Forschungszentrum Karlsruhe
in der Helmholtz-Gemeinschaft

Wissenschaftliche Berichte
FZKA 6878

Subunit Selectivity of the Crosstalk between GR and AP-1

R. Ünal

Institut für Toxikologie und Genetik

September 2003

Forschungszentrum Karlsruhe
in der Helmholtz-Gemeinschaft
Wissenschaftliche Berichte
FZKA 6878

Subunit Selectivity of the Crosstalk between GR and AP-1

Resat ÜNAL

Institut für Toxikologie und Genetik

Von der Fakultät für Chemie und Biowissenschaften
der Universität Karlsruhe (TH)
genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe
2003

Impressum der Print-Ausgabe:

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)

ISSN 0947-8620

Subunit Selectivity of the Crosstalk between GR and AP-1

Zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

an der Fakultät für Chemie und Biowissenschaften

der Universität Karlsruhe (TH)

genehmigte

DISSERTATION

von

Resat ÜNAL

aus Ankara, TÜRKEI

2003

Dekan: Prof. Dr. Metzler

1. **Gutachter:** Prof. Dr. Peter Herrlich
2. **Gutachter:** PD. Dr. Hubert Schorle

Tag der mündlichen Prüfung: 08.05.2003

ZUSAMMENFASSUNG

Activating Protein 1 (AP-1) ist ein Transkriptionsfaktor, dem eine wesentliche Bedeutung im Hinblick auf Zellwachstum, Differenzierung, Tumorenstehung und Entwicklung nachgewiesen wurde. Er besteht aus mehreren Proteinen und gehört zu einer Familie von Transkriptionsfaktoren, die ihre Funktion mittels Dimerisierung ausübt. Die Heterogenität der Dimerkomposition im AP-1 Komplex ist hoch, was auf die Exprimierung verschiedener AP-1 Untereinheiten zur gleichen Zeit zurückzuführen ist.
AP-1 besteht aus den Mitgliedern der *Jun* (*c-Jun*, *JunB* and *Jun D*), der *Fos* (*c-Fos, FosB, Fra-1, Fra2*) und der *CREB/ATF* Familie. Die Signalkaskade mittels der mitogen aktivierten Proteinkinase MAPK (engl. Mitogen Activated Protein Kinase) reguliert das Ausmaß der Transaktivierung durch Jun, Fos und ATF-2 in einer stimulusspezifischen Weise. Die Behandlung der Fibroblasten beispielsweise mit dem Wachstumsfaktor „Platelet derived growth factor" (PDGF), mit Serum, welches ebenfalls Wachstumsfaktoren enthält, oder dem Phorbolester TPA aktiviert bevorzugt die extrazellulär regulierten Kinasen der MAPK-Familie, die die Jun/Fos Aktivität in hohem Maße stimuliert. Die Behandlung der gleichen Zellen mit Stress induzierenden Stimuli wie mit Ultraviolettbestrahlung (UVC) oder alkylierenden Agenzien führt hingegen vornehmlich zur Aktivierung der Kaskade, die durch die Mitglieder der Jun N-terminalen Kinase (JNK)/Stress-aktivierte Proteinkinase (SAPK) der MAPK-Familie vertreten sind, die schließlich den Jun/ATF-Komplex heraufreguliert.
Abhängig von der Dimerkomposition binden verschiedene AP-1-Dimere an unterschiedlichen AP-1-Bindestellen. Die Bindung von Jun/Fos erfolgt beispielsweise bevorzugt an heptamerischen und jene von Jun/ATF-2 an oktamerischen AP-1-Bindestellen.

Glukokortikoide (GK) finden aufgrund ihrer antiinflammatorischen und immunsuppressiven Wirkung eine breite therapeutische Anwendung. Sie sind an der Regulation vieler physiologischer Prozesse beteiligt. Der Einfluss der Glukokortikoide in der Zelle wird durch ihre Rezeptorbindung an den Glukokortikoidrezeptor (GR) vermittelt. Studien bezüglich der Interaktion zwischen AP-1 und GR haben herausgestellt, dass die Transkription der Zielgene von AP-1 durch die GR-Aktivität gehemmt wird.
Die vorliegende Arbeit verfolgte zum einen das Ziel, transgene Mausmodelle zu etablieren, in welchem Zielzellen entweder bevorzugt die Dimerisierung von c-Jun-Proteinen mit c-Fos-Proteinen oder mit ATF-2-Proteinen aufweisen. Bisher konnten chimäre Mauslinien etabliert werden, deren Verpaarung zur Herstellung homozygot transgener Mäuse führen wird. Diese werden dann einer phenotypischen und histologischen Analyse unterzogen, um mögliche Abweichungen in den mutanten Mäusen im Vergleich zu Wildtypmäusen zu charakterisieren.
Zum anderen wurde ein bereits etabliertes Zellkulturmodell in stabil transfizierten Fibroblasten herangezogen, um mehr Aufschluss darüber zu bekommen, ob die c-Jun/c-Fos-Dimere (m0-Zelllinie) oder die c-Jun/ATF2-Dimere (m0.1-Zelllinie) an der GR-mediierten Hemmung der Transkription der Zielgene von AP-1 beteiligt sind. Im ersten Schritt konnte gezeigt werden, dass die TPA-Stimulierung der m0-Fibroblasten die Zunahme der Transkription des AP-1-Zielgens Fra-I zur Folge hatte, wohingegen die UVC-Behandlung gar keine Fra-I-Transkription indizierte. Die Stimulierung der beiden Fibroblastenzelllinien mit GK und adenoviral E1A wird derzeit durchgeführt.

