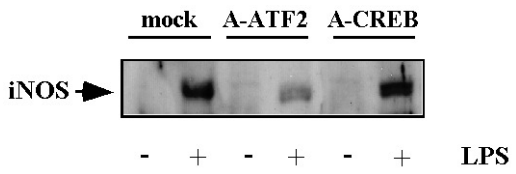


Fig. 68. ATF2 is involved in LPS-induced iNOS expression.
Lentiviral gene transfer of A-ATF2. Viruses were pseudotyped with the vesicular stomatitis virus and used to infect BV-2 cells. As a control mock-infected cells were analyzed. The cells were serum starved for twenty-four hours then incubated with LPS (0.1 µg/ml) for further twenty-four hours. Whole cell extracts were prepared and then analyzed by Western blotting using iNOS specific antibody. The infection was performed by G. Thiel.

Next, the expression of ATF2 upon LPS stimulation of BV-2 cells was tested. The cells were stimulated with LPS and then subjected to Western blot analysis using antibodies directed against both ATF2 and the phosphorylated and active form of ATF2. The results show that LPS strikingly induced the expression of the phosphorylated form of ATF2. In contrast, ATF2 is constitutively expressed in BV-2 cells and no changes in its expression were observed after stimulation with LPS (Fig. 69A). To test the kinetics of LPS-induced ATF2 expression, BV-2 cells were incubated with 0.1 µg/ml LPS for 1, 2, 4 and 6 hours. The induction of the phosphorylated form of ATF2 was observed after 2 hours and this induction lasts 6 hours following stimulation (Fig. 69B).

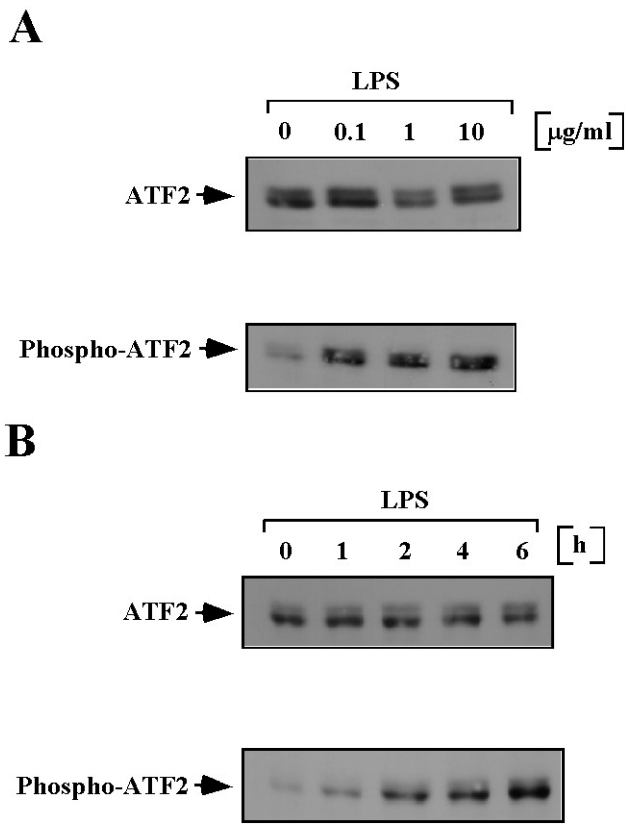


Fig. 69. LPS induces the phosphorylation of ATF2 in BV-2 microglial cells.
The BV-2 cell line was serum starved for twenty-four hours and then incubated with LPS (0.1, 1, 10 µg/ml) for 3 h (A) or incubated with LPS (0.1 µg/ml) for 1, 2, 4 and 6 hours (B). Nuclear extracts were prepared and then analyzed by Western blotting using specific antibodies against ATF2 and Phospho-ATF2.

C.7.1.1.4. c-Jun is part of the signaling pathway involved in LPS-induced iNOS expression in a microglial cell line

To check whether LPS induces c-Jun expression in BV-2 microglial cells, the cells were stimulated with LPS and then subjected to Western blot analysis using antibodies directed against both c-Jun and the phosphorylated and active form of c-Jun. The results show that LPS induced the expression of the phosphorylated form of c-Jun. In contrast, c-Jun is constitutively expressed in BV-2 cells (Fig. 70A). To study the kinetics of LPS-induced c-Jun expression, BV-2 cells were incubated with 0.1 µg/ml LPS for 1, 2, 4 and 6 hours. The induction of the phosphorylated form of c-Jun was observed after 2 hours (Fig. 70B).

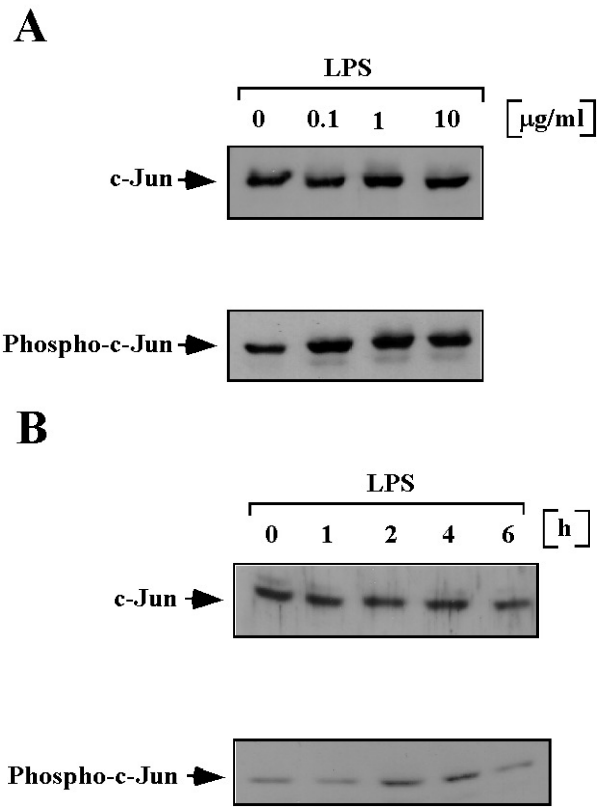


Fig. 70. LPS induces phosphorylation of c-Jun in BV-2 microglial cells.
The BV-2 cells were serum starved for twenty-four hours and then incubated with LPS (0.1, 1, 10 µg/ml) for 3 h (A) or incubated with LPS (0.1 µg/ml) for 1, 2, 4 and 6 hours (B). Nuclear extracts were prepared and then analyzed by Western blotting using specific antibodies against c-Jun and Phospho-c-Jun.

C.7.1.2. The constitutively active CREB mutant C2/CREB stimulates iNOS expression in microglial cells

It has been reported that cAMP-elevating agents such as forskolin and dibutyryl cAMP slightly increased nitrate production and the iNOS mRNA and protein contents and that these

agents markedly enhanced the cytokine-induced expression of iNOS in vascular smooth muscle cells (Koide et al., 1993). Additionally, it has been shown that the iNOS promoter contains several regulatory elements including NF-κB, C/EBP, AP-1 and CRE (Lowenstein et al., 1993; Xie et al., 1993; Keinanen et al., 1999; Taylor and Geller, 2000). To test whether CREB induces iNOS expression in microglial cells, the cells were infected with lentiviruses expressing the constitutively active mutant C2/CREB and then Western blot analysis was performed. The data show that C2/CREB is able to induce iNOS expression in BV-2 cells (Fig. 71).

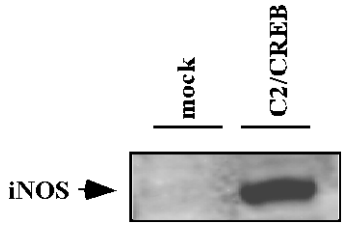


Fig. 71. C2/CREB induces iNOS expression in BV-2 cells.
Lentiviral gene transfer of C2/CREB. Viruses were pseudotyped with the vesicular stomatitis virus and used to infect BV-2 cells. As a control mock-infected cells were analyzed. Whole cell extracts were prepared and then analyzed by Western blotting using an antibody against iNOS. The infection was performed by G. Thiel.

C.7.2. Regulation of the immediate early genes *c-Jun* and *c-Fos* in gonadotrope cells

C.7.2.1. Buserelin, a gonadotropin-releasing hormone analogue, induces c-Jun and c-Fos expression in αT3M3 cells

The pituitary gonadotroph is the major site of action of gonadotropin-releasing hormone (GnRH). The activation of GnRH receptor in cultured pituitary cells and αT3-1 cell line has been shown to be associated with increased expression and levels of mRNA transcripts of *c-fos* and *c-jun* (Cesnjaj et al., 1994). Therefore, the regulation of c-Jun and c-Fos by GnRH in the gonadortope cell line αT3M3 was studied. These cells express the GnRH receptor as well as the M3 muscarinic acetylcholine receptor. The αT3M3 cells were stimulated with the GnRH analogue buserelin and Western blot analysis was performed. The results show that buserelin transiently induced the expression of c-Jun, Phospho-c-Jun and c-Fos. The induction was observed after 2 hours of incubating the cells with buserelin (Fig. 72).

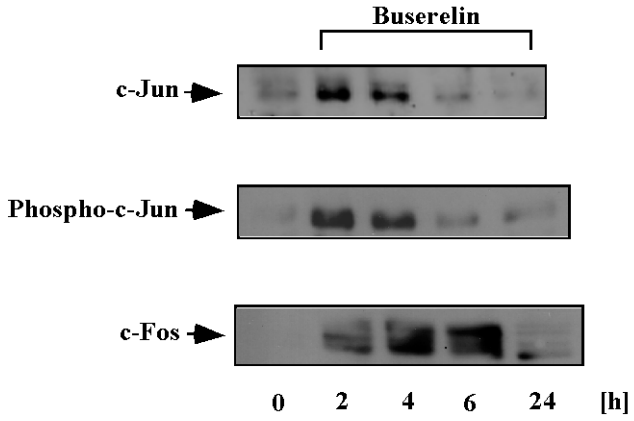


Fig. 72. Buserelin induces the levels of c-Jun, Phospho-c-Jun and c-Fos proteins in αT3M3 cells. αT3M3 cells were serum starved for twenty-four hours and then incubated with buserelin (10 nM) for different times (0, 2, 4, 6, 24 h) as indicated. Nuclear extracts were prepared and then analyzed by Western blotting using antibodies directed against c-Jun, Phospho-c-Jun or c-Fos.

C.7.2.2. Carbachol induces c-Jun and c-Fos expression in αT3M3 cells

It has been shown that the muscarinic acetylcholine receptor agonist carbachol induced c-Jun and c-Fos mRNA in a glial cell line (1321N1) (Trejo and Brown, 1991), therefore, the effect of carbachol on c-Jun and c-Fos expression in αT3M3 cells was tested. The results show that carbachol transiently induced the expression of c-Jun, Phospho-c-Jun and c-Fos. The induction was observed after 2 hours of incubating the cells with carbachol (Fig. 73).

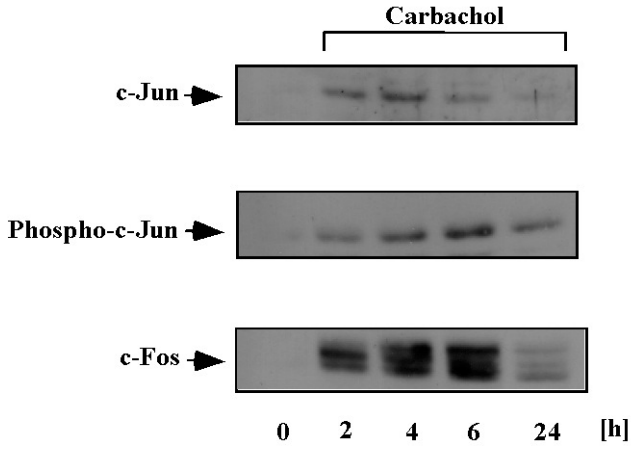


Fig. 73. Carbachol induces the levels of c-Jun, Phospho-c-Jun and c-Fos proteins in αT3M3 cells. αT3M3 cells were serum starved for twenty-four hours and then incubated with carbachol (1 mM) for different times (0, 2, 4, 6, 24 h) as indicated. Nuclear extracts were prepared and then analyzed by Western blotting using antibodies directed against c-Jun, Phospho-c-Jun or c-Fos.

C.7.2.3. Chelation of intracellular calcium reduced buserelin-stimulated both c-Jun and c-Fos expression

It has been reported that GnRH receptor occupancy is linked with an increase in intracellular calcium through mobilization of two distinct pools in both αT3-1 cells and rat pituitary gonadotrope (Horn et al., 1991; Stojilkovic et al., 1994; McArdle et al., 1996). To investigate the requirement of intracellular calcium mobilization in buserelin-stimulated c-Jun and c-Fos expression, the cell-permeable calcium chelator BAPTA-AM was used. Different concentrations of BAPTA-AM were used to eliminate intracellular calcium and the results show that BAPTA-AM inhibited buserelin-induced c-Jun, Phospho-c-Jun and c-Fos expression in a dose dependent manner. The expression of c-Jun and Phospho-c-Jun was inhibited by 15 μM of BAPTA-AM, while 25 μM of BAPTA-AM were needed to inhibit c-Fos expression (Fig. 74).

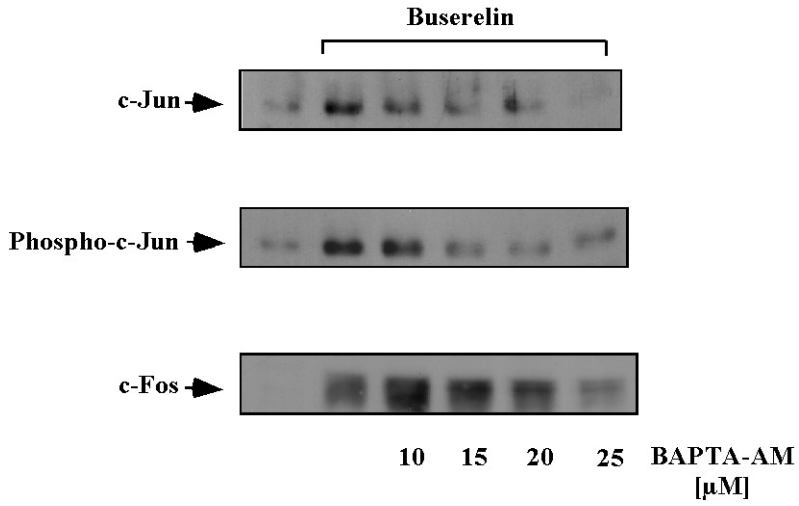


Fig. 74. BAPTA-AM inhibits buserelin-induced c-Jun, Phospho-c-Jun and c-Fos expression in αT3M3 cells.
αT3M3 cells were serum starved for twenty-four hours then pretreated for forty-five min with different concentrations of BAPTA-AM as indicated and then incubated with buserelin (10 nM) for 2 h. Nuclear extracts were prepared and then analyzed by Western blotting using c-Jun, Phospho-c-Jun or c-Fos specific anitbodies.

C.7.2.4. Calcium/calmodulin kinases mediate buserelin-induced c-Jun and c-Fos expression in αT3M3 cells

Because the intracellular effects of calcium are mediated by calcium/calmodulin-dependent protein kinases, the involvement of these kinases in the regulation of c-Jun and c-Fos expression in response to buserelin stimulation was tested. This was accomplished by using the Ca^{2+}/calmodulin-dependent protein kinases inhibitor KN-62. The results show that pretreatment of the αT3M3 cells with KN-62 impaired buserelin-induced c-Jun, Phospho-c-Jun and c-Fos expression (Fig. 75).

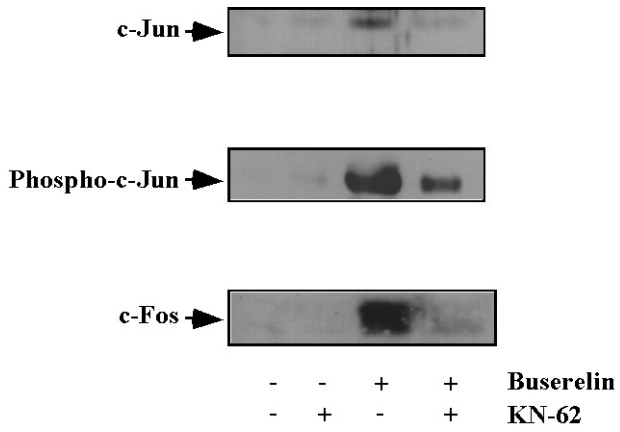


Fig. 75. KN-62 inhibits buserelin-induced c-Jun, Phospho-c-Jun and c-Fos expression in αT3M3 cells.
αT3M3 cells were serum starved for twenty-four hours then pretreated for forty-five min with KN-62 (10 μM) and then incubated with buserelin (10 nM) for 2 h. Nuclear extracts were prepared and then analyzed by Western blotting using c-Jun, Phospho-c-Jun or c-Fos specific antibodies.

C.7.2.5. Calcium/calmodulin kinases marginally influence carbachol-induced c-Jun and c-Fos expression in αT3M3 cells

The involvement of calcium/calmodulin-dependent protein kinases in the regulation of c-Jun and c-Fos expression in response to carbachol stimulation was also tested by using Ca^{2+}/calmodulin-dependent protein kinases inhibitor KN-62. The results show that pretreatment of the αT3M3 cells with KN-62 only marginally reduced carbachol-induced c-Jun, Phospho-c-Jun and c-Fos expression (Fig. 76).

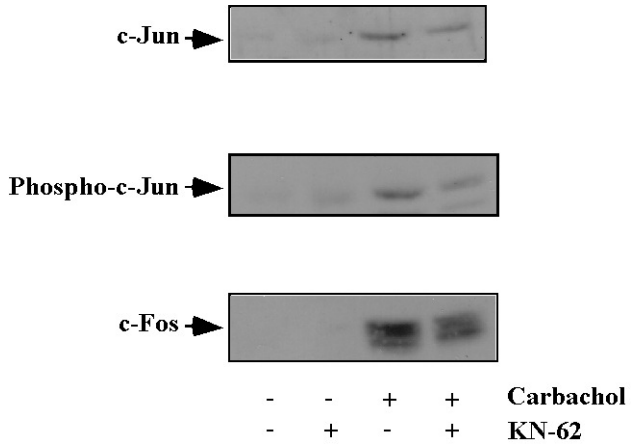


Fig. 76. KN-62 only marginally impaired carbachol-induced c-Jun, Phospho-c-Jun and c-Fos expression in αT3M3 cells.
αT3M3 cells were serum starved for twenty-four hours then pretreated for forty-five min with KN-62 (10 μM) and then incubated with carbachol (1 mM) for 3 h. Nuclear extracts were prepared and then analyzed by Western blotting using c-Jun, Phospho-c-Jun or c-Fos specific antibodies.

C.7.2.6. Buserelin- and carbachol-induced c-Jun and c-Fos expression require the activation of ERK

It has been shown that GnRHa, a GnRH agonist, activates ERK in pituitary organ culture (Mitchell et al., 1994) and the αT3-1 gonadotrope cell line (Sundaresan et al., 1996; Reiss et al., 1997). To evaluate whether buserelin and carbachol stimulate c-Jun and c-Fos expression via activation of ERK, αT3M3 cells were preincubated for 6 h with the MAPKK inhibitor PD98059. This compound inhibits the phosphorylation of the MAPKK, thus blocking the activation of ERK (Dudley et al., 1995). The results show that PD98059 blocked buserelin- and carbachol-induced c-Jun, Phospho-c-Jun and c-Fos expression in αT3M3 cells (Fig. 77A, B).

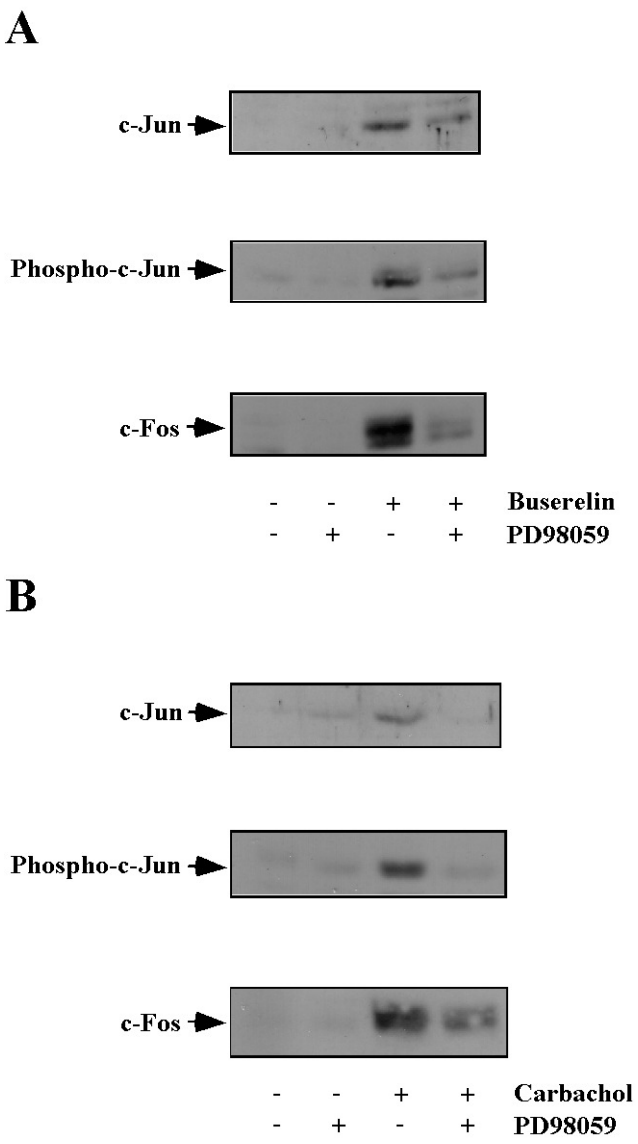


Fig. 77. PD98059 impairs buserelin- and carbachol-induced c-Jun, Phospho-c-Jun and c-Fos expression in αT3M3 cells.
αT3M3 cells were serum starved for twenty-four hours then pretreated for 6 h with PD98059 (50 μM) and then incubated with either buserelin (10 nM) for 2 h (A) or carbachol (1 mM) for 3 h (B). Nuclear extracts were prepared and then analyzed by Western blotting using c-Jun, Phospho-c-Jun or c-Fos specific antibodies.

D. Discussion

D.1. CREB-mediated gene transcription

Transcription of a number of cellular genes is activated by the second messenger cAMP that acts through specific protein kinases. Induction of cellular genes by cAMP requires first, the presence of a consensus cAMP response element (CRE) (Comb et al., 1986; Montminy et al., 1986; Delegeane et al., 1987; Hurst and Jones, 1987; Hardy and Shenk, 1988; Lin and Green, 1988; Sassone-Corsi, 1988; Sassone-Corsi et al., 1988b) and second, the catalytic subunit of PKA (Montminy and Bilezikjian, 1987; Riabowol et al., 1988; Mellon et al., 1989). PKA phosphorylates the bZIP transcription factor CREB that binds to CRE motifs and activates the transcription of these genes (Yamamoto et al., 1988; Jones and Jones, 1989). In this study several genes that are proposed to be regulated by CREB and/or other bZIP transcription factors were investigated, including GTP cyclohydrolase I (GTPCHI) gene, glucose-6-phosphatase (G6P) gene, TNFα gene, asparagine synthetase (AS) gene, secretogranin II (Sg II) gene, Egr-1 gene, c-Fos gene and tyrosine hydroxylase (TH) gene. As shown in Fig. 81, some of these genes have a canonical CRE within their regulatory regions, while others have a consensus sequence that resembles the CRE. The asparagine synthetase gene contains NSRE site, which has some similarity with the CRE.

T G A C G T C A	CRE consensus
T G A C G T C A	SgII CRE (-67/-60)
T G A C G T C A	TH CRE (-44/-37)
T G A C G **C G** A	GTPCHI CRE (-88/-81)
T **T** A C G T **A** A	G6P CRE1 (-161/152)
T **T G** C **A** T C A	G6P CRE2 (-136/-129)
T G A **G C** T C A	TNFα CRE (-106/-99)
T **C** A C G T C A	Egr-1 CRE (-138/-131)
T G A C G T **T T**	c-Fos CRE (-67/-60)
T G A **T** G **A A** A	AS NSRE (-68/-60)

Fig. 81. Sequence comparison of the CRE-like sequences in mammalian promoters.
Comparison of CRE-like sequences within different mammalian promoters. Different promoters are shown, SgII (accession # AF037451), TH (accession # X53503), GTPCHI (accession # BC025415), G6P (accession # BC020700), TNFα (accession # X02910), Egr-1 (accession # AJ245926), c-Fos (accession # X06769) and AS (accession # BC014621). The mismatches between the consensus CRE and the CRE-like sequences of the other promoters are shown in bold.

GTP cyclohydrolase I is the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin, which is an essential cofactor for the aromatic amino acid hydroxylases; phenylalanine, tyrosine and tryptophan hydroxylases (Whitworth, 1994) and also for nitric oxide synthases (Gross et al., 2000). The expression of GTP cyclohydrolase I is regulated at the transcriptional level by extracellular signaling molecules, including the proinflammatory cytokines interferon-γ (IFN-γ), interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα) (D'Sa et al., 1996; Plüss et al., 1996, 1997; Frank et al., 1998; Geller et al., 2000; Vann et al., 2000; Golderer et al., 2001). The transcription of the GTP cyclohydrolase I is stimulated by agents that elevate the intracellular levels of cAMP (Plüss et al., 1996, 1997; Hirayama et al., 2001), suggesting that CREB may be responsible for transactivation of the GTP cyclohydrolase I gene. Moreover, A CRE-like genetic element has been identified within the GTP cyclohydrolase I promoter (Kapatos et al., 2000). The bZIP transcription factors CREB, ATF2 and CREB2/ATF4 have been proposed to connect elevated intracellular cAMP concentrations with enhanced GTP cyclohydrolase I gene transcription (Kapatos et al., 2000; Hirayama et al., 2001). The transcriptional activation of a GTP cyclohydrolase I promoter/luciferase reporter gene was measured instead of *in vitro* transcription factor/DNA binding, because although DNA-binding is required for a subsequent transcriptional activation, enhanced binding activity of a transcription factor to DNA, monitored by an *in vitro* binding assay, does not

necessarily prove enhanced transcriptional activation potential of this protein. Overexpression of C2/CREB in HepG2 human hepatoma cells strongly activated the transcription of a GTP cyclohydrolase I promoter/reporter gene, containing an intact CRE-like motif. Furthermore, mutations within the CRE-like sequence significantly impaired transcriptional activation by C2/CREB. Although the CRE-like sequence is mutated, transcription of the GTP cyclohydrolase I reporter gene was still slightly induced. The slightly induced transcription might be due to the presence of a conserved family of coactivators of CREB termed TORCs (Transducers of Regulated CREB activity). These coactivators enhance CRE-dependent transcription via a phosphorylation-independent interaction with the bZIP DNA binding/dimerization domain of CREB (Conkright et al., 2003). Additionally, nanomolar concentrations of the expression vector encoding the modified catalytic subunit of PKA (NLSCα) strongly enhanced transcription of the GTP cyclohydrolase I promoter/luciferase reporter gene. Only 100 ng expression plasmid/plate were used, while usually 1-5 µg expression plasmid/plate were needed to affect gene transcription (Grewal et al., 2000; Streeper et al., 2000, 2001). Taken together, these data show that the GTP cyclohydrolase I gene is regulated by CREB and that the CRE-like motif within the regulatory region of the human GTP cyclohydrolase I gene functions as a *bona fide* CRE.

The TNFα gene promoter is known to contain a CRE-like site within its regulatory region and it has been suggested this CRE-like element is implicated in the regulation of TNFα gene expression (Leitman et al., 1999). Overexpression of the constitutively active mutant C2/CREB transactivated the TNFα reporter gene in HepG2 cells. Additionally, overexpression of the modified catalytic subunit of PKA (NLSCα) enhanced transcription of a TNFα reporter gene that contains two copies of the CRE/AP1 site as regulatory sequence, indicating that the TNFα gene is regulated by CREB in HepG2 cells.

The Egr-1 gene, encoding a zinc finger transcription factor, also contains a CRE-like site within its regulatory region (Tsai-Morris et al., 1988). CREB binding to the CRE is involved in the regulation of Egr-1 gene expression in response to certain stimuli (Lee et al., 1995; Rolli et al., 1999; Bernal-Mizrachi et al., 2000). Overexpression of C2/CREB transactivated the Egr-1 reporter gene in HepG2 cells.

Glucose-6-phosphatase catalyzes the final step of the metabolic pathways that are central to hepatic glucose production, i.e. gluconeogenesis and glycogen breakdown (Van Schaftingen and Gerin, 2002). Hepatic glucose-6-phosphatase gene expression is regulated by a variety of extracellular signaling molecules, including glucose, insulin, glucocorticoids and cAMP.

Conflicting results were published concerning the stimulation of glucose-6-phosphatase promoter activity by elevated intracellular levels of cAMP. It has been reported that the cell permeable analogue of cAMP, dibutyryl cAMP, did not significantly stimulate transcription of a glucose-6-phosphatase promoter/luciferase reporter gene in H4IIIE hepatoma cells (Schmoll et al., 1996), while a later report by the same group showed that dibutyryl cAMP stimulated glucose-6-phosphatase promoter activity on the order of 2-fold (Schmoll et al., 1999). It had been suggested that two distinct genetic elements are important for cAMP-induced glucose-6-phosphatase gene expression (Lin et al., 1997; Schmoll et al., 1999). The mutant C2/CREB transactivated the glucose-6-phosphatase reporter genes containing either CRE1 or CRE2 or both CREs and this activation is shown to be mediated via the two reported genetic elements: the sequence from –161 to –152 termed "CRE1" (Schmoll et al., 1999) and the sequence from –136 to –129 termed "CRE2"(Lin et al., 1997) of the human glucose-6-phosphatase gene promoter. These two genetic elements function as *bona fide* CREs. Streeper and his group demonstrated that PKA stimulated reporter gene transcription under control of CRE1, while CRE2 region contains an accessory factor binding site that likely enhances the effect of cAMP mediated through CRE1. Thus, it was suggested that in the glucose-6-phosphatse gene promoter only CRE1, but not CRE2 functions as a *bona fide* CRE. The authors used 5 µg of expression vector encoding the catalytic subunit of PKA. In the study reported here, a modified catalytic subunit of PKA was used. Transfection of nanomolar amounts of the expression vector encoding the modified catalytic subunit of PKA (NLSCα) strongly activated the transcription of reporter genes containing either CRE1 or CRE2 or both CREs. In contrast, transfection of nanomolar concentrations of an expression plasmid encoding the wild-type of the catalytic subunit of PKA did not show any effect on reporter gene transcription. These experiments involving overexpression of NLSCα confirm the previous finding that both CRE1 and CRE2 function as *bona fide* CREs. Activation of glucose-6-phosphatase promoter/luciferase reporter gene transcription following coexpression of NLSCα together with CREBS133A, a CREB mutant lacking the major PKA phosphorylation site, was also observed. Phosphorylation of the serine 133 residue of CREB is the predominant mechanism to enhance the transcriptional activation potential of CREB via recruitment of the coactivator CREB binding protein (CBP) and its paralogue p300 to the promoter. Accordingly, in coexpression studies of NLSCα together with CREBS133A instead of wild-type CREB a clear reduction of reporter gene transcription was observed. However, expression of CREBS133A is still contributed to reporter gene transcription in the presence of

NLSCα, suggesting that either other residues of CREBS133A or other promoter bound proteins involved in the regulation of CREB-mediated transcription are phosphorylated. Furthermore, PKA can directly phosphorylate CBP or other components of the transcriptional machinery downstream from CREB (Xu et al., 1998; Zanger et al., 1999). Nevertheless, here the experiments confirmed that both CRE-like sequences within the glucose-6-phosphatase gene function as genetic elements to mediate transactivation via CREB.

D.2. Role of ATF2 in regulating the transcription of CRE-containing reporter genes

ATF2 has been described to function as CRE-binding protein, connecting cellular stimulation with transcription of CRE-containing genes (Maekawa et al., 1989). ATF2 has been proposed to be involved in cAMP-enhanced GTP cyclohydrolase I gene transcription in human neuroblastoma cells (Hirayama et al., 2001). In HepG2 cells, overexpression of C2/ATF2 induced GTP cyclohydrolase I reporter gene transcription. Additionally, mutations within the CRE-like sequence of the GTP cyclohydrolase I gene significantly impaired transcriptional activation by C2/ATF2. These data indicate that the GTP cyclohydrolase I gene is a *bona fide* target of ATF2 and that the CRE-like site within the GTP cyclohydrolase I promoter is functional. Competition experiments between C2/CREB and dominant-negative bZIP mutants or C2/ATF2 and dominant-negative bZIP mutants revealed that CREB and ATF2 do not heterodimerize but they rather compete for binding to the CRE-like motif of the GTP cyclohydrolase I promoter. ATF2 contains at its N-terminus an activation domain. The transactivation capacity of the N-terminal domain of ATF2 can be enhanced through phosphorylation by the MAPK members p38 and JNK/SAPK (Davis, 2000; Chang and Karin, 2001; Kyriakis and Avruch, 2001). Overexpression of a constitutively active mutant of the MAPKK [MKK6(E)], together with the expression of the p38 MAPK, stimulated transcription of a GTP cyclohydrolase I promoter/luciferase reporter gene. It has been reported that p38 MAPK is constitutively active in the liver (Mendelson et al., 1996). However, here it is shown that the constitutively active protein kinase MKK6 was inactive towards the GTP cyclohydrolase I reporter gene, unless the concentration of the p38 protein kinase was experimentally elevated, suggesting that HepG2 hepatoma cells do not contain a constitutive p38 MAPK activity. Employment of dominant-negative bZIP mutants confirmed that ATF2 and c-Jun heterodimerize and that CREB, CREB2/ATF4 and C/EBP do not heterodimerize with ATF2.

The TNFα gene has been described to be activated by ATF2 (Tsai et al., 1996). Overexpression of C2/ATF2 in HepG2 cells induced the transcription of a TNFα reporter gene containing only the CRE/AP1 motif of the TNFα gene regulatory region. It has been suggested that in embryonic stem cells-derived cardiac myocytes the p38 MAPK positively regulates TNFα biosynthesis, while JNK represses TNFα gene expression (Minamino et al., 1999). In contrast, the JNK pathway has been shown to be responsible for TNFα production in mast cells (Ishizuka et al., 1997). Overexpression of a constitutively active mutant of the MAPKK [MKK6(E)], together with the expression of the p38 MAPK, stimulated TNFα promoter/luciferase reporter gene transcription. Additionally, a reporter gene was also activated that contains only the CRE/AP1 motif of the TNFα gene as regulatory sequence. Taken together, the activation of p38 MAPK and subsequently the activation of the transcription factor ATF2 are involved in the regulation of TNFα gene transcription in HepG2 cells.

Egr-1 gene transcription has been reported to be regulated by ATF2, using a cAMP response element within the proximal Egr-1 promoter, coupling a phosphorylated ATF2 with enhanced Egr-1 biosynthesis (Rolli et al., 1999). Overexpression of C2/ATF2 only weakly stimulated the Egr-1 promoter/luciferase reporter gene in HepG2 cells.