ABSTRACT

It has been reported that AP-1 plays important roles in cellular growth, differentiation, tumor formation and development. AP-1 consists of several proteins associating as dimers. There is a high heterogeneity in dimer composition of the AP-1 complex which is caused by the fact that multiple AP-1 subunits can be expressed at the same time. The AP-1 transcription complex is composed of the members of the *Jun* (*c-Jun*, *JunB* and *Jun D*), *Fos* (*c-Fos, FosB, Fra-1, Fra2*) and *CREB/ATF* families. MAPK pathways regulate both the amounts and transactivating capacities of the Jun, Fos and ATF-2 proteins in a stimulus specific manner: treatment of fibroblasts with PDGF, serum or phorbol esters predominantly activates the Extracellular-Regulated Kinase (ERK) members of the MAPK family, which leads to strong stimulation of Jun/Fos activity via *de novo* synthesis of c-Jun/c-Fos, Jun B/c-Fos, and Jun B/Fos B. Treatment of the same cells with stress-inducing stimuli like ultraviolet light or alkylating agents predominantly activates the Jun N-terminal Kinase (JNK)/Stress Activated Protein Kinase (SAPK) members of the MAPK family which preferentially, enhance Jun/ATF-activity. Depending on the composition of the dimer, different sequence elements are preferentially recognized. Jun/Fos dimers bind to heptameric AP-1 binding sites while Jun/ATF dimers bind octameric AP-1 binding sites.

Glucocorticoids (GC) are widely used as anti-inflammatory and immunosuppressive drugs. Glucocorticoid hormones are involved in the regulation of many physiological processes. The effect of glucocorticoids in the cell is transmitted through its receptor, the glucocorticod receptor (GR). It is reported that the interaction between GR and AP-1 results in the inhibition of AP-1 mediated transcription by GR.

One of the aims in this project was to generate transgenic mice, predominantly carrying c-Jun with altered dimerization specificities where c-Jun dimerizes with c-Fos or ATF-2. Throughout the course of this work only chimaeric animals which would enable to get homozygous transgenic mice, were obtained. Phenotypical and histological analysis would be performed with transgenic mice in order characterize the differences between the mutated and wild type mice.

On the other hand a cell culture model, which already was established by the stable transfection of fibroblasts with c-Fos favouring c-Jun (m0) or ATF-2 favouring c-Jun (m0.1) mutant constructs, was used in order to address which of the above mentioned dimers participate in the inhibition of GR mediated AP-1 transcriptional activity. This work shows that TPA stimulation but not UVC stimulation of m0-fibroblasts transcriptionally activates Fra-1 which is an AP-1 target gene. The experiments where the fibroblasts are stimulated with glucocorticoids and adenoviral E1A are currently in progress.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AF-2	trans-Activation Function domain “
AP-1	Activating Protein 1
ATF	Activating Transcription Factor
bp	base pair
bZip	Basic Leucine Zipper
cDNA	Complementary DNA
CEF	Chicken Embryonic Fibroblast
c-Fos	Cellular Fos
c-Jun	Cellular Jun
Cre	causes recombination
CRE	cAMP Responsive Element
CREB	cAMP Responsive Element Binding
d.p.c	*days post coitus*
DBD	DNA-Binding domain
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E	Embryonic day
EDTA	Ethylenediamine-N,N-tetraacetate
ER	Estrogen Receptor
ERK	Extracellular-Regulated Kinase
et al.	Lat. *et ali*, and others
FCS	Fetal calf serum
g	gram
GalDBD-Jun	Galactosidase-DBD-Jun
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone-acetyltransferases
HDAC	histon deacetylases

Hsp	heat schock protein
HSV-tk	Herpes Simplex Virus Thymidine Kinase
IκB	Inhibitor of NF-κB
Ik-B	Inhibitory Protein of NFkB
IL	Interleukin
JNK	c-Jun N-terminal Kinase
kD	Kilodalton
LBD	Ligand Binding domain
loxP-	Locus of crossover P1
LTNL	LoxP-HSV-tk-PGK-neo-LoxP
µ	micro-(10^{-6})
µg	microgram
µl	microliter
µM	micromolar
m	milli-(10^{-3})
M	Molar
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse embryonic fibroblast
mg	milligram
min	minute (s)
ml	milliliter
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
mRNA	Messenger RNA
n	nano-(10^{-9})
NF-κB	Nuclear factor kappa B
ng	nanogram
nGRE	negative Glucocorticoid Responsive element
O/N	Overnight
°C	Degrees Celsius
OD	Optical density

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RAR	Retinoic Acid Receptor
RHD	Rel homology domain
RNA	Ribonucleic acid
rpm	rotation per minute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SAPK	Stress Activated Protein Kinase
SRE	Serum Responsive Element
SW1/SNF	Switch/Sucrose nonfermentable proteins
TBE	Tris-boric acid-EDTA
TBP	TATA-Box Binding Protein
TEMED	N,N,N',N' tetramethylene-diamine
TFIIB or TFIID	Transcription Factor complex IIB or IID
TRE	TPA Responsive Element
U	Unit (s)
UV	Ultra-violet light
V	Volt

1. INTRODUCTION

1.1 Cross-talk between glucocorticoid receptor and AP-1

The cross-talk between the glucocorticoid receptor (GR) and AP-1 has been discovered more than 10 years ago. Glucocorticoid receptor acts as a bona fide transcription factor, that influences the activity of another transcription factor, without an apparent need for its direct DNA contact. Today it is clear that the cross-talk ability of GR is essential for mouse development, while the activation of target promoters carrying a glucocorticoid response element (GRE), is surprisingly, dispensable for survival under animal house conditions. It has also been shown that the cross-talk function is responsible for almost all regulatory actions of cortisol in the immune system (Herrlich P., 2001)