The genes encoding the granin proteins contain CRE motifs in their 5`-upstream regions. Conflicting results were published concerning cAMP-mediated transcriptional regulation of the secretogranin II gene. While the adenylyl cyclase activator forskolin increased secretogranin mRNA levels in chromaffin cells (Fischer-Colbrie et al., 1990), it caused a decrease in PC12 pheochromocytoma cells (Thompson et al., 1992, 1994). In CATH.a catecholaminergic cells, overexpression of C2/ATF2 transactivated the secretogranin II promoter/luciferase reporter genes that contain either one or four copies of CRE motif, suggesting that the secretogranin II gene is a target gene for ATF2. Competition experiments using dominant-negative bZIP mutants confirms the previous observation that CREB and ATF2 compete for the same DNA-binding site, the CRE, but they do not heterodimerize with each other.

The tyrosine hydroxylase promoter is known to contain a CRE motif within its regulatory region (Lazaroff et al., 1995; Tinti et al., 1997). It has been shown that in PC12 cells the transcription of the tyrosine hydroxylase is enhanced by activation of ATF2, which directly acts on the CRE in the tyrosine hydroxylase promoter (Suzuki et al., 2002). On the contrary, in CATH.a catecholaminerigc cells overexpression of the mutant C2/ATF2 did not affect the

tyrosine hydroxylase reporter gene. These data are compatible with *in vitro* DNA-protein binding and antibody supershift experiments showing that antibodies directed against ATF2 were unable to change the pattern of DNA-protein complexes bound to the CRE of the tyrosine hydroxylase gene (Lim et al., 2000). Additionally, the c-Fos gene promoter contains a CRE motif within its regulatory region (Berkowitz et al., 1989). Moreover, CREB and its closely related family members are shown to be general mediators of stimulus-dependent transcription of c-Fos gene (Ahn et al., 1998). Overexpression of the mutant C2/ATF2 only marginally activated a c-Fos promoter/luciferase reporter gene. Taken together, the data obtained by using C2/ATF2 lead to the conclusion that C2/ATF2 seems to select between different CRE elements.

D.3. c-Jun transactivates CRE-containing reporter genes

The bZIP transcription factors c-Jun and ATF2 are known to form heterodimers and bind to the consensus CRE within specific genes. Overexpression of C2/c-Jun transactivated GTP cyclohydrolase I promoter/luciferase reporter gene in HepG2 cells. Additionally, point mutations within the CRE-like sequence of the GTP cyclohydrolase I gene significantly impaired transcriptional activation mediated by C2/c-Jun. Taken together, these data suggest that the GTP cyclohydrolase I gene is a target gene for c-Jun and that the CRE-like motif present within the GTP cyclohydrolase I promoter is functional. Additionally, Overexpression of the mutant C2/c-Jun transactivated Egr-1 reporter gene. Competition experiments using dominant-negative bZIP mutants show that CREB and c-Jun do not heterodimerize but they compete for binding to the CRE element. The transcription factors c-Jun and ATF2 are activated by phosphorylating kinases such as JNK, which in turn, is controlled by MEKK1. Expression of a truncated form of MEKK1 (MEKK1Δ) was shown to induce the GTP cyclohydrolase I promoter activity. Dominant-negative mutants of c-Jun and ATF2 inhibited MEKK1Δ-induced reporter gene transcription, indicating that either c-Jun or ATF2 or heterodimers of these bZIP proteins are responsible for the transcriptional effect of MEKK1Δ expression.

It has been shown that a heterodimer consisting of c-Jun and ATF2 is essential to mediate stimulus-transcription coupling of the TNFα gene (Falvo et al., 2000). Moreover, the ATF2/c-Jun heterodimer binds to the CRE-element within the TNFα gene (Tsai et al., 1996).

Overexpression of C2/c-Jun transactivated the TNFα reporter gene that contains only the CRE/AP1 motif of the TNFα gene as regulatory sequence. Furthermore, overexpression of a truncated form of MEKK1 (MEKK1Δ) induced the TNFα promoter/luciferase reporter gene transcription in HepG2 cells. Additionally, a reporter gene that contains only the CRE/AP1 motif of the TNFα gene as regulatory sequence was also activated. Taken together, the activation of JNK and subsequently the activation of the transcription factors c-Jun and ATF2 are involved in the regulation of TNFα gene transcription in HepG2 cells.

D.4. CREB2/ATF4 regulates transcription of specific-reporter genes

The transcription factor CREB2 was originally described as a repressor of CREB and CRE-mediated transcription (Karpinski et al., 1992), however, no transcriptional repression domain could be found in the CREB2 molecule (Schoch et al., 2001). Rather, there are today good evidences that CREB2 functions as a transcriptional activator (Liang and Hai, 1997; He et al., 2001; Schoch et al., 2001; Luo et al., 2003; Thiel et al., 2005a). It has been shown by DNA/protein binding experiments that CREB2/ATF4 is recruited by the GTP cyclohydrolase CRE-like motif (Kapatos et al., 2000). Furthermore, overexpression of CREB2/ATF4 in PC12 cells transactivates the GTP cyclohydrolase promoter in the presence of the cAMP analogue 8-Br-cAMP (Kapatos et al., 2000). In HepG2 cells, expression of CREB2/ATF4 was not able to transactivate the GTP cyclohydrolase I reporter gene. Additionally, CREB2/ATF4 did not transactivate the TNFα or the Egr-1 promoters, which both contain a CRE-like sequence within their regulatory region. These three reporter genes are transactivated by the mutant C2/CREB. To explain these observations it is important to mention that CREB2/ATF4 and C2/CREB contain an identical transcriptional activation domain and differ only in their bZIP DNA-binding and dimerization domains. Thus, the differences between CREB2/ATF4 and C2/CREB in transactivating reporter genes containing CRE-like motifs can be attributed to their different DNA-binding abilities. In contrast, nanomolar concentrations of the expression vector encoding CREB2/ATF4 strongly induced transcription of asparagine synthetase promoter/luciferase reporter gene via the nutrient-sensing response unit, which has some similarities to the CRE sequence. Taken together, these data indicate that CREB2/ATF4 has different transactivation potential towards reporter genes containing CRE-like motifs.

D.5. CREB2/ATF4, ATF5 and CHOP but not CREB regulate asparagine synthetase gene transcription

Asparagine synthetase is expressed in most mammalian cells as a housekeeping enzyme responsible for the biosynthesis of asparagine from aspartate and glutamine (Andrulis et al., 1987). It has been shown that cells lacking sufficient asparagine synthetase activity, asparagine deprivation results in cell cycle arrest (Patterson and Maxwell, 1970; Greco et al., 1989) and induction of apoptosis (Story et al., 1993; Bussolati et al., 1995). The expression of asparagine synthetase activity has been shown to be enhanced by amino acid deprivation (Arfin et al., 1977), and this regulation is transcriptional in nature (Guerrini et al., 1993; Hutson et al., 1997). The asparagine synthetase promoter contains a nutrient-sensing response element that have some similarity with the CRE sequence, this element has been proposed to be responsible for the activation of asparagine synthetase gene transcription by amino acids deprivation (Barbosa-Tessmann et al., 2000; Kilberg and Barbosa-Tessmann, 2002). Additionally, the bZIP proteins C/EBPβ and ATF4 have been proposed to play a role in the transcriptional activation of the asparagine synthetase in response to nutrient deprivation (Siu et al., 2001, 2002). Depriving HepG2 cells of amino acids strongly induced asparagine synthetase biosynthesis. Furthermore, experiments involving the dominant-negative bZIP mutants showed that ATF4 and C/EBPα are the major regulators of amino acid deprivation-induced asparagine synthetase gene transcription, while CREB and c-Jun do not play any role in this process. It has been shown that ATF2 phosphorylation is necessary to induce *CHOP* expression by amino acid starvation (Averous et al., 2004). Dominant-negative mutant of ATF2 did not significantly affect the asparagine synthetase gene transcription induced by amino acid deprivation, suggesting that ATF2 does not play a prominent role in connecting amino acid deprivation with enhanced asparagine synthetase gene transcription. The bZIP transcription factor ATF5, which is closely related to CREB2/ATF4, is shown in this study to contain a constitutively active transcriptional activation domain. Overexpression of ATF5 induced asparagine synthetase gene transcription, suggesting that ATF5 is a positive regulator of amino acid deprivation-induced asparagine synthetase gene transcription. The C/EBP homologous protein (CHOP) has been demonstrated to act as a negative regulator of C/EBP proteins (Ron and Habener, 1992). Furthermore, CHOP is shown to downregulate CREB2/ATF4-dependent transcription (Gachon et al., 2001). In the study reported here CHOP blocked the upregulation of asparagine synthetase reporter gene transcription induced by either amino acid deprivation or overexpression of CREB2/ATF4 or ATF5, suggesting that

CHOP functions as a shut-off-device for amino acid deprivation-induced asparagine synthetase gene transcription. In contrast, CHOP failed to block the transcriptional activation of CRE-containing reporter genes induced by C2/CREB. Although additional details regarding interactions of different proteins and the definition of precise cascade of molecular events by which amino acids regulate gene expression remain to be established, the results shown here underscore the contribution of bZIP transcription family members to nutrient regulation of gene expression in mammalian cells.

D.6. Role of C/EBPα in the regulation of specific reporter genes

The C/EBPα molecule contains a Val^{296} residue, which is of main importance to restrict the interactions to C/EBP sites. C/EBPα is known to strongly bind to CRE sequences when Val^{296} is mutated to alanine (Miller et al., 2003). Competition experiments using the dominant-negative bZIP mutants revealed that CREB and C/EBPα recognize different genetic elements. The dominant-negative C/EBP mutant A-C/EBP did not block C2/CREB-induced transcription of the GTP cyclohydrolase I reporter gene. Rather, an activation of reporter gene transcription was observed, suggesting that A-C/EBP removes inhibitory constraints on CRE-regulated transcription units. These data are complemented by *in vitro* DNA/protein binding experiments showing that A-C/EBP did not compete with CREB bound to its cognate site (Greenwel et al., 2000; Menard et al., 2002). Furthermore, expression of C/EBPα and the constitutively active mutant C2/C/EBPα in HepG2 cells strongly induced C/EBP-responsive reporter gene, while the GTP cyclohydrolase I reporter gene was only slightly induced.
Additionally, overexpression of C/EBPα in CATH.a catecholaminergic cells only weakly induced the secretogranin II reporter gene transcription. The marginal stimulation in the transcription of both GTP cyclohydrolase I and secretogranin II reporter genes by C/EBPα argues against a functional role of C/EBPα protein in the control of CRE-containing genes.

As a conclusion the bZIP proteins CREB and ATF2 compete for binding to CRE sequences and do not heterodimerize. CREB2/ATF4, ATF5 and C/EBP bind to the NSRE sequence and do not heterodimerize with CREB or ATF2.

D.7. Expression of genes embedded in the chromatin in two different cell lines

In the first part of the thesis the transcriptional activation of specific reporter genes by bZIP proteins was investigated by performing transient transfection experiments. In the second part of the thesis the expression of endogenous genes was analysed: first, by studying the regulation of iNOS gene by bZIP proteins and second, by investigating the biosynthesis of the bZIP proteins c-Jun and c-Fos in response to extracellular stimuli.

D.7.1. Regulation of iNOS gene expression in BV-2 microglial cells

Inducible nitric oxide synthase (iNOS) was first identified in macrophages stimulated with IFN-γ and bacterial LPS (Lowenstein et al., 1992). It has been found that glial cells in the CNS produce NO in response to the induction of iNOS by bacterial LPS and a series of cytokines including IL-1β, TNFα and IFN-γ (Murphy et al., 1993; Feinstein, et al., 1994; Brosnan et al., 1997). Astrocytes in the healthy brain do not express iNOS, however, after ischemic, traumatic, neurotoxic or inflammatory damage the reactive astrocytes express iNOS in the mouse, rat and human (Galea at al., 1992; Hu et al., 1995; Pahan et al., 1997a, b, 1998). LPS is the ligand for Toll-like receptor 4 (TLR4), which is shown to be expressed in BV-2 microglial cells (Jung et al., 2005) and it has been reported that LPS induces iNOS expression in BV-2 cells (Watters et al., 2002). Moreover, microarray analysis shows that in LPS-stimulated BV-2 cells iNOS gene was upregulated on the order of 105.9 fold (Thomas et al., 2005). It has been reported that multiple signaling pathways are activated in macrophages upon LPS exposure (Denlinger et al., 1996). NF-κB has been shown to be a critical regulator of iNOS transcription by LPS (Xie et al., 1994). Furthermore, the MAPK cascades have been shown to signal the induction of iNOS in glial cells (Bhat et al., 1998, 1999). Depending on the cell type and the stimulation used, p38 MAPK have been shown to have either an upregulatory role (Chen et al., 1999; Chen and Wang, 1999; Bhat et al., 2002), down-regulatory role (Guan et al., 1997; Chan and Riches, 2001; Chan et al., 2001) or no role (Chan et al., 1999; Cho et al., 2002) in iNOS expression. Here the p38 MAPK specific inhibitor SB203580 is shown to inhibit LPS-induced iNOS expression in microglia. Furthermore, infection of the microglial cells with lentiviruses expressing the MKP-1, which is known to dephosphorylate p38 MAPK, also inhibited iNOS expression in response to LPS. Previous

studies have shown that JNK pathway contributes to the upregulation of iNOS expression (Guan et al., 1999; Hua et al., 2002; Lahti et al., 2003). A JNK specific inhibitor L-JNK1 inhibited LPS-induced iNOS expression in microglial cells. The activity of L-JNK1 have to be verified to confirm that JNK plays a role in LPS-induced iNOS expression, maybe by expressing the collagenase promoter, which is a target gene for c-Jun, and then inducing the cells with LPS and using L-JNK1 as an inhibitor. Furthermore, a dominant-inhibitory mutant of MKP-1, which is known to dephosphorylate JNK, also inhibited iNOS expression in response to LPS. The bZIP transcription factor ATF2 is phosphorylated and subsequently activated by both JNK and p38 MAPKs, while c-Jun is phosphorylated only via the JNK pathway, indicating that ATF2 and c-Jun could be involved in the signaling pathways leading to the activation of iNOS expression in response to LPS stimulation. Dominant-inhibitory mutant of ATF2 (A-ATF2) inhibited LPS-induced iNOS expression in microglia. Moreover, experiments employing antibodies directed against the phosphorylated forms of ATF2 or c-Jun show that the phosphorylated and active forms of ATF2 and c-Jun are induced in response to LPS stimulation, indicating that ATF2 and c-Jun are involved in the induction of iNOS in response to LPS stimulation. A schematic representation of the proposed signaling pathways implicated in LPS-induced iNOS expression in microglia is shown is Fig. 82.

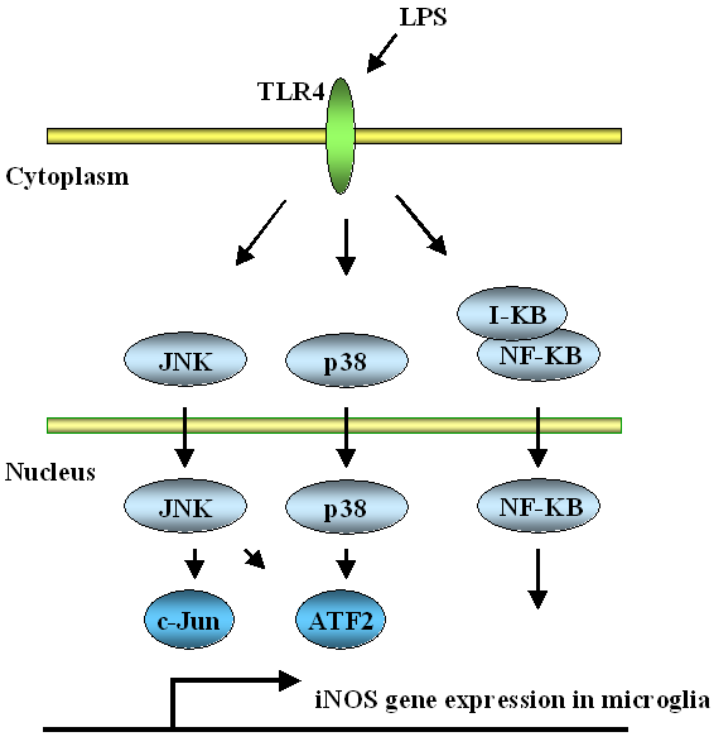


Fig. 82. Schematic representation of the proposed signaling pathways inducing iNOS expression in response to LPS in microglia.
LPS binds to TLR4 and activation of different signaling pathways, including NF-κB and the MAPKs p38 and JNK, leads to the activation of iNOS gene expression.

Additionally, it has been reported that cAMP either enhances or reduces iNOS expression depending on the cell type and the stimuli used. cAMP-elevating agents, including cAMP analogues, norepinephrine and prostaglandins, suppressed LPS-induced iNOS expression in rat microglia (Minghetti et al., 1997), kupffer cells (Mustafa and Olson, 1998) and hepatocytes (Smith et al., 1997). In contrast, cAMP induced iNOS expression in rat vascular smooth muscle cells and mesengial cells (Imai et al., 1994; Eberhardt et al., 1998). Infection of the BV-2 microglial cells with lentiviruses expressing the constitutively active CREB mutant C2/CREB induced iNOS expression in these cells. The adenylyl cyclase activator forskolin induced iNOS expression in BV-2 microglial cells. This experiment was unfortunately not reproducible more than two times, the reason is unclear but further investigations are required to clarify the involvement of CREB in iNOS expression. Taken together, these data indicate that the bZIP proteins CREB, ATF2 and c-Jun belong to the key regulators of iNOS gene expression in microglial cells.