Glucocorticoids belong to the first-identified steroid hormones, are synthesized in and secreted from the adrenal cortex. Glucocorticoid hormones are involved in the regulation of many physiological processes including the control of carbohydrate and lipid metabolism, modulation of immune responses and various physiological responses in the brain including stress responses and behaviour. The level of glucocorticoids is tightly controlled by feedback regulation of glucocorticoid synthesis via the hypothalamic-pituitary-adrenal (HPA) axis. In combination with other hormones, especially glucagon, at birth are glucocorticoids involved in the activation of key hepatic gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. Glucocorticoids are important during embryonic lung development and may be involved in epithelial cell functions. In the perinatal period glucocorticoids help to promote surfactant synthesis in alveoler epithelial cells of the developing lung (Cole T. *et al.*, 1995). The most important and therapeutically significant function of glucocorticoids is their anti-inflammatory and immune-suppressive action through the inhibition of synthesis of cytokines and chemokines and repression of genes encoding surface receptors and adhesion molecules, that are functional in the recruitment and migration of lymphocytes. The inhibition of the action of the transcription factor NFkB, upon the activation of GRs by glucocorticoids and the downregulation of the expression of adhesion molecules like intercellular adhesion molecule I, vascular-cell adhesion molecule I and E-selectin which are under

the positive control of NFkB, is a good example to explain the repression of adhesion molecules by glucocorticoids (Barnes and Karin., 1997).

The effect of glucocorticoids in the cell is transmitted through its receptor, the glucocorticod receptor (GR). The GR belongs to the family of nuclear receptors (Mangelsdorf and Evans., 1995). It acts as ligand dependent transcription factor that influences the expression of genes in several ways. In the absence of glucocorticoids, GR is located in the cytoplasm in a form bound to chaperones, e.g Hsp90 and Hsp56, which keep the protein in a conformation ready to accept the lipophilic hormone ligand. The translocation of the GR into the nucleus, where it acts as transcription factor, takes place upon hormone binding (Kang *et al.*, 1999). The protein structure of GR is characteristic for the nuclear receptors. It contains a central DNA-Binding domain (DBD) which is highly conserved among steroid receptors. The DBD consists of two zinc-finger motives, where each zinc atom is coordinated in a tetrahedral arrangement by four cysteines (Freedman *et al.*, 1988). The amino acids of the first zinc finger are responsible for the sequence-specific binding of the receptor to the DNA and those of the second zinc finger are important for the dimerisation of GR molecules (Dahlman-Wright *et al.*, 1991). The DBD is connected to the LBD via a hinge region. The sequences for nuclear translocation are located in the hinge region (Picard and Yamamoto. 1987). The ligand binding domain (LBD) whose ligand interaction appears to be a determinant for the accessibilitiy of the transactivation domain AF-2 (Hu and Lazar, 1999) is located C-terminal. The LBD is composed of 12 amphipathic alpha helices and one beta sheet structure. Upon ligand binding, conformational changes occur in helices 10, 11 and 12, which provide accessibility for the ligand. Through the interaction between helices 12 and 3, new protein surfaces are provided, which are necessary for the interaction with coactivators (Moras and Gronemeyer., 1998). The N- terminus domain is carrying the ligand-independent AF-1.

The ligand-bound GR modulates the expression of target genes via different mechanisms. It may bind as a homodimer to specific promoter regions of target genes and activate their transcription or may repress the transcriptional activation of certain target genes.

GR and two groups of physiologically important transcription factors, AP-1 and nuclear factor-kB, have been reported to mutually interfere with each others activity. On the other hand, studies have supported a positive role for the AP-1 transcription factor in regulating GR gene expression in certain fibroblast cells. However, the cross-talk between GR and AP-1 is a more complex process, and the outcome is determined by the composition of the AP-1 subunits, alterations in local DNA topology and relative positions of GRE and AP-1 sites within the promoter (Diamond *et al.*, 1990., Miner and Yamamoto, 1992, Periyasamy and Sanchez, 2002)

Depending on the nature of the GRE, GR binding can result in activation or repression of genes containing GR-binding sites. Alternatively, GR can also modulate the expression of genes through a GRE-independent mechanism, such as protein-protein interactions of GR with other sequence-specific DNA binding factors or coactivators.

1.2 Transactivation

The transactivation of hormone-regulated target genes via the GR occurs upon the binding of GR as a homodimer to the specific promoter regions of the target genes. This cis-acting element on the promoters are called glucocorticoid responsive elements (GRE) (Beato *et al.*, 1995). Tyrosine aminotransferase which is playing a role in gluconeogenesis (Jantzen *et al.*, 1987) and the human metallothionein IIa genes (Karin *et al.*, 1984) are such positively regulated target genes.

The GR may interact with the members of the basal transcription machinery in DNA bound form, it may directly interact with basal transcription factor TFIIB and two members of the basal transcription factor TFIID, TATA-Box Binding Protein (TBP) and TAFII30 (Horwitz *et al.*, 1996). Although these interactions are playing a role in transcriptional activation, the main activity is based on the interactions of GR with coactivators. It is possible to classify the coactivators into two groups, which are members of switch/sucrose (SW1/SNF) nonfermentable proteins and members of histone-acetyltransferases (HAT) . Members of both the SW1/SNF and HAT families

are able to change the chromatin structure and promote transcription. They partially may also influence directly the activity of the basal transcriptional machinery or the generation of the preinitiation complex via protein-protein interactions (Glass and Rosenfeld, 2000).