D.7.2. Expression of c-Jun and c-Fos in αT3M3 gonadotrope cells

Both the biosynthesis and the secretion of the gonadotropins are under the regulation of the gonadotropin-releasing hormone (GnRH). When GnRH binds to its seven-transmembrane receptor (Tsutsumi et al., 1992), it induces interaction of the receptor with the heterodimeric Gq protein, which leads to the activation of phospholipase C and formation of inositol 1,4,5-triphosphate and diacylglycerol, leading to elevation of intracellular Ca^{2+} and activation of protein kinase C (Huckle and Conn, 1988; Horn et al., 1991; Reinhart et al., 1992). It has been shown that activation of GnRH receptor in cultured pituitary cells and αT3-1 cell line is associated with increased expression and levels of mRNA of the transcription factors *c-fos* and *c-jun* (Cesnjaj et al., 1994). The GnRH analogue buserelin and the muscarinic acetylcholine receptor agonist carbachol induced the expression of c-Jun and c-Fos in αT3M3 cells. It has been reported that Ca^{2+} is a critical mediator of the induction of gonadotropin secretion by GnRH (Chang et al., 1988; Huckle and Conn, 1988; Naor et al., 1988). Elimination of intracellular calcium by treating the αT3M3 cells with the cell-permeable intracellular calcium chelator BAPTA-AM inhibited buserelin-induced c-Jun and c-Fos expression. Many effects of Ca^{2+} in cells are mediated by protein phosphorylations catalyzed by calcium/calmodulin-dependent protein kinases. The Ca^{2+}/calmodulin-dependent protein kinases inhibitor KN-62 was shown here to inhibit buserelin-induced c-Jun and c-Fos expression in αT3M3 cells, suggesting that the calcium/calmodulin-dependent signaling pathway may be implicated in the induction of c-Jun and c-Fos expression in gonadotrope cells. KN-62 slightly impaired carbachol-induced c-Jun and c-Fos expression in αT3M3 cells. In the gonadotrope-derived αT3-1 cell line, GnRH has been shown to activate multiple protein kinases including ERK (Roberson et al., 1995; Weck et al., 1998; Benard et al., 2001), JNK (Levi et al., 1998; Mulvaney and Roberson, 2000) and p38 MAPK (Roberson et al., 1999). The MAPKK (MEK) inhibitor PD98059 inhibited buserelin- and carbachol-induced c-Jun and c-Fos expression, suggesting that and the activation of ERK is required for the induction of c-Jun and c-Fos expression in αT3M3 cells. Further studies are required to investigate the role of other members of MAPK signaling pathways and the requirement of calcium signals in the regulation of the immediate early genes c-Jun and c-Fos in response to extracellular stimulation.

E. Summary

The basic region leucine zipper (bZIP) transcription factors are proteins that bind as dimers to specific sequences within promoter genes to activate or repress the transcription of these genes. The best known member of the bZIP family is the cAMP response element (CRE) binding protein CREB that binds to the sequence 5`-TGACGTCA-3´ termed CRE. To investigate gene regulation by bZIP transcription factors constitutively active and dominant-negative bZIP mutants were used. The results show that genes encoding GTP cyclohydrolase I, secretogranin II, tumor necrosis factor α (TNFα) and Egr-1 are regulated by CREB and ATF2 via CRE motifs or related sequences. CREB and ATF2 do not heterodimerize but they rather compete for DNA-binding. In contrast, the bZIP proteins CREB2/ATF4, ATF5 and C/EBP heterodimerize with each other but not with CREB or ATF2. CREB2/ATF4, ATF5 and C/EBP transactivate the asparagine synthetase gene but has no or marginal impact on CRE-controlled genes. Furthermore, CREB2/ATF4, ATF5 and C/EBP mediate the activation of the asparagine synthetase gene transcription induced by amino acid deprivation.

In a second series of experiments the regulation of the inducible nitric oxide synthase (iNOS) has been investigated as a result of Toll-like receptor 4 stimulation. The results show that stress-activated protein kinases and the bZIP proteins ATF2 and/or c-Jun are involved in the upregulation of iNOS gene following stimulation.

Finally, the regulation of the c-Jun and c-Fos encoding genes has been investigated in pituitary cells. Stimulation of the gonadotropin-releasing hormone receptor or the muscarinic acetylcholine receptor type III stimulated c-Jun and c-Fos expression indicating that bZIP transcription factor synthesis is controlled by extracellular signals.

F. References

Agre, P., Johnson, P. F. and McKnight, S. L., Cognate DNA binding specificity retained after leucine zipper exchange between HCN4 and C/EBP, *Science*, **1989**, 246, 922-926.

Ahn, S., Olive, M., Aggarwal, S., Krylov, D., Ginty, D. D. and Vinson, C., A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of *c-fos*, *Mol. Cell. Biol.*, **1998**, 18, 967-977.

Andrews, N. C. and Faller, D. V., A micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells, *Nucleic Acid Res.*, **1991**, 19, 249.

Andrulis, I. L., Chen, J. and Ray, P. N., Isolation of human cDNAs for asparagine synthetase and expression in Jensen rat sarcoma cells, *Mol. Cell. Biol.*, **1987**, 7, 2435-2443.

Angel, P. and Karin, M., The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation, *Biochim. Biophys. Acta.*, **1991**, 1072, 129-157.

Angelastro, J. M., Mason, J. L., Ignatova, T. N., Kukekov, V. G., Stengren, G. B., Goldman, J. E. and Greene, L. A., Downregulation of activating transcription factor 5 is required for differentiation of neural progenitor cells into astrocytes, *J Neurosci.*, **2005**, 25, 3889-3899.

Antonson, P. and Xanthopoulos, K. G., Molecular cloning, sequence, and expression of the human gene encoding CCAAT/enhancer binding protein α (C/EBPα), *Biochem. Biophys. Res. Commun.*, **1995**, 215, 106-113.

Arfin, S. M., Simpson, D. R., Chiang, C. S., Andrulis, I. L. and Hatfield, G. W., A role for asparaginyl-tRNA in the regulation of asparagine synthetase in a mammalian cell line, *Proc. Natl. Acad. Sci. USA*, **1977**, 74, 2367-2369.

Averous, J., Bruhat, A., Jousse, C., Carraro, V., Thiel, G. and Fafournoux, P., Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation, *J. Biol. Chem.*, **2004**, 279, 5288-5297.

Aznar, S., Tonder, N., Bele, S., Kiessling, M., Zimmer, J. and Gass, P., c-JUN, KROX-24, and c-FOS expression in hippocampal grafts placed in excitotoxic hippocampal lesions of the rat, *Exp Neurol.*, **1995**, 136, 205-211.

Bakker, O. and Parker, M. G., CAAT/enhancer binding protein is able to bind to ATF/CRE elements, *Nucl. Acids Res.*, **1991**, 19, 1213-1217.

Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Siu, F., Schuster, S. M., Nick, H. S. and Kilberg, M. S., Activation of the human asparagine synthetase gene by amino acid response and the endoplasmic reticulum stress response pathways occurs by common genomic elements, *J. Biol. Chem.*, **2000**, 275, 26976-26985.

Barone, M. V., Crozat, A. Y., Tabaee, A., Philipson, L. and Ron, D., CHOP (GADD153) and its oncogenic variant, TLS-CHOP, differ in their ability to induce G1/S arrest, *Genes Dev.*, **1994**, 8, 453-464.

Barron, K. D., The microglial cell. A historical review, *J Neurol Sci.*, **1995**, 134, 57-68.

Batchvarova, N., Wang, X. Z. and Ron, D., Inhibition of adipogenesis by the stress-induced protein CHOP (gadd153), *EMBO J.*, **1995**, 14, 4654-4661.

Bedogni, B., Pani, G., Colavitti, R., Riccio, A., Borrello, S., Murphy, M., Smith, R., Eboli, M. L. and Galeotti, T., Redox regulation of cAMP-responsive element-binding protein and induction of manganous superoxide dismutase in nerve growth factor-dependent cell survival, *J. Biol. Chem.*, **2003**, 278, 16510-16519.

Behrens, A., Sibilia, M. and Wagner, E. F., Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation, *Nat. Genet.*, **1999**, 21, 326-329.

Benard, O., Naor, Z. and Seger, R., Role of dynamin, Src, and Ras in the protein kinase C-mediated activation of ERK by gonadotropin-releasing hormone, *J. Biol. Chem.*, **2001**, 276, 4554-4563.

Benbrook, D. M. and Jones, N. C., Different binding specificities and transactivation of variant CRE´s by CREB complexes, *Nucl. Acids Res.*, **1994**, 22, 1463-1469

Berkowitz, L. A., Riabowal, K. T. and Gilman, M. Z., Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse *c-fos* promoter, *Mol. Cell. Biol.*, **1989**, 9, 4272-4281.

Bernal-Mizrachi, E., Wice, B., Inoue, H. and Permutt, M. A., Activation of serum response factor in the depolarization induction of Egr-1 transcription in pancreatic islet β-cells, *J. Biol. Chem.*, **2000**, 275, 25681-25689.

Bhat, N. R., Zhang, P., Lee, J. C. and Hogan, E. L., Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-α gene expression in endotoxin-stimulated primary glial cultures, *J. Neurosci.*, **1998**, 18, 1633-1641.

Bhat, N. R., Zhang, P. and Bhat, A. N., Cytokine induction of inducible nitric oxide synthase in an oligodendrocyte cell line: role of p38 mitogen-activated protein kinase activation, *J. Neurochem.*, **1999**, 72, 472-478.

Bhat, N. R., Feinstein, D. L., Shen, Q. and Bhat, A. N., p38 MAPK-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells, *J. Biol. Chem.*, **2002**, 277, 29584-29592.

Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K. and Tjian, R., Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1, *Science*, **1987**, 238, 1386-1392.

Bonny, C., Oberson, A., Negri, S., Sauser, C. and Schorderet, D. F., Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death, *Diabetes*, **2001**, 50, 77-82.

Brenner, B., Koppenhoefer, U., Weinstock, C., LinderKamp, O., Lang, F. and Gulbins, E., Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153, *J. Biol. Chem.*, **1997**, 272, 22173-22181.

Brewer, J. W., Cleveland, J. L. and Hendershot, L. M., A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells, *EMBO J.*, **1997**, 16, 7207-7216.

Brosnan, C. F., Lee, S. C. and Liu, J., Regulation of inducible nitric oxide synthase expression in human glia: implications for inflammatory central nervous system diseases, *Biochem Soc. Trans*., **1997**, 25, 679-683.

Bruhat, A., Jousse, C., Wang, X. Z., Ron, D., Ferrara, M. and Fafournoux, P., Amino acid limitation induces expression of *CHOP*, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels, *J. Biol. Chem.*, **1997,** 272, 17588-17593.

Bussolati, O., Belletti, S., Uggeri, J., Gatti, R., Orlandini, G., Dall'Asta, V. and Gazzola, G. C., Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells, *Exp. Cell Res.*, **1995**, 220, 283-291.

Cao, Z., Umek, R. M. and McKnight, S. L., Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells, *Genes Dev.*, **1991**, 5, 1538-1552.

Carlson, S. G., Fawcett, T. W., Bartlett, J. D., Bernier, M. and Holbrook, N. J., Regulation of the C/EBP-regulated gene gadd153 by glucose deprivation, *Mol. Cell. Biol.*, **1993**, 13, 4736-4744.

Cesnjaj, M., Catt, K. J. and Stojilkovic, S. S., Coordinate activations of calcium and protein kinase-C in the expression of primary response genes in pituitary gonadotrophs, *Endocrinology*, **1994**, 135, 692-701.

Chan, E. D., Winston, B. W., Uh, S. T., Wynes, M. W., Rose, D. M. and Riches, D. W., Evaluation of the role of mitogen-activated protein kinases in the expression of inducible

nitric oxide synthase by IFN-gamma and TNF-alpha in mouse macrophages, *J Immunol.*, **1999**, 162, 415-422.

Chan, E. D., Morris, K. R., Belisle, J. T., Hill, P., Remigio, L. K., Brennan, P. J. and Riches, D. W., Induction of inducible nitric oxide synthase-NO by lipoarabinomannan of Mycobacterium tuberculosis is mediated by MEK1-ERK, MKK7-JNK, and NF-kappaB signaling pathways*, Infect Immun.*, **2001**, 69, 2001-2010.

Chan, E. D. and Riches, D. W., IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line, *Am J Physiol. Cell Physiol.*, **2001**, 280, C441-C450.

Chang, J. P., Stojilkovic, S. S., Graeter, J. S. and Catt, K. J., Gonadotropin-releasing hormone stimulates luteinizing hormone secretion by extracellular calcium-dependent and -independent mechanisms, *Endocrinology*, **1988**, 122, 87-97.

Chang, L. and Karin, M., Mammalian MAP kinase signalling cascades, *Nature*, **2001**, 410, 37-40.

Chen, C., Chen, Y. H. and Lin, W. W., Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages, *Immunology*, **1999**, 97, 124-129.

Chen, C. C. and Wang, J. K., p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages, *Mol. Pharmacol.*, **1999**, 55, 481-488.

Cho, M. K., Suh, S. H. and Kim, S. G., JunB/AP-1 and NF-kappa B-mediated induction of nitric oxide synthase by bovine type I collagen in serum-stimulated murine macrophages, *Nitric Oxide*, **2002**, 6, 319-332.

Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Freidman, A. D., Nakabeppu, Y., Kelly, T. J. and Lane, M. D., Differentiation-induced gene expression in

3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of twp adipocyte-specific genes, *Genes Dev.*, **1989**, 3, 1323-1335.

Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R. and Goodman, R., Phosphorylated CREB binds specifically to the nuclear protein CBP, *Nature*, **1993**, 365, 855-859.

Cibelli, G., Jüngling, S., Schoch, S., Gerdes, H. H. and Thiel. G., Identification of a functional cAMP response element in the secretogranin II gene, *Eur. J. Biochem.*, **1996**, 236, 171-179.

Clegg, C. H., Ran, W., Uhler, M. D. and McKnight, G. S., A mutation in the catalytic subunit of protein kinase A prevents myristylation but does not inhibit biological activity, *J. Biol. Chem.*, **1989**, 264, 20140–20146.

Clerk, A. and Sugden, P. H., Cell stress induced phosphorylation of ATF-2 and c-Jun transcription factors in rat ventricular myocytes, *Biochem. J.*, **1997**, 325, 801-810.

Comb, M., Birnberg, N. C., Seascholtz, A., Herbert, E. and Goodman, H. M., A cyclic-AMP- and phorbol ester-inducible DNA element, *Nature*, **1986**, 323, 353-356.

Conkright, M. D., Canettieri, G., Screaton, R., Guzman, E., Miraglia, L., Hogenesch, J. B. and Montminy, M., TORCs: Tranducers of Regulated CREB activity, *Mol. Cell.*, **2003**, 12, 413-423.

Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C., SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1, *FEBS Lett.*, **1995**, 364, 229-233.

Curran, T. and Franza, B. R. J., Fos and Jun: The AP-1 connection, *Cell*, **1988**, 55, 395-397.

D'Amico, M., Hulit, J., Amanatullah, D. F., Zafonte, B. T., Albanese, C., Bouzahzah, B., Fu, M., Augenlicht, L. H., Donehower, L. A., Takemaru, K., Moon, R. T., Davis, R., Lisanti, M. P., Shtutman, M., Zhurinsky, J., Ben-Ze'ev, A., Troussard, A. A., Dedhar, S. and Pestell, R. G., The integrin-linked kinase regulates the cyclin D1 gene through glycogen synthase kinase

3β and cAMP responsive element-binding protein dependent pathways, *J. Biol. Chem.*, **2000**, 275, 32649-32657.

Da Silva, J., Pierrat, B., Marry, J.-L. and Lesslauer, W., Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes, *J. Biol. Chem.*, **1997**, 272, 28373-28380.

Davis, R. J., Signal transduction by the JNK group of MAP kinases, *Cell*, **2000**, 103, 239-252.

Delegeane, A. M., Ferlands, L. H. and Mellon, P. L., Tissue-specific enhancer of the human glycoprotein hormone alpha-subunit gene: dependence on cyclic AMP-inducible elements, *Mol. Cell. Biol.*, **1987**, 7, 3994-4002.

Deng, T. and Karin, M., c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK, *Nature*, **1994**, 371, 171-175.

Denlinger, L. C., Fisette, P. L., Garis, K. A., Daugherty, S. K., Bertics, P. J. and Proctor, R. A., in *Novel Therapeutic Strategies in the Treatment of Sepsis* (Morrison, D. C. and Ryan, J. L., eds), Marcel Dekker, Inc., New York, **1996**, pp227-252.

Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R. J., JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain, *Cell*, **1994**, 76, 1025-1037.

De Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. and Bos, J. L., Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP, *Nature*, **1998**, 396, 474-477.

Desdouets, C., Matesic, G., Molina, C. A., Foulkes, N. S., Sassone-Corsi, P., Brechot, C. and Sobczak-Thepot, J., Cell cycle regulation of cyclin A gene expression by the cAMP-responsive transcription factors CREB and CREM, *Mol. Cell. Biol.*, **1995**, 15, 3301-3309.

Diehl, A. M., Johns, D. C., Yang, S., Lin, H., Yin, L. A., Matelis, L. A. and Lowrence, J. H., Adenovirus-mediated transfer of CCAAT/Enhancer binding protein alpha identifies a

dominant antiproliferative role for this isoform in hepatocytes, *J. Biol. Chem.*, **1996**, 271, 7343-7353.

Distel, R. J., Ro, H.-S., Rosen, B. S., Groves, D. L. and Spiegelman, B. M., Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: Direct participation of *c-fos*, *Cell*, **1987**, 49, 835-844.

Dixit, V. M., Marks, R. M., Sarma, V. and Prochownik, E. V., The antimitogenic action of tumor necrosis factor is associated with increase AP-1/*c-jun* proto-oncogene transcription, *J. Biol. Chem.*, **1989**, 264, 16905-16909.

D'Mello, S. R., Turzai, L. M., Gioio, A. E. and Kaplan, B. B., Isolation and structural characterization of the bovine tyrosine hydroxylase gene, *J Neurosci Res.*, **1989**, 23, 31-40.

Dony, C. and Gruss, P., Proto-oncogene c-fos expression in growth regions of fetal bone and mesodermal web tissue, *Nature*, **1987**, 328, 711-714.

Downey, J. S. and Han, J., Cellular activation mechanisms in septic shock, *Front Biosci.*, **1998**, 3, d468-476.

D'Sa, C., Hirayama, K., West, A., Hahn, M., Zhu, M. and Kapatos, G., Tetrahyrobiopterin biosynthesis in C6 glioma cells: induction of GTP cyclohydrolase I gene expression by lipopolysaccharide and cytokine treatment, *Mol Brain Res.*, **1996**, 41, 105-110.

Dudely, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R., A synthetic inhibitor of the mitogen-activated protein kinase cascade, *Proc. Natl. Acad. Sci. USA*, **1995**, 92, 7686-7689.

Eberhardt, W., Pluss, C., Hummel, R. and Pfeilschifter, J., Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1beta and cAMP in rat mesangial cells, *J Immunol.*, **1998**, 160, 4961-4969.

Estes, S. D., Stoler, D. L. and Anderson, G. R., Normal fibroblasts induce the C/EBPβ and ATF-4 bZIP transcription factors in response to anoxia, *Exp Cell Res.*, **1995**, 220, 47-54.

Estus, S., Zaks, W. J., Freeman, R. S., Gruda, M., Bravo, R. and Johnson, E. M., Jr. Altered gene expression in neurons during programmed cell death: identification of *c-jun* as necessary for neuronal apoptosis, *J. Cell. Biol.*, **1994**, 127, 1717-1727.

Falvo, J. V., Uglialoro, A. M., Brinkman, B. M. N., Merika, M., Parekh, B. S., Tsai, E. Y., Morielli, A. D., Peralta, E. G., Maniatis, T., Thanos, D. and Goldfeld, A. E., Stimulus-specific assembly of enhancer complexes on tumor necrosis-α gene promoter, *Mol. Cell. Biol.*, **2000**, 20, 2239-2247.

Fawcett, T. W., Martindale, J. L., Guyton, K. Z., Hai, T. and Holbrook, N. J., Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response, *Biochem J.*, **1999**, 339, 135-141.

Feinstein, D. L., Galea, E., Roberts, S., Berquist, H., Wang, H. and Reis, D. J., Induction of nitric oxide synthase in rat C6 glioma cells, *J. Neurochem.*, **1994**, 62, 315-321.

Ferreri, K., Gill, G. and Montminy, M., The cAMP regulated transcription factor CREB interacts with a component of the TFIID complex, *Proc. Natl Acad. Sci. USA*, **1994**, 91, 1210-1213.

Fischer-Colbrie, R., Gutierrez, J., Hsu, C.-M., Iacangelo, A. and Eiden, L. E., Sequence analysis, tissue distribution and regulation by cell depolarization, and second messengers of bovine secretogranin II (Chromogranin C) mRNA, *J. Biol. Chem.*, **1990**, 265, 9208-9213.

Flodby, P., Barlow, C., Kalefjord, H., Ahrlund-Richer, L. and Xanthopolous, K. G., Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/Enhancer binding protein, *J. Biol. Chem.,* **1996**, 271, 24753-24760.

Fornace, A., Alamo, I. and Hollander, M., DNA damage-inducible transcripts in mammalian cells, *Proc. Natl. Acad. Sci. USA*, **1988**, 85, 8800-8804.

Foster, J. D., Pederson, B. A. and Nordlie, R. C., Glusose-6-phosphatase structure, regulation, and function: an update, *Proc Soc Exp Biol Med.*, **1997**, 215, 314-332.

Frank, D. A. and Greenberg, M. E., CREB: a mediator of long-term memory from mollusks to mammals, *Cell*, **1994**, 79, 5-8.

Frank, S., Kolb, N., Werner, E. R. and Pfeilschifter, J., Coordinated induction of inducible nitric oxide synthase and GTP-cyclohydrolase I is dependent on inflammatory cytokines and interferon-γ in HaCaT keratinocytes: Implications for the model of cutaneous wound repair, *J Invest Dermatol.*, **1998**, 111, 1065-1071.

Freytag, S. O. and Geddes, T. J., Reciprocal regulation of adipogenesis by Myc and C/EBP alpha, *Science*, **1992**, 256, 379-382.

Friedman, A. D., Landschulz, W. H. and McKnight, S. L., CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells, *Genes Dev.*, **1989**, 3, 1314-1322.

Friedman, A. D. and McKnight, S. L., Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene, *Genes Dev.*, **1990**, 4, 1416-1426.

Gachon, F., Gaudray, G., Thebault, S., Basbous, J., Koffi, J. A., Devaux, C. and Mesnard, J., The cAMP response element binding protein-2 (CREB-2) can interact with the C/EBP-homologous protein (CHOP), *FEBS Lett.*, **2001**, 502, 57-62.

Galea, E., Feinstein, D. L. and Reis, D. J., Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures, *Proc. Natl. Acad. Sci. USA*, **1992**, 89, 10945-10949.

Geller, D. A., Di Silvio, M., Billiar, T. R. and Hatakeyama, K., GTP cyclohydrolase I is coinduced in hepatocytes stimulated to produce nitric oxide, *Biochem. Biophys. Res. Commun.*, **2000**, 276, 633-641.

Gerber, H. P., Georgiv, O., Harshman, K. and Schaffner, W., In vitro transcription complementation assay with mini extracts of transiently transfected COS-1 cell, *Nucleic Acid Res.*, **1992**, 20, 5855-5856.

Gius, D., Cao, X., Rauscher III, F. J., Cohen, D. R., Curran, T. and Sukhatme, V. P., Transcriptional activation and repression by Fos are independent functions: the C terminus represses immediate-early gene expression via CArG elements, *Mol. Cell. Biol.*, **1990**, 10, 4243-4255.

Golderer, G., Werner, E. R., Heufler, C., Strohmaier, W., Gröbner, P. and Werner-Felmayer, G., GTP cyclohydrolase I mRNA: novel splice variants in the slime mould *Physarum polycephalum* and in human monocytes (THP-1) indicate conservation of mRNA processing, *Biochem J.*, **2001**, 355, 499-507.

Gonzalez, G. A., Yamamoto, K. K., Fischer, W. H., Karr, D., Menzel, P., Biggs, W. W. W., Vale, W. W. and Montminy, M. R., A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence, *Nature*, **1989**, 337, 749-752.

Grayson, D. R., Costa, R. H., Xanthopoulos, K. G. and Darnell, J. E., One factor recognizes the liver-specific enhancers in alpha 1-antitrypsin and transthyretin genes, *Science*, **1988**, 239, 786-788.

Greco, A., Gong, S. S., Ittmann, M. and Basilico, C., Organization and expression of the cell cycle gene, ts11, that encodes asparagine synthetase, *Mol. Cell. Biol.*, **1989**, 9, 2350-2359.

Greenwel, P., Tanaka, S., Penkov, D., Zhang, W., Olive, M., Moll, J., Vinson, C., Di Liberto, M. and Ramirez, F., Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins, *Mol. Cell. Biol.*, **2000**, 20, 912-918.

Grewal, S. S., Fass, D. M., Yao, H., Ellig, C. L., Goodman, R. H. and Stork, P. J. S., Calcium and cAMP signals differentially regulate cAMP-responsive element-binding protein function via a Rap1 extracellular signal-regulated kinase pathway, *J. Biol. Chem.*, **2000**, 275, 34433-34441.

Groot, M., Boxer, L. M. and Thiel, G., Nerve growth factor- and epidermal growth factor-regulated gene transcription in PC12 pheochromocytoma and INS-1 insulinoma cells, *Eur. J. Cell Biol.*, **2000**, 79, 924-935.

Gross, S. S., Jones, C. L., Hattori, Y. and Raman, C. S., Tetrahydrobiopterin: An essential cofactor of nitric oxide synthase with an elusive role, *In: Lancaster JR, editor. Nitric oxide. Orlando: Academic Press.*, **2000**, pp 167-185.

Guan, Z., Baier, L. D. and Morrison, A. R., p38 mitogen-activated protein kinase downregulates nitric oxide and upregulates prostaglandin E_2 biosynthesis stimulated by interleukin-1β, *J. Biol. Chem.*, **1997**, 272, 8083-8089.

Guerrini, L., Gong, S. S., Mangasarian, K. And Basilico, C., *Cis*- and *trans*-acting elements involved in amino acid regulation of asparagine sythetase gene expression, *Mol. Cell. Biol.*, **1993**, 13, 3202-3212.

Gupta, S., Campbell, D., Derijard, B. and Davis, R. J., Transcription factor ATF2 regulation by the JNK signal transduction pathway, *Science*, **1995**, 267, 389-393.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. and Davis, R. J., Selective interaction of JNK protein kinase isoforms with transcription factors, *EMBO J.*, **1996**, 15, 2760-2770.

Guyton, K. Z., Xu, Q. and Holbrook, N. J., Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element, *Biochem J.*, **1996**, 314, 547-554.

Hai, T. and Curran, T., Cross-family dimerization of transcription factors fos/jun and ATF/CREB alters DNA binding specificity, *Proc. Natl. Acad. Sci. USA*, **1991**, 88, 3720-3724.

Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M. and Rubin, L. L., A *c-jun* dominant negative mutant protects sympathetic neurons against programmed cell death, *Neuron*, **1995**, 14, 927-939.

Han, J., Lee, J.-D., Jiang, Y., Li, Z., Feng, L. and Ulevitch, R. J., Characterization of the structure and function of a novel MAP kinase kinase (MKK6), *J. Biol. Chem.*, **1996**, 271, 2886-2891.

Hansen, M. B., Mitchelmore, C., Kjaerulff, K. M., Rasmussen, T. E., Pedersen, K. M. and Jensen, N. A., Mouse Atf5: Molecular cloning and two novel mRNAs, genomic organization, and odorant sensory neuron localization, *Genomics*, **2002**, 80, 344-350.

Hardy, S. and Shenk, T., Adenoviral control regions activated by E1A and the cAMP response element bind to the same factor, *Proc. Natl. Acad. Sci. USA*, **1988**, 85, 4171-4175.

Harootunian, A. T., Adams, S. R., Wen, W., Meinkoth, J. L., Taylor, S. S. and Tsien, R. Y., Movement of the free catalytic subunit of cAMP-dependent protein kinase into and out of the nucleus can be explained by diffusion, *Mol. Biol. Cell.*, **1993**, 4, 993-1002.

Hatakeyama, K., Harada, T., Suzuki, S., Watanabe, Y. and Kagamiyama, H., Purification and characterization of rat liver GTP cyclohydrolase I. Cooperative binding of GTP to the enzyme, *J. Biol. Chem.*, **1989**, 264, 21660-21664.

Hatakeyama, K., Inoue, Y., Harada, T. and Kagamiyama, H., Cloning and sequencing of cDNA encoding rat GTP cyclohydrolase I. The first enzyme of the tetrahydrobiopterin biosynthetic pathway, *J. Biol. Chem.*, **1991**, 266, 765-769.

He, C. H., Gong, P., Hu, B., Stewart, D., Choi, M. E., Choi, A. M. K. and Alam, J., Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation, *J. Biol. Chem.*, **2001**, 276, 20858-20865.

Hendricks-Taylor, L. R. and Darlington, G. J., Inhibition of cell proliferation by C/EBP occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen, *Nucleic Acids Res.*, **1995**, 23, 4726-4733.

Hettmann, T., Barton, K. and Leiden, J. M., Microphthalmia due to p53-mediated apoptosis of anterior lens epithelial cells in mice lacking the CREB-2 transcription factor, *Dev Biol.*, **2000**, 222, 110-123.

Hilberg, F., Aguzzi, A., Howells, N. and Wagner, E. F., *c-jun* is essential for normal mouse development and hepatogenesis, *Nature*, **1993**, 365, 179-181.

Hill, C. S. and Treisman, R., Transcriptional regulation by extracellular signals: mechanisms and specificity, *Cell*, **1995**, 80, 199-211.

Hirai, S. and Yaniv, M., Jun DNA-binding is modulated by mutations between the leucines or by direct interaction of fos with TGACTCA sequence, *New Biol.*, **1989**, 1, 181-191.

Hirayama, K., Shimooji, M., Swick, L., Meyer, A. and Kapatos, G., Characterization of GTP cyclohydrolase I gene expression in the human neuroblastoma SKN-BE(2)M17: enhanced transcription in response to cAMP is conferred by the proximal promoter, *J Neurochem.*, **2001**, 79, 576-587.

Hoffmeyer, A., Grosse-Wilde, A., Flory, E., Neufeld, B., Kunz, M., Rapp, U. R. and Ludwig, S., Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor α gene expression in T lymphocytes, *J. Biol. Chem.*, **1999**, 274, 4319-4327.

Holt, J. T., Gopal, T. V., Moulton, A. D. and Nienhuis, A. W., Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation, *Proc. Natl. Acad. Sci. USA*, **1986**, 83, 4794-4798.

Horn, F., Bilezikjian, L. M., Perrin, M. H., Bosma, M. M., Windle, J. J., Huber, K. S., Blount, A. L., Hille, B., Vale, W. and Mellon, P. L., Intracellular responses to gonadotropin-releasing hormone in a clonal cell line of the gonadotrope lineage, *Mol. Endocrinol.*, **1991**, 5, 347-355.

Hornbuckle, L. A., Edgerton, D. S., Ayala, J. E., Svitek, C. A., Oeser, J. K., Neal, D. W., Cardin, S., Cherrington, A. D. and O'Brien, R. M., Selective tonic inhibition of G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression by insulin in vivo, *Am. J. Physiol. Endocrinol. Metab.*, **2001**, 281, E713-E725.

Hu, B. R., Fux, C. M., Martone, M. E., Zivin, J. A. and Ellisman, M. H., Persistent phosphorylation of cyclic AMP responsive element-binding protein and activating transcription factor-2 transcription factors following transient cerebral ischemia in rat brain, *Neuroscience*, **1999**, 89, 437-452.

Hu, S. X., Sheng, W. S., Peterson, P. K. and Chao, C. C., Differential regulation by cytokines of human astrocyte nitric oxide production, *Glia*, **1995**, 15, 491-494.

Hua, L. L., Zhao, M. L., Cosenza, M., Kim, M. O., Huang, H., Tanowitz, H. B., Brosnan, C. F. and Lee, S. C., Role of mitogen-activated protein kinases in inducible nitric oxide synthase and TNFalpha expression in human fetal astocytes, *J Neuroimmunol.*, **2002**, 126, 180-189.

Huang, Z. M., Thewke, D., Gong, Q. Q., Schlichter, D. and Wicks, W. D., Functional recognition of the neuronal tyrosine hydroxylase cAMP regulatory element in different cell types, *Brain Res Mol Brain Res.*, **1991**, 11, 309-319.

Huckle, W. R. and Conn, P. M., Molecular mechanism of gonadotropin releasing hormone action. II. The effector system, *Endocrine Rev.*, **1988**, 9, 387-395.

Hurst, H. C. and Jones, N. C., Identification of factors that interact with the E1A-inducible adenovirus E3 promoter, *Genes Dev.*, **1987**, 1, 1132-1146.

Hurt, H. C., Transcription factors 1: bZIP proteins, *Protein profile.*, **1995**, 2, 101-168.

Hutson, R. G., Kitoh, T., Amador, D. A. M., Cosic, S., Schuster, S. M. and Kilberg, M. S., Amino acid control of asparagine synthetase gene: relation to asparaginase resistance in human leukemia cells, *Am. J. Physiol. Cell Physiol.*, **1997**, 272, C1691-C1699.

Huttner, W. B. and Natori, S., Helper proteins for neuroendocrine secretion, *Curr. Biol.,* **1995**, 5, 242-245.

Imai, T., Hirata, Y., Kanno, K. and Marumo, F., Induction of nitric oxide synthase by cyclic AMP in rat vascular smooth muscle cells, *J Clin Invest.*, **1994**, 93, 543-549.

Ionescu, A. M., Schwarz, E. M., Vinson, C., Puzas, J. E., Rosier, R., Reynolds, P. R. and O'Keefe, R. J., PTHrP modulates chondrocyte differentiation through AP-1 and CREB signaling, *J. Biol. Chem.*, **2001**, 276, 11639-11647.

Ishizuka, T., Terada, N., Gerwins, P., Hamelmann, E., Oshiba, A., Fanger, G. R., Johnson, J. L. and Gelfand, E. W., Mast cell tumor necrosis α production is regulated by MEK kinases, *Proc. Natl. Acad. Sci, USA*, **1997**, 94, 6358-6363.

Jean, D. and Bar-Eli, M., Regulation of tumor growth and metastasis of human melanoma by the CREB transcription factor family, *Mol. Cell. Biochem.*, **2000**, 212, 19-28.

Jean, D. and Bar-Eli, M., Targeting of ATF-1/CREB transcription factors by signaling chain Fv fragment in human melanoma: potential modality for cancer therapy, *Crit. Rev. Immunol.*, **2001**, 21, 275-286.

Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S. and Han, J., Characterization of the structure and function of a new mitogen-activated protein kinase (p38b), *J. Biol. Chem.*, **1996**, 271, 17920-17926.