The SW1/SNF complex has been identified in yeast (Neigeborn and Carlson, 1984, Stern *et al.*, 1984). It is also present in Drosophila (Papoulas *et al.*, 1998) and vertebrates as a multiprotein complex (Muchardt and Yaniv, 1993, Chiba *et al.*, 1994). It is responsible for an ATP-dependent repositioning of nucleosomes. ATP-dependent repositioning of nucleosomes results in a local alteration in chromatin structure. Through the interaction of GR and BRG1, a component of the human SW1/SNF1 complex, the chromatin structure around the GRE will be altered so that the sequence-specific transcription factors or the factors of the basal transcription machinery have a better access to the promoter region (Fryer and Archer, 1998). The pre-initiation complex will be formed easier and more stable, and the transcription of the target gene will be more efficient (Wilson *et al.*, 1996).

The members of the histone-acetyltransferase family alter the acetylation status of chromatin via their intrinsic histone acetyltransferase activity (Torchia *et al.*, 1998). The acetylation of the histones relaxes the chromatin structure and so the transcription factors may bind easier to the promoter regions, which ends up with a higher efficiency in transcriptional activity (Berger, 1999). The acetylation status is controlled by two enzyme activities of histone acetyl-transferases (HAT) and histon deacetylases (HDAC) (Wade and Wolffe 1997, Xu *et al.*, 1999). CBP/p300 (Bannister and Kouzarides, 1996, Ogryzko *et al.*, 1996), SRC-1 (Onate *et al.*, 1995) and TIF-2/GRIP-1 (Voegel *et al.*, 1996., Hong *et al.*, 1996) are coactivators with HAT activity.

CBP was identified as a protein interacting with the transcription factor CREB, whereas p300 was identified as a key protein which was functional for the action of the adenoviral oncoprotein E1A (Whyte *et al.*, 1989), Eckner *et al.*, 1994). Both proteins are highly homologous, and are involved in many common cellular functions (Arany *et al.*, 1994). CBP and p300 serve as coactivators for many transcription factors (McKenna *et al.*, 1999). Through direct interaction with RNA helicase A,

which binds to RNA Polymerase II, CBP/p300 mediates transcriptional activation (Nakajima *et al*., 1997). By the HAT activity of CBP/p300 the chromatin structure is relaxed and the generation of the pre-initiation complex facilitated.

SRC-1 and TIF-2 interact through a LXXLL sequence motive (Heery *et al*., 1997) with the AF-2 region in C-terminus of GR. Additionally, SRC-1 can interact with CBP, which results in the synergestic function of both SRC-1 and CBP (Smith *et al*., 1996).

GRIP-1 had been identified as a protein interacting with GR by the yeast two-hybrid system (Hong *et al*., 1996). It interacts with the hormone binding domain of GR. It was shown that GRIP-1 interacted with the hormone binding domains (HBD) of three different steroid receptors in a hormone regulated manner in yeast. GRIP-1 exhibited a strong transcriptional activation activity when tethered to the enhancer element of a reporter gene by fusion to a suitable DBD indicating that it can interact functionally with the transcription machinery (Walfish *et al*., 1997., Hong *et al*., 1997).

1.3 Transrepression

The anti-inflammatory and immune-suppressive effect of glucocorticoids is thought to be due to the repression of the genes that are involved in the regulation of immune and inflammatory reactions (Barnes., 1998). The genes encoding cytokines, cell adhesion molecules, and enzymes which are involved in inflammatory reactions are belonging to these genes. Most of the pro-inflammatory genes are under the control of the transcription factors AP-1 and NF-kB, which might mean that the repression of AP-1 and NF-kB via GR might be the molecular basis of the anti-inflammatory and immune-suppressive effect of glucocorticoids.

The mechanism of repression via GR differ from one gene to the other. The expression of many genes is repressed because of the interference of GR with transcription factors which are involved in the positive regulation of the target gene. GR may also block the transcription of certain genes via synthesis of inhibitory

proteins or via blocking the signal transduction cascades. In case of the repression of NFkB target genes, delayed onset of inhibition of target genes occurs, because here the synthesis of IkB-alpha was found to be activated by glucocorticoids (Auphan *et al.*, 1995, Scheinman *et al.*, 1995) indicating that new protein synthesis was required.

1.3.1 Repression via the synthesis of an inhibitor

The repression of NF-kB activity through GR is an example for the repression of transcriptional activation via the synthesis of an inhibitor by GR. NF-kB proteins contain a N-terminus RelA homology domain which is responsible for DNA binding, dimerisation, nuclear translocation and interaction with other proteins (Dumont *et al.*, 1998). In most cells, Ik-B blocks the nuclear localisation signal sequences of NF-kB and prevents the translocation of NF-kB into the nucleus. Upon stimulation of the cells with cytokines, UV, oxidants etc. Ik-B is phosphorylated, ubiquitinated and finally proteosomally degraded. This degradation leads to the translocation of NF-kB into the nucleus, and ends up with the transcriptional activation of NF-kB regulated target genes. Most of the NF-kB target genes which are immunologically relevant, are under the negative control of GR. One of the models which is explaining this situation, is the IkB model (Scheinmann *et al.*, 1995). According to this model, increased levels of cortisone lead to the synthesis of increased levels of IkB which ends up with the transcriptional repression of NFk-B regulated genes. However it also has been shown that in many cell types, the NFk-B repression through GR is independent of IkB (Heck *et al.*, 1997). A transcriptionally inactive GR mutant did not show IkB induction, whereas it was able to repress NFkB activity. So IkB induction probably is not the general mechanism for NFkB repression.