Johnson, P. F., Landschulz, W. H., Graves, B. J. and McKnight, S. L., Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses, *Genes Dev.*, **1987**, 1, 133-146.

Johnson, R. S., Spiegelman, B. M. and Papaioaannou, V., Pleiotropic effects of a null mutation in the *c-fos* proto-oncogene, *Cell*, **1992**, 71, 577-586.

Johnson, R. S., Van Lingen, B., Papaioannou, V. E. and Spiegelmann, B. M., A null mutation at the *c-jun* locus causes embryonic lethality and retarded cell growth in culture, *Genes Dev.*, **1993**, 7, 1309-1317.

Jones, R. H. and Jones, N. C., Mammalian cAMP-responsive element can activate transcription in yeast and binds a yeast factor(s) that resembles the mammalian transcription factor ANF, *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 2176-2180.

Jooss, K. U., Funk, M. and Muller, R., An autonomous N-terminal transactivation domain in Fos protein plays a crucial role in transformation, *EMBO J.*, **1994**, 13, 1467-1475.

Jousse, C., Bruhat, A., Ferrara, M. and Fafournoux, P., Evidence of multiple signaling pathways in the regulation of gene expression by amino acids in human cell lines, *J Nutr.*, **2000**, 130, 1555-1560.

Jung, D. Y., Lee, H., Jung, B.-Y., Ock, J., Lee, M.-S., Lee, W.-H. and Suk, K., TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-β as a decision marker, *J Immunol.*, **2005**, 174, 6467-6476.

Jüngling, S., Cibelli, G., Czardybon, M., Gerdes, H. H. and Thiel, G., Differential regulation of chromogranin B and synapsin I gene promoter activity by cyclic AMP and cyclic AMP-dependent protein kinase, *Eur. J. Biochem.*, **1994**, 226, 925-935.

Kapatos, G., Stegenga, S. L. and Hirayama, K., Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat GTP cyclohydrolase I gene, *J. Biol. Chem.*, **2000**, 275, 5947-5957.

Karin, M., The regulation of AP-1 activity by mitogen-activated protein kinases, *J. Biol. Chem.*, **1995**, 270, 16483-16486.

Karin, M. and Hunter, T., Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus, *Curr Biol.*, **1995**, 5, 747-757.

Karpinski, B. A., Morle, G. D., Huggenvik, J., Uhler, M. D. and Leiden, J. M., Molecular cloning of human CREB-2: An ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element, *Proc. Natl. Acad. Sci. USA*, **1992**, 89, 4820-4824.

Kaufmann, K., Bach, K. and Thiel, G., Extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1, *Biol. Chem.*, **2001**, 382, 1077-1081.

Kawai, T., Matsumoto, M., Takeda, K., Sanjo, H. and Arika, S., ZIP kinase, a novel serine/threonine kinase which mediates apoptosis, *Mol. Cell. Biol.*, **1998**, 18, 1642-1651.

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. and Graybiel, A. M., A family of cAMP-binding proteins that directly activate Rap1, *Science*, **1998**, 282, 2275-2279.

Keinanen, R., Vartiainen, N. and Koistinaho, J., Molecular cloning and characterization of the rat inducible nitric oxide synthase (iNOS) gene, *Gene*, **1999**, 234, 297-305.

Kilberg, M. S. and Barbosa-Tessmann, I. P., Genomic sequences necessary for transcriptional activation by amino acid deprivation of mammalian cells, *J. Nutr.*, **2002**, 132, 1801-1804.

Klar, J., Sandner, P., Müller, M. W. H. and Kurtz, A., Cyclic AMP stimulates renin gene transcription in juxtaglomerular cell, *Pflügers Arch Eur J Physiol*, **2002**, 444, 335-344.

Koide, M., Kawahara, Y., Nakayama, I., Tsuda, T. and Yokoyama, M., Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells, *J. Biol. Chem.*, **1993**, 268, 24959-24966.

Kreutzberg, G. W., Microglia: a sensor for pathological events in the CNS, *Trends Neurosci.*, **1996**, 19, 312-318.

Kwok, R. P. S., Lundbald, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E., Creen, M. R. and Goodman, R. H., Nuclear protein CBP is a coactivator for the transcription factor CREB, *Nature*, **1994**, 370, 223-226.

Kyriakis, J. M. and Avruch, J., Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation, *Physiol Rev.*, **2001**, 81, 807-869.

Lahti, A., Jalonen, U., Kankaanranta, H. and Moilanen, E., c-Jun NH_2-terminal kinase inhibitor anthra(1,9-cd)pyrazol-6(2H)-one reduces inducible nitric-oxide synthase expression by destabilizing mRNA in activated macrophages, *Mol Pharmacol.*, **2003**, 64, 308-315.

Lamph, W. W., Wamsley, P., Sassone-Corsi, P. and Verma, I. M., Induction of proto-oncogene Jun/AP-1 by serum and TPA, *Nature (Lond.)*, **1988**, 334, 629-631.

Landschulz, W. H., Johnson, P. F. and McKnight, S. L., The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins, *Science*, **1988**, 240-1759-1764.

Langlois, W. J., Sasaoka, T., Saltiel, A. R. and Olefsky, J. M., Negative feedback regulation and desensitization of insulin- and epidermal growth factor-stimulated p21ras activation, *J. Biol. Chem.*, **1995**, 270, 25320-25323.

Lazaroff, M., Patankur, S., Yoon, S. O. and Chikaraishi, D. M., The cyclic AMP response element directs tyrosine hydroxylase expression in catecholaminergic central and peripheral nervous system cell lines from transgenic mice, *J. Biol. Chem.*, **1997**, 270, 21579-21589.

Lee, H. J., Mignacca, R. C. and Sakamoto, K. M., Transcriptional activation of egr-1 by granulocyte-macrophage colony-stimulating factor but not interleukin 3 requires phosphorylation of cAMP response element-binding protein (CREB) on serine 133, *J. Biol. Chem.*, **1995**, 270, 15979-15983.

Lee, R. J., Albanese, C., Stenger, R. J., Watanabe, G., Inghirami, G., Haines, G. K., Webster, M., Muller, W. J., Brugge J. S., Davis, R. J. and Pestell, R. G., pp60(v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and JNK kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60(v-src) signaling in breast cancer cells, *J. Biol. Chem.*, **1999**, 274, 7341-7350.

Leitman, D, C., Ribeiro, R. C. J., Mackow, E. R., Baxter, J. D. and West, B. L., Identification of a tumor necrosis factor-responsive element in the tumor necrosis factor alpha gene, *J. Biol. Chem.*, **1991**, 266, 9343-9346.

Lekstrom-Himes, J. and Xanthopoulos, K. J., Biological role of the CCAAT/enhancer-binding protein family of transcription factors, *J. Biol. Chem.,* **1998**, 273, 28545-28548.

Levi, N. L., Hanoch, T., Benard, O., Rozenblat, M., Harris, D., Reiss, N., Naor, Z. and Seger, R., Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary alpha T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42, *Mol. Endocrinol.*, **1998**, 12, 815-824.

Li, X.-Y. and Green, M. R., Intramolecular inhibition of activating transcription factor-2 function by its DNA-binding domain, *Genes Dev.*, **1996**, 10, 517-527.

Li, Y. and Van de Werve, G., Distinct hormone stimulation and counteraction by insulin of the expression of the two components of glucose 6-phosphatase in HepG2 cells, *Biochem. Biophys. Res. Commun.*, **2000**, 272, 41-44.

Liang, G. and Hai, T., Characterization of human activating transcription factor-4, a transcriptional activator that interacts with multiple domains of cAMP-responsive element-binding protein (CREB)-binding protein (CBP), *J. Biol. Chem.*, **1997**, 272, 24088-24095.

Lim, J., Yang, C., Hong, S. J. and Kim, K.-S., Regulation of tyrosine hydroxylase gene transcription by the cAMP-signaling pathway: Involvement of multiple transcription factors. *Mol. Cell. Biochem.*, **2000**, 212, 51-60.

Lin, A., Minden, A., Martinetto, H., Claret, F. X., Lange-Carter, C., Mercurio, F., Johnson, G. L. and Karin, M., Identification of a dual specificity kinase that activates the jun kinases and p38-Mpk2, *Science*, **1995**, 268, 286-290.

Lin, B., Morris, D. W. and Chou, J. Y., The role of HNF1alpha, HNF3gamma, and cyclic AMP in glucose-6-phosphatase gene activation, *Biochemistry*, **1997**, 36, 14096-14106.

Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J., $cPLA_2$ is phosphorylated and activated by MAP kinase, *Cell*, **1993**, 72, 269-278.

Lin, Y. S. and Green, M. R., Interaction of a common transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters, *Proc. Natl. Acad. Sci. USA*, **1988**, 85, 3396-3400.

Liu, Y., Gorospe, M., Yang, C. and Holbrook, N. J., Role of mitogen-activated protein tyrosine phosphatase during the cellular response to genotoxic stress, *J. Biol. Chem.*, **1995**, 270, 8377-8380.

Livingstone, C., Patel, G. and Jones, N., ATF-2 contains a phosphorylation-dependent transcriptional activation domain, *EMBO J.*, **1995**, 14, 1785-1797.

Long, F., Schipani, E., Asahara, H., Kronenberg, H. and Montminy, M., The CREB family of activators is required for endochondral bone development, *Development (Camb.)*, **2001**, 128, 541-550.

Lonze, B. E. and Ginty, D. D., Function and regulation of CREB family transcription factors in the nervous system, *Neuron*, **2002**, 35, 605-623.

Lowenstein, C. J., Glatt, C. S., Bredt, D. S. and Snyder, S. H., Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme, *Proc. Natl. Acad. Sci. USA*, **1992**, 89, 6711-6715.

Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W. and Murphy, W. J., Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide, *Proc. Natl. Acad. Sci. USA*, **1993**, 90, 9730-9734.

Luo, S., Baumeister, P., Yang, S., Abcouwer, S. F. and Lee, A. S., Induction of grp68/BiP by translational block. Activation of the Grp78 promoter by ATF4 through an upstream ATF/CRE site independent of the endoplasmic reticulum stress elements, *J Biol Chem.*, **2003**, 278, 37375-37385.

Luthman, H. and Magnusson, G. M., High efficiency polyoma DNA transfection of chloroquine treated cells, *Nucleic Acids Res.*, **1983**, 11, 1295-1308.

Mabuchi, T., Kitagawa, K., Kuwabara, K., Takasawa, K., Ohtsuki, T., Xia, Z., Storm, D., Yanagihara, T., Hori, M. and Matsumoto, M., Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate in vitro and ischemia in vivo, *J. Neurosci.*, **2001**, 21, 9204-9213.

MacDougald, O. A. and Lane, M. D., Transcriptional regulation of gene expression during adipocyte differentiation, *Annu. Rev. Biochem.*, **1995**, 64, 345-373.

Maekawa, T., Sakura, H., Kanei-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J.-I., Yoshida, M. and Ishii, S., Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in the brain, *EMBO J.*, **1989**, 8, 2023-2028.

Maekawa, T., Bernier, F., Sato, M., Nomura, S., Singh, M., Inoue, Y., Tokunaga, T., Imai, H., Yokoyama, M., Reimold, A., Glimcher, L. H. and Ishii, S., Mouse ATF-2 null mutants display features of a severe type of meconium aspiration syndrome, *J. Biol. Chem.*, **1999**, 274, 17813-17819.

Mai, F. C., Qiao, J., Tsygankova, O. M., Meinkoth, J. L., Quilliam, L. A. and Cheng, X., Differential signaling by cyclic AMP. Opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation, *J. Biol. Chem.*, **2002**, 277, 11497-11504.

Mayr, B. and Montminy, M. R., Transcriptional regulation by the phosphorylation-dependent factor CREB, *Nature Rev. Mol. Cell. Biol.*, **2001**, 2, 599-609.

McArdle, C. A., Forrest-Owen, W., Davidson, J. S., Fowkes, R., Bunting, R., Mason, W. T., Poch, A. and Kratzmeier, M., Ca^{2+} entry in gonadotrophs and alpha T3-1 cells: does store-dependent Ca^{2+} influx mediate gonadotrophin-releasing hormone action?, *J. Endocrinol.*, **1996**, 149, 155-169.

McBride, K. and Nemer, M., The C-terminal domain of c-fos is required for activation of an AP-1 site specific for jun-fos heterodimers, *Mol. Cell. Biol.*, **1998**, 18, 5073-5081.

Mellon, P. L., Clegg, C. H., Correll, L. A. and McKnight, G. S., Regulation of transcription by cyclic AMP-dependent protein kinase, *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 4887-4892.

Menard, C., Hein, P., Paquin, A., Savelson, A., Yang, X. M., Lederfein, D., Barnabe-Heider, F., Mir, A. A., Sterneck, E., Peterson, A. C., Johnson, P. F., Vinson, C. and Miller, F. D., An essential role for a MEKC/EBP pathway during growth factor-regulated cortical neurogenesis, *Neuron*, **2002**, 36, 597-610.

Mendelson, K. G., Contois, L.-R., Tevosian, S. G., Davis, R. J. and Paulson, K. E., Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver, *Proc. Natl. Acad. Sci. USA*, **1996**, 93, 12908-12913.

Merrill, J. E. and Benveniste, E. N., Cytokines in inflammatory brain lesions: helpful and harmful, *Trends Neurosci.*, **1996**, 19, 331-338.

Metz, R., Bannister, A. J., Sutherland, J. A., Hagemeier, C., O'Rourke, E. C., Cook, A., Bravo, R. and Kouzarides, T., c-Fos-induced activation of a TATA-box-containing promoter involves direct contact with TATA-box-binding protein, *Mol. Cell. Biol.*, **1994**, 14, 6021-6029.

Mielnicki, L. M., Hughes, R. G., Chevray, P. M. and Pruitt, S. C., Mutated Atf4 suppresses c-Ha-ras oncogene transcript levels and cellular transformation in NIH3T3 fibroblasts, *Biochem. Biophys. Res. Commun.*, **1996**, 228, 586-595.

Miller, M., Shuman, J. D., Sebastian, T., Dauter, Z. and Johnson, P. F., Structural basis for DNA recognition by the basic region leucine zipper transcription factor CCAAT/enhancer binding protein α, *J. Biol. Chem.*, **2003**, 278, 15178-15184.

Minamino, T., Yujiri, T., Papst, P. J., Chan, E. D., Johnson, G. L. and Terada, N., MEKK1 suppresses oxidative stress-induced apoptosis of embryonic stem cell-derived cardiac myocytes, *Proc. Natl. Acad. Sci. USA*, **1999**, 96, 15127-15132.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérijard, B., Davis, R. J., Johnson, G. L. and Karin, M., Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK, *Science*, **1994**, 266, 1719-1794.

Minghetti, L. Nicolini, A., Polazzi, E., Creminon, C., Maclouf, J. and Levi, G., Inducible nitric oxide synthase expression in activated rat microglial cultures is downregulated by exogenous prostaglandin E2 and by cyclooxygenase inhibitors, *Glia*, **1997**, 19, 152-160.

Mitchell, R., Sim, P. J., Leslie, T., Jojnson, M. S. and Thomson, F. J., Activation of MAP kinase associated with the priming effect of LHRH, *J. Endocrinol.*, **1994**, 140, R15-R18.

Mithieux, G., New knowledge regarding glucose-6-phosphatase gene and protein and their roles in the regulation of glucose metabolism, *Eur J Endocrinol.*, **1997**, 136, 137-145.

Montminy, M. R., Low, M. J., Tapia-Arancibia, L., Reichlin, S., Mandel, G. and Goodman, R. H., Cyclic AMP regulates somatostatin mRNA accumulation in primary diencephalic cultures and in transfected fibroblast cells, *J Neurosci.*, **1986**, 6, 1171-1176.

Montminy, M. R. and Bilezikjian, L. M., Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene, *Nature*, **1987**, 328, 175-178.

Morgan, J. I. and Curran, T., Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun, *Annu Rev Neurosci.*, **1991**, 14, 421-451.

Morooka, H., Bonventre, J. V., Pombo, C. M., Kyriakis, J. M. and Force, T., Ischemia and reperfusion enhance ATF-2 and c-Jun binding to cAMP response elements and to an AP-1 binding site from the c-jun promoter, *J. Biol. Chem.*, **1995**, 270, 30084-30092.

Muller, R. and Wagner, E. F., Differentiation of F9 teratocarcinoma stem cells after transfer of *c-fos* proto-oncogenes, *Nature*, **1984**, 311, 438-442.

Mulvaney, J. M. and Roberson, M. S., Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mitogen activated protein kinases by gonadotropin-releasing hormone, *J. Biol. Chem.*, **2000**, 275, 14182-14189.

Murphy, S., Simmons, M. L., Agullo, L., Garcia, A., Feinstein, D. L., Galea, E., Reis, D. J., Minc-Golomb, D. and Schwartz, J. P., Synthesis of nitric oxide in CNS glial cells, *Trends Neurosci.*, **1993**, 16, 323-328.

Mustafa, S. B. and Olson, M. S., Expression of nitric-oxide synthase in rat kupffer cells is regulated by cAMP, *J. Biol. Chem.*, **1998**, 273, 5073-5080.

Naor, Z., Capponi, A. M., Rossier, M. F., Ayalon, D. and Limore, R., Gonadotropin-releasing hormone-induced rise in cytosolic free Ca^{2+} levels: mobilization of cellular and extracellular Ca^{2+} pools and relationship to gonadotropin secretion, *Mol. Endocrinol.*, **1988**, 2, 512-520.

Nelson, D. L. and Cox, M. M., *Lehninger Principles of Biochemistry*, **2000**, Worth publishers.

Newman, J. R. S. and Keating, A. E., Comprehensive identification of human bZIP interactions with coiled-coil arrays, *Science*, **2003**, 300, 2097-2101.