1.3.2 Repression via inhibition of signal transduction

The repression of AP-1 through the inhibition of JNK activity is a model for the repression through the inhibition of signal transduction by GR (Caelles *et al.*, 1997).

AP-1 (Activator protein 1) is a sequence-specific transcription factor and acts as homo or heterodimers. c-Jun is one of the major members of AP-1 family. AP-1 activation occurs upon mitogen, oncoprotein, cytokine and UV irradiation mediated induction (Angel and Karin., 1991, Karin *et al.*, 1997). The activation of AP-1 occurs through post-translational modifications and increased synthesis of AP- 1 components. The transcriptional activation of c-Jun happens through the phosphorylation of its amino terminal serine 63 and 73 residues by JNK (c-Jun amino teminal kinase) (Smeal *et al.*, 1991). JNK activity is necessary for the recruitment of the coactivator CBP.

The induced GR inhibits JNK and represses the activation of c-Jun. The repression on JNK is independent of transcriptional activity of hormon receptors. A rapid and transcription-independent inhibition of phosphorylation and of JNK activity has been reported (Caelles *et al.*, 1997, Gonzales *et al.*, 2000). Ligand-induced GR or an associated factor seems to down-modulate JNK activity. JNK repression could represent the predominant immediate mechanism of GR action.

1.3.3 Repression via GR binding sites of target genes

The competition for overlapping binding sites might also end up with the repression of the transcriptional activation of target genes. Prolactin is an example for it. The repression on this promoter is the result of the competition between GR and other transcription factors for binding to the same binding site (Sakai *et al.*, 1988). The promoter contains a GR binding site which differs from the classical GR binding site, so that the binding to this sequence does not result in transcriptional activation. These binding sites are called negative GREs and do not have the same consensus sequence as in classical GREs. The nGREs in the promoter of the prolactin gene is overlapping with the binding sites for the hypophyse-specific transcription factor Pit-1/GHF-1 and the ubiquitin factor XTF which together are responsible for the expression of the gene (Subramaniam *et al.*, 1997). The presence of glucocorticoids and binding of ligand-bound GR to nGREs prevents the transcriptional activation by these transcription factors. The repression of the human bone-specific osteocalcin gene takes place by a similar mechanism. Here, the binding site of the basal transcription factor TFIID

overlaps with a nGRE. (Celeste *et al.*, 1986, Strömstedt *et al.*, 1991, Meyer *et al.*, 1997). The generation of the transcription initiation complex is prevented by the GR which results in the repression of the target gene.

Murine proliferin repression occurs through another mechanism. The proliferin promoter does not contain overlapping binding sites. However, AP-1 and GR binding sites are in a very close neighborhood. This kind of a regulatory element is called composite GRE. The GR binds to the promoter but is able only to regulate the transcription in the presence of AP-1. When AP-1 consists of c-Jun/c-Fos, transcription will be upregulated. When it consists of a c-Jun homodimer, it is downregulated (Diamond *et al.*, 1990., Pearce and Yamamoto, 1993).

1.3.4 Repression via the modulation of the transcription function of other transcription factors

Most of the immunoregulatory genes, which are repressed via GR , are under the positive control of the transcription factors AP-1 and NFkB and do not contain binding sites for GR in their promoters. The repression in these genes is probably occurring through protein-protein interaction between GR and the positive regulating transcription factor (Jonat *et al.*, 1990, Lucibello *et al.*, 1990).

The human collagenase I gene is one of the genes which is repressed on this way (Schüle *et al.*, 1990). Collagenase I is a metalloprotease and is participating in the tissue degradation caused by rheumatoid arthritis. It is positively transregulated by the transcription factor AP-1. The activation through AP-1 occurs via physiological agents like growth factors and nonphysiological agents like UV irradiation or treatment with phorbol esters such as TPA. The activation of collagenase I occurs upon the binding of AP-1 to the TRE on the collagenase promoter (Angel *et al.*, 1987, Lee *et al.*, 1987). Collagenase I does not contain binding sites for GR and the repression of AP-1 through GR does not depend on a competition of AP-1 with GR for the binding site on the promoter (König *et al.*, 1992). AP-1 is also able to repress GRE-bound GR activation. By using mutants where the dimerization of GR was

blocked, it has been shown that while the DNA binding and activation of glucocorticoid regulated promoters require GR dimerization, repression is a function of GR monomers (Heck *et al.*, 1994). Although there is no DNA binding required for repression, the DBD domains and the N-terminal and C-terminal parts of the proteins are necessary for transcriptional repression (Schüle *et al.*, 1990., Kerppola *et al.*, 1993). All these data indicate that there is a protein-protein interaction between GR and AP-1 which is resulting in a formation of an inactive DNA bound complex. A physical interaction between GR and AP-1 is reported (Touray *et al.*, 1991, Yang-Yen *et al.*, 1990).

1.4 Mechanisms of cross-talk between glucocorticoid receptor and AP-1

One of the proposed models for the mechanism of cross-talk between glucocorticoid receptor and AP-1 was the competition for a limiting coactivator. CBP/p300 coactivators seemed good candidates. A severe embryonic-lethal phenotype of CBP knock-out mice (Yao *et al.*, 1998) and the demonstration of the significance of a CBP dose by the existance of a human syndrome, Rubinstein-Taybi Syndrome caused by CBP haploid insufficiency (Petrij *et al.*, 1995), indicated that CBP is a limiting component. The hypothesis was that through the binding of CBP to GR, the amount of free coactivator interacting with AP-1 would decrease and would reduce the transcriptional activation mediated by AP-1. However it is unlikely that the limiting abundance of CBP accounts for the repressive action of GR. Overexpression of CBP did not obliterate the inhibition of IL-6 promoter activity nor that of GalDBD-Jun fusion dependent reporter (De Bosscher *et al.*, 2000). Cross-talk with dissociating mutants of the GR and the effect of dissociating ligands for RAR and GR which do not permit coactivator binding and target gene activation by respective receptors, are not explainable by competition for CBP (Fanjul *et al.*, 1994, Chen *et al.*, 1995, Vayssiere *et al.*, 1997).

The cross-talk between the GR and other transcription factors is mutual and would be compatible with the formation of a complex involving the interfering transcription factor and possible additional partner proteins. This direct formation of a complex

suggested by e.g coprecipitation experiments (Jonat *et al.*, 1990) has been supported by genomic footprints and chromatin immunoprecipitations. Nuclear receptors and possibly numereous other transcription factors participating in cross-talk type of modulation share relevant properties. The mutual nature of cross-talk favours the existance of a protein complex with similar functional features in both directions. NFkB and AP-1 are inhibited after the assembly of the pre-initiation complex (Nissen and Yamamoto, 2000). Thus probably the assembly of coactivators and TBP-associated factors is not destroyed during inhibition of transcription. Cross-talk does not involve the recruitment of classical corepressors such as SMRT. Conformational changes where the DNA-element influences the structure of the transcription factors, supported by the structural analyses that DNA binding elements influence factor structure (Lefstin and Yamamoto., 1998, van Tilborg *et al.*, 2000) are plausible observations. The composition of AP-1 determines the positive or negative cross-talk with GR (Diamond *et al.*, 1990, Teurich and Angel 1995). Based on this observations, a working model of cross-talk might be derived. According to this working model, the modulating factor is associated with one or several factors X which exert their yet unknown molecular function only when tethered to an active transcription complex. The modulating tethering factor has presumably not yet been switched into an active conformation and thus may not assemble coactivators on its own. Specificity will be achieved by both the modulating factor and the associated X.

1.5 Activator Protein 1 (AP-1) and Subunits of AP-1

AP-1 (Activator Protein 1) is a family of dimeric transcription factors. It had been described as a TPA inducible transcription factor that adresses specific sequences of the metallothionein gene and the 72 base pair repeat of the Simian virus 40 enhancer region. It plays a central role in the regulatory network of eucaryotic gene expression (Angel and Karin, 1991). It has been reported that AP-1 plays important roles in cellular growth, differentiation, tumor formation and development (Curran and Franza, 1988; Angel and Karin, 1991).

AP-1 consists of several proteins associating as dimers. There is a high heterogeneity in dimer composition of the AP-1 complex which is caused by the fact that multiple AP-1 subunits can be expressed at the same time. The AP-1 transcription complex is composed of members of the *Jun* (*c-Jun*, *JunB* and *Jun D*), *Fos* (*c-Fos, FosB, Fra-1, Fra2*) and *CREB/ATF* families. These are characterized by the presence of a leucine zipper required for dimerization, and the basic region, involved in the recognition of DNA motifs, the heptameric AP-1 binding site TRE (TPA Responsive Element) (De Cesare *et al.*, 1995) where Jun/Fos(like) dimers bind to or the octameric CRE (cAMP Responsive element) where Jun/ATF(like) dimers bind to (Hai *et al.*, 1989). The octomeric element only differs by one nucleotide from the heptameric AP-1 binding site.

1.5.1 *c-Jun*

The *c-Jun* gene is located on human chromosome 1 at region p31-32, and on murine chromosome 4, subregion C5-7. It is an intronless, immediate early gene and encodes the c-Jun proto-oncoprotein. The c-Jun proto-oncoprotein is a major component of the AP-1 family of transcription factors. It is able to homodimerize, but is commonly associated with other transcription factor partners (van Dam *et al.*, 1998). c-Jun dimers are thought to play a decisive role in embryonal development (Hilberg *et al.*,1993), in cell proliferation and tumorigenesis (Vogt, 1994), in the cellular response to genotoxic stress (Devary *et al.*, 1992; Schreiber *et al.* 1995) and in apoptosis (Ham *et al.*, 1995). Dimerization is a prerequisite for DNA binding (Smeal *et al.*, 1989) and enhances also the nuclear translocation (Chida *et al.*, 1999). The major transactivation domain of Jun is located at the N-terminus of the protein. Jun is preferentially dimerizing with Fos and Fos related transcription factors within the AP-1 complex (Cohen *et al.*, 1989), however can also dimerize with some members of CREB/ATF family of proteins. The consensus sequence for CREB/ATF proteins is referred to as CRE. Jun is highly responsive to external stimuli. Its regulation is also integrated into the cell cycle. It undergoes activating phosphorylation at the M-G1 transition. Jun is able to transactivate the promoter of cyclin D1, elevated c-Jun activity may be important in advancing the cell through G1 (Vogt P., 2001).