Nishikura, K. and Murray, J. M., Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells, *Mol. Cell. Biol.*, **1987**, 7, 639-649

Nomura, N., Zu, Y.-L., Maekawa, T., Tabata, S., Akiyama, T. and Ishii, S., Isolation and characterization of a novel member of the gene family encoding the cAMP response element-binding protein CRE-BP1, *J. Biol. Chem.*, **1993**, 268, 4259-4266.

North, M. J., A practical approach, *In: Proteolytic Enzymes*. *IRL press Oxford*, **1969**, pp. 117-119.

Ofir, R., Dwarki, V. J., Rashid, D. and Verma, I. M., Phosphorylation of the C terminus of fos protein is required for transcriptional transrepression of the c-fos promoter. *Nature*, **1990**, 348, 80-82.

Pahan, K., Namboodiri, A. M. S., Sheikh, F. G., Smith, B. T. and Singh, I., Increasing cAMP attenuates induction of inducible nitric-oxide synthase in rat primary astrocytes, *J. Biol. Chem.*, **1997a**, 272, 7786-7791.

Pahan, K., Sheikh, F. G., Namboodiri, A. M. S. and Singh, I., Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages, *J. Clin. Invest*., **1997b**, 100, 2671-2679.

Pahan, K., Sheikh, F. G., Namboodiri, A. M. S. and Singh, I., N-acetyl cysteine inhibits induction of no production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes, *Free Radic. Biol. Med.*, **1998**, 24, 39-48.

Pang, L., Sawada, T., Decker, S. J. and Saltiel A. R., Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor, *J. Biol. Chem.*, **1995**, 270, 13585-13588.

Parker, D., Ferreri, K., Nakajima, T., LaMorte, V. J., Evans, R., Koerber, S. C., Hoeger, C. and Montminy, M. R., Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism, *Mol. Cell. Biol.*, **1996**, 16, 694-703.

Pati, D., Meistrich, M. L. and Plon, S. E., Human Cdc34 and Rad6B ubiquitin-conjugating enzymes target repressors of cyclic AMP-induced transcription for proteolysis, *Mol. Cell. Biol.*, **1999**, 19, 5001-5013.

Patterson, M. K. Jr., and Maxwell, M. D., Effects of L-asparagine deprivation on the cell cycle of the Jensen sarcoma, *Cancer Res.*, **1970**, 30, 1064-1067.

Pawson, T., Protein modules and signaling networks, *Nature*, **1995**, 373, 573-580.

Perry, V. H., Andersson, P. B. and Gordon, S., Macrophages and inflammation in the central nervous system, *Trends Neurosci.*, **1993**, 16, 268-273.

Persengiev, S. P., Devireddy, L. R. and Green, M. R., Inhibition of apoptosis by ATFx: a novel role for a member of the ATF/CREB family of mammalian bZIP transcription factors, *Genes Dev.*, **2002**, 16, 1806-1814.

Peters, C. S., Liang, X., Li, S., Kannan, S., Peng, Y., Taub, R. and Diamond, R. H., ATF-7, a novel bZIP protein, interacts with the PRL-1 protein-tyrosine phosphatase, *J. Biol. Chem.*, **2001**, 276, 13718-13726.

Petrij, F., Giles, R. H., Dauwerse, H. G., Saris, J. J., Hennekam, R. C., Masuno, M., Tommerup, N., Van Ommen, G. J., Goodman, R. H., Peters, D. J. M. and Breuning, M. H., Rubinstein-Taybi syndrome caused by mutations in the transcriptional coactivator CBP, *Nature*, **1995**, 376, 348-351.

Pinter, J. H., Janovick, J. A. and Conn, P. M., Gonadotropin-releasing hormone receptor concentration differentially regulates intracellular signaling pathways in GGH3 cells, *Pituitary*, **1999**, 2, 181-190.

Plüss, C., Werner, E. R., Blau, N., Wachter, H. and Pfeilschifter, J., Interleukin 1β and cAMP trigger the expression of GTP cyclohydrolase I in rat renal mesangial cells, *Biochem J.*, **1996**, 318, 665-671.

Plüss, C., Werner, E. R., Wachter, H. and Pfeilschifter, J., Differential effect of dexamethasone on interleukin 1β- and cyclic AMP-triggered expression on GTP cyclohydrolase I in rat renal mesanglial cells, *Brit. J. Pharmacol.*, **1997**, 122, 534-538.

Pugh, P. C. and Margiotta, J. F., Nicotinic acetylcholine receptor agonists promote survival and reduce apoptosis of chick ciliary ganglion neurons, *Mol Cell Neurosci.*, **2000**, 15, 113-122.

Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. and Woodgett, J. R., Phosphorylation of c-jun mediated by MAP kinases, *Nature*, **1991**, 353, 670-674.

Quinn, P. G., Distinct activation domains within cAMP response element-binding protein (CREB) mediate basal and cAMP-stimulated transcription, *J. Biol. Chem.*, **1993**, 268, 16999-17009.

Radhakrishnan, I., Perez-Alvarado, G. C., Parker, D., Dyson, H. J., Montminy, M. R. and Wright, P. E., Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions, *Cell*, **1997**, 91, 741-752.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J., Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine, *J. Biol. Chem.*, **1995**, 270, 7420-7426.

Ramji, D. P. and Foka, P., CCAAT/enhancer-binding proteins: structure, function and regulation, *Biochem J.*, **2002**, 365, 561-575.

Rauscher, F. J., Sambucetti, L. C., Curran, T., Distel, R. J. and Spiegelman, B. M., Common DNA-binding site for Fos protein complexes and transcription factor AP-1, *Cell*, **1988**, 52, 471-480.

Reimold, A. M., Grusby, M. J., Kosarars, B., Fries, J. W. U., Mori, R., Maniwa, S., Clauss, I. M., Collins, T., Sidman, R. L., Glimcher, M. J. and Glimcher, L. H., Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice, *Nature*, **1996**, 379, 262-265.

Reinhart, J., Mertz, L. M. and Catt, K. J., Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptors, *J. Biol. Chem.*, **1992**, 267, 21281-21284.

Reiss, N., Llevi, L. N., Shacham, S., Harris, D., Seger, R. and Naor, Z., Mechanism of mitogen-activated protein kinase activation by gonadotropin-releasing hormone in the pituitary of alphaT3-1 cell line: differential roles of calcium and protein kinase C, *Endocrinology*, **1997**, 138, 1673-1682.

Rhoades, K. L., Golub, S. H. and Economou, J. S., The regulation of the human tumor necrosis factor α promoter region in macrophages, T cell, and B cell lines, *J. Biol. Chem.*, **1992**, 267, 22102-22107.

Riabowol, K. T., Fink, J. S., Gilman, M. Z., Walsh, D. A., Goodman, R. H. and Feramisco, J. R., The catalytic subunit of cAMP-dependent protein kinase induces expression of genes containing cAMP-responsive enhancer elements, *Nature*, **1988**, 336, 83-86.

Riccio, A., Ahn, S., Davenprot, C. M., Blendy, J. A. and Ginty, D. D., Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons, *Science*, **1999**, 286, 2358-2361.

Roberson, M. S., Misra-Press, A., Laurance, M. E., Stork, P. J. S. and Maurer, R. A., A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone α-subunit promoter by gonadotropin-releasing hormone, *Mol. Cell. Biol.*, **1995**, 15, 3531-3539.

Roberson, M. S., Zhang, T., Li, H. and Mulvaney, J. M., Activation of the p38 mitogen-

activated protein kinase pathway by gonadotropin-releasing hormone, *Endocrinology*, **1999**, 140, 1310-1318.

Roesler, W. J., Vandenbark, G. R. and Hansen, R. W., Cyclic AMP and the induction of eukaryotic gene transcription, *J. Biol. Chem.*, **1988**, 263, 9063-9066.

Rolli, M., Kotlyarov, A., Sakamoto, K. M., Gaestel, M. and Neininger, A., Stress-induced stimulation of early growth gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase-2 independent manner, *J. Biol. Chem.*, **1999**, 274, 19559-19564.

Ron, D. and Habener, J. F., CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription, *Genes Dev.*, **1992**, 6, 439-453.

Rosa, P. and Gerdes, H. H., The granin protein family: Markers for neuroendocrine cells and tools for the diagnosis of neuroendocrine tumors, *J. Endocrinol. Invest.,* **1994**, 17, 207-225.

Rosenberg, D., Groussin, L., Bertagna, X. and Bertherat, J., cAMP pathway alterations from the cell surface to nucleus in adrenocortical tumors, *Endocri. Res.*, **2002a**, 28, 765-775.

Rosenberg, D., Groussin, L., Julian, E., Perlemoine, K., Bertagna, X. and Bertherat, J., Role of the PKA-regulated transcription factor CREB in development and tumorigenesis of endocrine tissues, *Ann. N. Y. Acad. Sci.*, **2002b**, 968, 65-74.

Rudolph, D., Tafuri, A., Gass, P., Hammerling, G. J., Arnold, B. and Schutz, G., Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein, *Proc. Natl. Acad. Sci. USA*, **1998**, 95, 4481-4486.

Ruther, U., Garber, C., Komitowski, D., Muller, R. and Wagner, E. F., Deregulated c-fos expression interferes with normal bone development in transgenic mice, *Nature*, **1987**, 325, 412-416.

Ryseck, R. P. and Bravo, R., c-JUN, JUN B, JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins, *Oncogene*, **1991**, 6, 533-542.

Sadowski, I., Bell, B., Broad, P. and Hollis, M., GAL4 fusion vectors for expression in yeast or mammalian cells, *Gene*, **1992**, 118, 137-141.

Saklatvala, J., Rawlinson, L., Waller, R. J., Sarsfield, S., Lee, J. C., Morton, L. F., Barnes, M. J. and Farndale, R. W., Role for p38 mitogen-activated protein kinase in platelet aggregation caused by collagen or a thromboxane analogue, *J. Biol Chem.*, **1996**, 271, 6586-6589.

Sassone-Corsi, P., Coupling gene expression to cAMP signaling: role of CREB and CREM, *Int. J. Biochem. Cell Biol.*, **1988**, 30, 27,38.

Sassone-Corsi, P., Sisson, J. C. and Verma, I. M., Transcriptional autoregulation of the proto-oncogene fos, *Nature*, **1988a**, 334, 314-319.

Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. L. and Verma, I. M., Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element, *Genes Dev.*, **1988b**, 2, 1529-1538.

Sassone-Corsi, P., Ransone, L. J. and Verma, I. M., Cross-talk in signal transduction: TPA-inducible factor Jun/AP-1 activates cAMP-responsive enhancer elements, *Oncogene*, **1990**, 5, 427-431.

Scammell, J. G., Granins- Marker of the regulated secretory pathway, *Trends Endocrinol. Metabil.*, **1993**, 4, 14-18.

Schlingensiepen, K.-H., Schlingensiepen, R., Kunst, M., Klinger, I., Gerdes, W., Seifert, W. and Brysch, W., Opposite functions of *jun*-B and *c-jun* in growth regulation and neuronal differentiation, *Dev. Genet.*, **1993**, 14, 305-312.

Schmoll, D., Allan, B. B. and Burchell, A., Cloning and sequencing of the 5´ region of the human glucose-6-phosphatase gene: transcriptional regulation by cAMP, insulin and glucocorticoids in H4IIE hepatoma cells, *FEBS Lett.*, **1996**, 383, 63-66.

Schmoll, D., Wasner, C., Hinds, C. J., Allan, B. B., Walther, R. and Burchell, A., Identification of a cAMP response element within the glucose-6-phosphatase hydrolytic subunit gene promoter which is involved in the transcriptional regulation by cAMP and glucocorticoids in H4IIE hepatoma cells, *Biochem J.,* **1999**, 338, 457-463.

Schoch, S., Cibelli, G., Magin, A., Steinmüller, L. and Thiel, G., Modular structure of cAMP response element binding protein 2 (CREB2), *Neurochem. Int.*, **2001**, 38, 601-608.

Schorpp-Kistner, M., Wang, Z. Q., Angel, P. and Wagner, E. F., JunB is essential for mammalian placentation, *EMBO J.*, **1999**, 18, 934-948.

Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Möhle-Steinlein, U., Tian, J., Karin, M., Angel, P. and Wagner, E. F., Control of cell cycle progression by c-Jun is p53-dependent, *Genes Dev.*, **1999**, 13, 607-619.

Silva, A. J., Kogan, J. H., Frankland, P. W. and Kida, S., CREB and memory, *Annu. Rev. Neurosci.*, **1998**, 21, 127-148.

Siu, F., Chen, C., Zhong, C. and Kilberg, M. S., CCAAT/enhancer-binding protein-beta is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene, *J. Biol. Chem.*, **2001**, 276, 48100-48107.

Siu, F., Bain, P. J., LeBlanc-Chaffin, R., Chen, H. and Kilberg, M. S., ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene, *J. Biol. Chem.*, **2002**, 277, 24120-24127.

Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M. and Karin, M., Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73, *Nature*, **1991**, 354, 494-496.

Smeyne, R. J., Schilling, K., Oberdick, J., Robertson, L., Luk, D., Curran, T. and Morgan, J. I., A fos-lac Z transgenic mouse that can be used for neuroanatomic mapping, *Adv Neurol.*, **1993**, 59, 285-291.

Smith, F. S., Ceppi, E. D. and Titheradge, M. A., Inhibition of cytokine-induced nitric oxide synthase expression by glucagon and cAMPin cultured hepatocytes, *Biochem J.*, **1997**, 326, 187-192.

Sommer, C., Gass, P. and Kiessling, M., Selective c-JUN expression in CA1 neurons of the gerbil hippocampus during and after acquisition of an ischemia-tolerant state, *Brain Pathol.*, **1995**, 5, 135-144.

Steinmüller, L. and Thiel, G., Regulation of gene transcription by a constitutively active mutant of activating transcription factor 2 (ATF2), *Biol. Chem.*, **2003**, 384, 667-672.

Stojilkovic, S. S., Reinhart, J. and Catt, K. J., Gonadotropin-releasing hormone receptors: structure and signal transduction pathways, *Endocri Rev.*, **1994**, 15, 462-499.

Story, M. D., Voehringer, D. W., Stephens, L. C. and Meyn, R. E., L-asparaginase kills lymphoma cells by apoptosis, *Cancer Chemother Pharmacol.*, **1993**, 32, 129-133.

Streeper, R. S., Svitek, C. A., Goldman, J. K. and O'Brien, R. M., Differential role of hepatocyte nuclear factor-1 in the regulation of glucose-6-phosphatase catalytic subunit gene transcription by cAMP in liver- and kidney-derived cell lines, *J. Biol. Chem.*, **2000**, 275, 12108-12118.

Streeper, R. S., Hornbuckle, L. A., Svitek, C. A., Goldman, J. K., Oeser, J. K. and O'Brien, R. M., Protein kinase A phosphorylates hepatocyte nuclear factor-6 and stimulates glucose-6-phosphatase catalytic subunit gene transcription, *J. Biol. Chem.*, **2001**, 276, 19111-19118.

Sun, H., Charles, C. H., Lau, L. F. and Tonks, N. K., MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo, *Cell*, **1993**, 75, 487-493.

Sun, H., Tonks, N. K. and Bar-Sagi, D., Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1, *Science*, **1994**, 266, 285-288.

Sun, P., Enslen, H., Myung, P. S. and Maurer, R. A., Differential activation of CREB by

Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity, *Genes Dev.*, **1994**, 8, 2527-2539.

Sundaresan, S., Colin, I. M., Pestell, R. G. and Jameson, J. L., Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone: evidence for the involvement of protein kinase C, *Endocrinology*, **1996**, 137, 304-311.

Sutherland, J. A., Cook, A., Bannister, A. J. and Kouzarides, T., Conserved motifs in Fos and Jun define a new class of activation domain, *Genes Dev.*, **1992**, 6, 1810-1819.

Suzuki, T., Yamakuni, T., Hagiwara, M. and Ichinose, H., Identification of ATF-2 as a transcriptional regulator for the tyrosine hydroxlase gene, *J. Biol. Chem.*, **2002**, 277, 40768-40774.

Takeda, J., Maekawa, T., Sudo, T., Seino, Y., Imura, H., Seito, N., Tanaka, C. and Ishii, S., Expression of the CRE-BP1 transcriptional regulator binding to the cyclic AMP response element in central nervous system, regenerating liver and human tumors, *Oncogene*, **1991**, 6, 1009-1014.

Tanaka, K., Nogawa, S., Nagata, E., Ito, D., Suzuki, S., Dembo, T., Kosakai, A. and Fukuuchi, Y., Persistent CREB phosphorylation with protection of hippocampal CA1 pyramidal neurons following temporary occlusion of the middle cerebral artery in the rat, *Exp. Neurol.*, **2000**, 161, 462-471.

Tanaka, T., Tsujimura, T., Takeda, K., Sugihara, A., Maekawa, A., Terada, N., Yoshida, N. and Akira, S., Targeted disruption of ATF4 discloses its essential role in the formation of eye lens fibres, *Genes Cells*, **1998**, 3, 801-810.

Taylor, B. S. and Geller, D. A., Molecular regulation of the human inducible nitric oxide synthase (iNOS) gene, *Shock*, **2000**, 13, 413-424.

Thiel, G. and Cibelli, G., Corticotropin-releasing factor and vasoactive intestinal polypeptide activate gene transcription through the cAMP signaling pathway in a catecholaminergic immortalized neuron, *Neurochem. Int.*, **1999**, 34, 183-191.

Thiel, G., Kaufmann, K., Magin, A., Lietz, M., Bach, K. and Cramer, M., The human transcriptional repressor protein NAB1: expression and biological activity, *Biochim Biophys Acta.*, **2000**, 1493, 289-301.