Mice lacking a functional *c-Jun* die between day 12.5 and 13.5 of embryonic development (Hilberg *et al.*, 1993). E12.5 *c-Jun*-defective foetuses are characterized by interventricular septum defects in the heart and incomplete separation of the aorta and the pulmonary artery, which leads to a persistent truncus arteriosus, thus indicating that c-Jun is essential for the formation of a normal cardiac outflow tract (Eferl *et al.*, 1999). The mutant foetesus show also abnormalities in the liver, which include areas of haemorrhaging and of generalized oedema as well as increased numbers of apoptotic hepatoblasts and haematopoietic cells (Eferl *et al.*, 1999).

1.5.2 ***Jun B***

Knock-out *Jun B* embryos die between day 8.5 and 10 of embryonic development based on vascular defects in the extraembryonic tissues. In mutant mice, yolk sac vascularization is impaired and the expression of proliferin, matrix metalloproteinase-9 and urokinase plasminogen activator are deregulated in the tropohoblasts. The embryonic lethality results from defects in placentation (Schorpp-Kistner *et al.*, 1999).

Jun B appears to be dispensable for hematopoietic differentiation, since lethally irradiated mice can be fully reconstituted with *Jun B* deficient foetal liver cells. Furthermore, mice specifically lacking JunB expression in the myeloid lineage develop a myeloid leukemia with increased numbers of segmented neutrophilis (Passegue *et al.*, 2001). However broad overexpression of Jun B did not result in an overt phenotype (Shorpp *et al.*, 1996), targeted overexpression of *Jun B* in T-lymphocytes of transgenic mice interferes with T helper cell differentiation (Li *et al.*, 1996). It is thought that Jun B induction may contribute to the differentiation of naive T-helper cells into functional subsets of T-Lymphocytes (Lee *et al.*, 1999).

1.5.3 *Jun D*

Jun D is one of the three mammalian Jun proteins that contribute to the AP-1 transcription factor complex. Jun D is similar to other Jun members in its interaction with Fos members and in DNA binding affinity (Nakabeppu *et al*., 1988), but differs from c-Jun in its N-terminal domain. Jun D contains the N-terminal serine residues as in c-Jun but lacks a functional JNK docking site and is a weaker transcriptional activator (Kallunki *et al*., 1996). High *Jun* D mRNA levels are observed in a range of tissues (Ryder *et al*., 1989), whereas the c-Jun and Jun B genes have restricted expression pattern (Wilkinson *et al*., 1989). JunD is shown to interact with menin, the product of *MEN1* tumor suppressor gene, supporting a role for Jun D in growth inhibition (Agarwal *et al*., 1999).

Mice lacking *Jun D* are viable and only mutant males show impaired growth, hormone imbalace and age-dependent defects in reproduction due to impaired spermatogenesis (Thepot *et al*., 2000).

1.5.4 *c-Fos*

The *c-Fos* gene is located on human chromososme 14 at region q21-31 (Barker *et al*., 1984) and on murine chrosome 12 subregion E-D. It is a 4 kb long gene containing 3 introns and transcribing a 2.2 kb mRNA. It encodes the c-Fos protein, containing a basic leucine zipper region in its central part, which is conserved among all Fos proteins and is required for binding to a TRE element which is the cis-acting element c-Jun/c-Fos dimers bind to. The expression of *c-fos* is tightly controlled in most tissues, the mRNA is expressed at low levels sponteneously. It increases due to a stimulation of cells by growth factors, phorbol esters, cytokines etc. Through the negative autoregulation of *c-Fos* promoter (Lucibello *et al*., 1989), premature termination of nascent transcripts (Lamb *et al*., 1990) and postranscriptional turnover of RNA (Shyu *et al*., 1989) mechanisms, mRNA levels are kept low in the absence of external stimuli.

c-Fos knock-out mice are viable and fertile. They show bone defects, lacking osteoclasts, leading to an osteopetrotic phenotype (Wang *et al.*, 1992). Extramedullary haematopoiesis and lymphopenia, which are secondary to the bone phenotype, are abnormalities of the hematopoietic system in mutant mice (Okada *et al.*, 1994).

1.5.5 *Fos B*

The *Fos B* gene is located on murine chromosome 7 subregion A1-B1, and it contains three introns. A serum response element (SRE) and an adjacent AP-1 like sequence (FAP) are present in the *Fos B* promoter. There are also several overlapping TATA-like elements present in the promoter of *Fos B* (Lazo et al., 1992). There is a high homology between *c-Fos* anf *Fos B*, which suggests that the gene originate from a common ancestor form. Like *c-Fos,* also *Fos B* expression is transiently inducible in most adult tissues by a number of growth factors whereas in contrast to *c-Fos*, the primary transcript derived from *Fos B* is subject to alternative splicing. Alternative splicing causes the generation of two mature mRNA forms of approximately 3.9 and 3 kilobases in the the cytoplasm (Yen *et al.*, 1991).

Mutant mice containing non-functional *Fos B* develop normally (Gruda *et al.*, 1996). Also overexpression of *Fos B* does not cause an overt phenoptype (Grigoriadis, 1993). Overexpression of the alternatively spliced form of *Fos B*, lacking the transactivation activity, but having the DNA binding and dimerization activity, interferes with normal cell differentiation (Mumberg *et al.*, 1991).

1.5.6 *Fra-1 and Fra-2*

Mice lacking *Fra-1* die on day 10 of embryonic development caused by defects in the placenta and the yolk sac (Schreiber *et al.*, 2000).

The phenotype of mice defective in *Fra-2* has not yet been reported. But due to ist broad expression pattern during late embryonic development it is thought that its deletion may cause defects in many tissues (Carrasco and Bravo., 1995).