Thomas, D. M., Francescutti-Verbeem, D. M. and Kuhn, D. M., Gene expression profile of activated microglia under conditions associated with dopamine neuronal damage, *FASEB J.*, **2005**, 20, 515-517.

Thompson, M. E., Zimmer, W. E., Wear, L. B., MacMillan, L. A., Thompson, W. J., Huttner, W. B., Hidaka, H. and Scammell, J. G., Differential regulation of chromogranin B/secretogranin I and secretogranin II by forskolin in PC12 cells, *Mol. Brain Res.*, **1992**, 12, 195-202.

Thompson, M. E., Valentine, D. L., Strada, S. J., Wagner, J. A. and Scammell, J. G., Transcriptional regulation of secretogranin II and chromogranin B by cyclic AMP in a rat pheochromocytoma cell line, *Mol. Pharmacol.*, **1994**, 46, 880-889.

Timchenko, N. A., Wilde, M., Nakanishi, M., Smith, J. R. and Darlington, G. J., CCAAT/enhancer binding protein (C/EBP) inhibits cell proliferation through p21 (WAF-/CIP-1/SDI-1) protein, *Genes Dev.*, **1996**, 10, 804-815.

Tinti, C., Yang, C., Seo, H., Conti, B., Kim, C., Joh, T. H. and Kim, K.-S., Structure/function relationship of the cAMP response element in tyrosine hydroxylase gene transcription, *J. Biol. Chem.*, **1997**, 272, 19158-19164.

Togari, A., Ishinose, H., Matsumoto, S., Fijita, K. and Nagatsu, T., Multiple mRNA forms of human GTP cyclohydrolase I, *Biochem. Biophys. Res. Commun.*, **1992**, 187, 359-365.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. and Hidaka, H., KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, *J. Biol. Chem.*, **1990**, 265, 4315-4320.

Trejo, J. and Brown, J. H., c-fos and c-jun are induced by muscarinic receptor activation of protein kinase C but are differentially regulated by intracellular calcium, *J. Biol. Chem.*, **1991**, 266, 7876-7882.

Tsai, E. Y., Jain, J., Pesavento, P. A., Rao, A. and Goldfeld, A. E., Tumor necrosis factor alpha gene regulation in activated T cells involves ATF2/Jun and NFATp, *Mol. Cell. Biol.*, **1996**, 16, 459-467.

Tsai-Morris, C.-H., Cao, X. and Sukhatme, V. P., 5` flanking sequence and genomic structure of Egr-1, a murine mitogen inducible zinc finger encoding gene, *Nucleic Acid Res.*, **1988**, 16, 8835-8846.

Tsien, R. Y., New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures, *Biochemisty*, **1980**, 19, 2396-2404.

Tsien, R. Y., A non-disruptive technique for loading calcium buffers and indicators into cells, *Nature*, **1981**, 290, 527-528.

Tsutsumi, M., Zhou, W., Millar, R. P., Mellon, P. L., Roberts, J. L., Flanagan, C. A., Dong, K., Gillo, B. and Sealfon, S. C., Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor, *Mol. Endocrinol.*, **1992**, 6, 1163-1169.

Uhler, M. D. and McKnight, G. S., Expression of cDNAs from two isoforms of the catalytic subunit of cAMP-dependent protein kinase, *J. Biol. Chem.*, **1987**, 262, 15202-15207.

Ubeda, M., Wang, X.-Z., Zinszner, H., Wu, I., Habener, J. and Ron, D., Stress-induced binding of the transcription factor CHOP to a novel DNA control element, *Mol. Cell. Biol.*, **1996**, 16, 1479-1489.

Ullrich, A. and Schlessinger, J., Signal transduction by receptors with tyrosine kinase activity, *Cell*, **1990**, 61, 203-212.

Umek, R. M., Friedman, A. D. and McKnight, S. L., CCAAT-enhancer binding protein: a component of a differentiation switch, *Science*, **1991**, 251, 288-292.

Vallejo, M., Ron, D., Miller, C. P. and Habener, J. F., C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to camp response elements, *Proc. Natl. Acad. Sci. USA*, **1993**, 90, 4679-4683.

Van Dam, H., Duyndam, M., Rottier, R., Bosch, A., De Vries-Smits, L., Herrlich, P., Zantema, A., Angel, P. and Van der Eb, A. J., Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus protein, *EMBO J.*, **1993**, 12, 479-487.

Van de Werve, G., Lange, A., Newgard, C., Mechin, M. C., Li, Y. and Berteloot, A., New lessons in the regulation of glucose metabolism taught by the glucose-6-phosphatse system, *Eur J Biochem.*, **2000**, 267, 1533-1549.

Vann, L. R., Twitty, S., Spiegel, S. and Milstien, S., Divergence in regulation of nitric-oxide synthase and its cofactor tetrahydrobiopterin by tumor necrosis factor-alpha. Ceramide potentiates nitric oxide synthesis without affecting GTP cyclohydrolase I activity, *J. Biol. Chem.*, **2000**, 275, 13275-13281.

Van Schaftingen, E. and Gerin, I., The glucose-6-phosphatase system, *Biochem. J.*, **2002**, 362, 513–532.

Verma, I. M. and Graham, W. R., The fos oncogene, *Adv Cancer Res.*, **1987**, 49, 29-52.

Vinson, C. R., Sigler, P. B. and McKnight, S. L., Scissors-grip model for DNA recognition by a family of leucine zipper proteins, *Science*, **1989**, 246, 911-916.

Vinson, C., Myakishev, M., Acharya, A., Mir, A. A., Moll, J. R. and Bonovich, M., Classification of human B-ZIP proteins based on dimerization properties, *Mol. Cell. Biol.*, **2002**, 22, 6321-6335.

Walton, M. W., Rehfuss, R. P., Chrivia, J. C., Lochner, J. E. and Goodman, R. H., A dominant repressor of cyclic adenosine 3´, 5´-Monophosphate (cAMP)-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter in vivo, *Mol Endocrinol.*, **1992**, 6, 647-655.

Walton, M., MacGibbon, G., Young, D., Sirimanne, E., Williams, C., Gluckman, P. and Dragunow, M., Do c-Jun, c-Fos and amyloid precursor protein play a role in neuronal death or survival?, *J. Neurosci. Res.*, **1998**, 53, 330-342.

Walton, M., Connor, B., Lawlor, P., Young, D., Sirimanne, E., Gluckman, P., Cole, G. and Dragunow, M., Neuronal death and survival in two models of hypoxic-ischemic brain damage, *Brain Res. Brain. Res. Rev.*, **1999**, 29, 137-168.

Wang, X. Z. and Ron, D., Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase, *Science*, **1996**, 272, 1347-1349.

Wang, X. Z., Kuroda, M., Sok, J., Batchvarova, N., Kimmel, R., Chung, P., Zinszner, H. and Ron, D., Identification of novel stress-induced genes downsream of *chop*, *EMBO J.,* **1998**, 17, 3619-3630.

Wang, Z. Q., Ovitt, C., Grigoriadis, A. E., Mohle-Steinlein, U., Ruther, U. and Wagner, E. F., Bone and haematopoietic defects in mice lacking c-fos, *Nature*, **1992**, 360, 741-745.

Watters, J. J., Sommer, J. A., Pfeiffer, Z. A., Prabhu, U., Guerra, A. N. and Bertics, P. J., A differential role for the mitogen-activated protein kinase in lipopolysaccharide signaling, *J. Biol. Chem.*, **2002**, 227, 9077-9087.

Weck, J., Fallest, P. C., Pitt, L. K. and Shupnik, M. A., Differential gonadotropin-releasing hormone stimulation of rat luteinizing hormone subunit gene transcription by calcium influx and mitogen-activated protein kinase-signaling pathways, *Mol. Endocrinol.*, **1998**, 12, 451-457.

Whitworth, J. A., Studies on the mechanisms of glucocorticoid hypertension in humans, *Blood Pressure*, **1994**, 3, 24-32.

Wiggin, G. R., Soloaga, A., Foster, J. M., Murray-Tait, V., Cohen, P. and Arthur, J. S., MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts, *Mol. Cell. Biol.*, **2002**, 22, 2871-2881.

Wilhelm, D., Van Dam, H., Herr, I., Baumann, B., Herrlich, P. and Angel, P., Both ATF-2 and c-Jun are phosphorylated by stress-activated protein kinases in response to UV irradiation, *Immunobiol.*, **1995**, 193, 143-148.

Williams, S. C., Cantwell, C. A. and Johnson, P. F., A family of C/EBP related proteins capable of forming covalently linked leucine zipper dimers *in vitro*, *Genes Dev.,* **1991**, 5, 1553-1567.

Wisdom, R. and Verma, I. M., Transformation by Fos proteins requires a C-terminal transactivation domain, *Mol. Cell. Biol.*, **1993**, 13, 7429-7438.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. and Mathison, J. C., CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein, *Science*, **1990**, 249, 1431-1433.

Xie, Q. W., Whisnant, R. and Nathan, C., Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide, *J Exp Med.*, **1993**, 177, 1779-1784.

Xie, Q. W., Kashiwabara, Y. and Nathan, C., Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase, *J. Biol. Chem.*, **1994**, 269, 4705-4708.

Xing, L., Gopal, V. K. and Quinn, P. G., cAMP response element-binding protein (CREB) interacts with transcription factors IIB and IID, *J. Biol. Chem.*, **1995**, 270, 17488-17493.

Xu, L., Lavinsky, R. M., Dasen, J. S., Flynn, S. E., McInerney, E. M., Mullen, T. M., Heinzel, T., Szeto, D., Korzus, E., Kurokawa, R., Aggarwal, A. K., Rose, D. W., Glass, C. K. and Rosenfeld, M, G., Signal specific co-activator domain requirements for Pit-1 activation, *Nature*, **1998**, 395, 301-306.

Yamada, T., Murayama, T. and Nomura, Y., Muscarinic acetylcholine receptors on rat thymocytes: their possible involvement in DNA fragmentation, *Jpn J Pharmacol.*, **1997a**, 73, 311-316.

Yamada, T., Yoshiyama, Y. and Kawaguchi, N., Expression of activating transcription factor-2 (ATF-2), one of the cyclic AMP response element (CRE) binding proteins, in Alzheimer disease and non-neurological brain tissues, *Brain Res.*, **1997b**, 749, 329-334.

Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H. and Montminy, M. R., Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB, *Nature*, **1988**, 334, 494-498.

Yang, X. and Karsenty, G., ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells, *J. Biol. Chem.*, **2004**, 279, 47109-47114.

Yarden, Y. and Ullrich, A., Growth factor receptor tyrosine kinases, *Annual Reviews in Biochemistry*, **1988**, 57, 443-468.

Zanger, K., Cohen, L. E., Hashimoto, K., Radovick, S. and Wondisford, F. E., A novel mechanism for cyclic adenosine 3',5'-monophosphate regulation of gene expression by CREB binding protein, *Mol. Endocrinol.*, **1999**, 13, 268-275.

Zhan, Q., Liebermann, D. A., Alamo, I., Hollander, M. C. and Ron, D., The *gadd* and *MyD* genes define a novel set of mammalian genes encoding acidic proteins that cooperatively suppress cell growth, *Mol. Cell. Biol.*, **1994**, 14, 2361-2371.

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L. and Ron, D., CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum, *Genes Dev.*, **1998**, 12, 982-995.

G. Zusammenfassung

„Basic Region Leucin Zipper“ (bZIP) Transkriptionsfaktoren sind Proteine, die an spezifische Sequenzen im Promotor binden und dadurch die Transkription dieser Gene entweder aktivieren oder hemmen. Das bekannteste Mitglied der bZIP-Familie ist das „cAMP responsive element“ (CRE)-bindende Protein CREB, welches an die CRE-Consensussequenz 5’-TGACGTCA-3’ bindet. Um die Gen-Regulation durch bZIP-Transkriptionsfaktoren zu untersuchen, wurden konstitutiv-aktive und dominant-negative bZIP-Mutanten verwendet. Die Ergebnisse zeigen, dass die GTP-Cyclohydrolase I, Sekretogranin II, Tumornekrosefaktor α und Egr-1 kodierende Gene durch CREB und ATF2 via CRE-Bindungsmotive oder ähnliche Sequenzen reguliert werden. CREB und ATF2 bilden keine Heterodimere, sondern konkurrieren um dieselbe DNA-Bindungsstelle. Im Gegensatz dazu bilden die bZIP-Proteine CREB2/ATF4, ATF5 und C/EBP zwar untereinander Heterodimere, jedoch dimerisieren sie nicht mit CREB oder ATF2. CREB2/ATF4, ATF5 und C/EBP haben keinen oder nur geringen Einfluss auf CRE-regulierte Gene. CREB2/ATF4, ATF5 und C/EBP transaktivieren das Asparaginsynthetasegen und regulieren dessen Transkription nach Entzug von Aminosäuren im Kulturmedium.

In einer zweiten Studie wurde die Regulation der induzierbaren Stickstoffmonoxidsynthase (iNOS) untersucht. Die Transkription des iNOS-Gens wird nach Stimulation des „toll-like“ Rezeptors 4 aktiviert. Die Ergebnisse zeigen, dass stressaktivierte Proteinkinasen und die bZIP Proteine ATF2 und/oder c-Jun die Aktivierung der iNOS-Gentranskription vermitteln.

Schließlich wurde die Regulation von c-Jun und c-Fos kodierenden Genen in pituitary cells untersucht. Die Stimulation des „Gonadotropin-Releasing Hormon Rezeptors“ (GnRHR) oder des muskarinischen-Acetylcholin-Rezeptors Typ III führte zur Aktivierung des c-Jun und des cFos-Gens. Dies zeigt, dass die Biosynthese der bZIP-Proteine c-Jun und cFos durch extrazelluläre Stimuli kontrolliert wird.

H. Publications and posters

1. Publications

1- Thiel, G., **Al Sarraj, J**., Vinson, C., Stefano, L., and Bach, K., Role of basic region leucine zipper transcription factors CREB, CREB2, activating transcription factor 2 and CAAT/enhancer binding protein α in cAMP response element-mediated transcription, *J Neurochem.*, 92, 321-336 (2005a).

2- Thiel, G., **Al Sarraj, J**. and Stefano, L., cAMP response element binding protein (CREB) activates transcription via two distinct genetic elements of the human glucose-6-phosphatase gene, *BMC Mol. Biol.*, 6, 2 (2005b).

3- **Al Sarraj, J**., Vinson, C., Han, J. and Thiel, G., Regulation of GTP cyclohydrolase I gene transcription by leucine zipper transcription factors, *J. Cell. Biochem.*, 96, 1003-1020 (2005a).

4- **Al Sarraj, J**., Vinson, C. and Thiel, G., Regulation of asparagine synthetase gene transcription by the basic region leucine zipper transcription factors ATF5 and CHOP, *Biol. Chem.*, 9, 873-879 (2005b).

5- Stefano, L., **Al Sarraj, J**., Roessler, G. O., Vinson, C., and Thiel, G., Up-regulation of tyrosine hydroxylase gene transcription by tetradecanoylphorbol acetate is mediated by the transcription factors Ets-like protein-1 (Elk-1) and Egr-1, *J Neurochem.*, 97, 92-104 (2006).

6- Bauer, I., **Al Sarraj, J**., Vinson, C., Larson, R. and Thiel, G., Interleukin-1β and tetradecanoylphorbol acetate induced biosynthesis of tumor necrosis factor α in human hepatoma cells involves the transcription factors ATF2 and c-Jun and stress-activated protein kinases, in press, (2006).

2. Posters presented at conferences

1- The basic region leucine zipper proteins CREB and activating transcription factor 2 (ATF2) stimulate GTP cyclohydrolase I gene transcription in human hepatoma cells, (**Jude Al Sarraj** and Gerald Thiel), *Presented at the 7th Joint meeting of the Signal Transduction Society (STS) in Weimar, Germany, November 06-08, 2003.*

2- Basic region leucine zipper transcription factors involved in the regulation of asparagine synthetase gene transcription via the nutrient-sensing response unit, (Gerald Thiel, **Jude Al Sarraj** and Karl Bach), *Presented at the 55. Mosbacher Kolloquium, Mosbach-Baden, Germany, April 01-03, 2004.*

3- Role of basic region leucine zipper transcription factors CREB, CREB2, activating transcription factor 2 and CAAT/enhancer binding protein α in cAMP response element-mediated transcription, (Gerald Thiel, **Jude Al Sarraj**, Luisa Stefano and Karl Bach), *Presented at the 3rd Weimar Conference on Genetics, Neurogenetics, Germany, September 29th-October 2nd, 2004.*

4- Regulation of asparagine synthetase gene transcription by the basic region leucine zipper transcription factors ATF5 and CHOP, (**Jude Al Sarraj**, Charles Vinson and Gerald Thiel), *Presented at the 9th Joint meeting of the Signal Transduction Society (STS) in Weimar, Germany, November 09-12, 2005.*

5- Upregulation of tyrosine hydroxylase gene transcription by tetradecanoylphorbol acetate is mediated by the transcription factors Elk-1 and Egr-1, (Luisa Stefano, **Jude Al Sarraj**, Oliver Roessler and Gerald Thiel), *Presented at the 9th Joint meeting of the Signal Transduction Society (STS) in Weimar, Germany, November 09-12, 2005.*

6- Interleukin-1β and 12-O-tetradecanoylphorbol acetate-induced biosynthesis of tumor necrosis factor α in human hepatoma cells involves the transcription factors ATF2 and c-Jun and stress-activated protein kinases, (Inge Bauer, **Jude Al Sarraj** and Gerald Thiel), *Presented at the 9th Joint meeting of the Signal Transduction Society (STS) in Weimar, Germany, November 09-12, 2005.*