1.5.7 ATF/CREB family members

CREB, ATF1, ATF2, ATFa, CREBP-2, ATF3, ATF4 and ATF6 belong to this family. They bind with high affinity to the octameric cyclic AMP-responsive element (CRE). CREB and ATF1 form homodimers or CREB/ATF1 heterodimers. The other ATF/CREB members combine both with themselves and specific Jun and/or Fos family members. For example, c-Jun forms stable dimers with ATF2, ATF3 and ATF-4 but not with ATF1 and CREB. c-Fos and Fra1 can heterodimerize with ATF4 but not with ATF2 and ATF3 (Hai and Curran., 1991, Chatton *et al.*, 1994).

1.6 Basic Leucine Zipper Region and c-.Jun/c-Fos, c-.Jun/ATF2 dimerization

The subunits of AP-1 belong to the family of basic leucine zipper (bZip) proteins, which share the same structural domains for dimerization and DNA binding: a basic region (b) and a leucine zipper (Zip) region.The basic region is the site of DNA binding, containing the actual DNA contact surface. The leucine zipper region is involved in the formation of homo- or heteromeric dimers, with other bZip proteins. Dimerization is a prerequisite for DNA binding (Papavassiliou *et al.*, 1995).

The Fos proteins do not associate with each other and therefore do not bind DNA by themselves. They can associate with any of the Jun proteins to generate stable heterodimers. It is thought that the interaction between c-Jun and c-Fos has important functional consequences.

The dimerization occurs by coiled-coil interaction through the leucine zipper domain and this interaction is a prerequisite for DNA binding. These associations are not limited to c-Jun and c-Fos but are also observed among other members of these families.

A number of leucine zipper proteins have been isolated on the basis of their ability to interact with the CRE (cAMP Responsive Element) and with the closely related ATF (Activating Transcription Factor) binding site, which is known to mediate the E1A-inducibility of several adenoviral genes. However only a subset of the ATF proteins, functionally related to CREB, is regulated by cAMP (Hoeffler, 1992). Heterodimerization with c-Jun or c-Fos but not CREB, has been demonstrated *in vitro*, for the ATF2, ATF3 and ATF4 proteins where DNA fragments containing the coding regions of the two CREB proteins CREB-1 and CREB-2 were obtained by PCR amplification from a human cDNA library. The fragments were linked to the bacteriophage T7 promoter to allow *in vitro* synthesis of CREB RNA. The synthetic RNAs were translated in a rabbit reticulocyte lysate and the products assayed by gel retardation for their ability to specifically interact with the CRE element (Benbrook and Jones, 1990; Ivashkiv *et al.*, 1990; Hai and Curran 1991. *In vivo* binding sites for the c-Jun-ATF2 heterodimer have been identified in several inducible promoters, including the site responsible for the E1A-mediated induction of the c-Jun promoter, and the virus-inducible enhancer of the beta-interferon gene (Ivashkiv *et al.*, 1990; van Dam *et al.,* 1993).

1.7 Leucine zipper mutants as tools for the analysis of dimer specific ‚Jun functions

The use of Jun homodimers do not help to understand the role of the Jun target genes involved in transformation since Jun homodimers are able to bind both the Jun/Fos recognition sites which are the classical TRE sites, or the Jun/ATF2 recognition sites which are the CRE sites. To understand the role of Jun target genes involved in transformation, mutants are made, where c-Jun proteins specifically bound to c-Fos or ATF2. In order to determine the dimerization specificity, certain amino acid residues adjacent to the hydrophobic interphase of the leucine zipper alpha helix- at the so called ´e ´and ´g ´positions were mutated. (van Dam *et al.*, 1998). The interactions between the amino acid residues are represented in the helical wheel model as shown in figure 1.

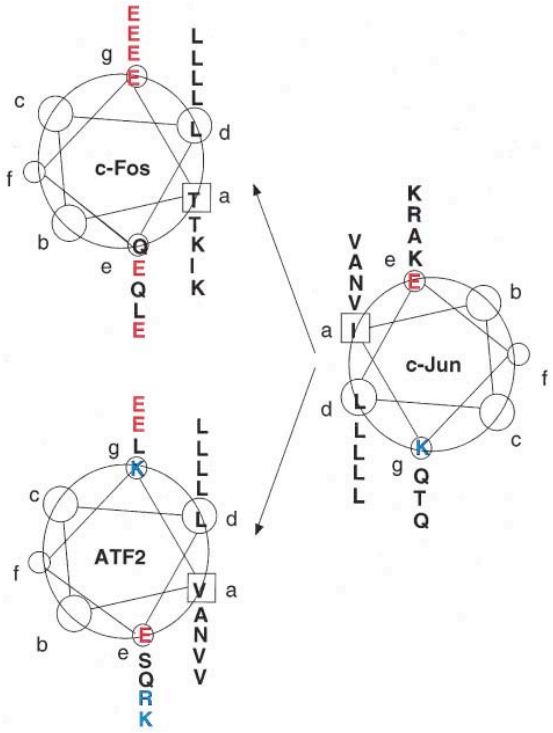


Figure 1: Schematic representation of the c-Jun, c-Fos and ATF2 leucine zipper domains by the helical wheel model. The ´e ´and ´g ´positioned aminoacids which are determinant in dimer specificity are located adjacent to the hydrophobic interface, which is formed by the residues at the ´d ´and the ´a´ positions (H van Dam and M. Castellazzi, 2001).

The residues located in ´ e ´and ´g ´positions of Fos proteins are strongly negatively charged whereas the Jun proteins are predominantly positively charged. Thus, the electrostatic interactions results in the generation of strongly stable Jun/Fos heterodimers but unstable Fos homodimers (Glover and Harrison, 1995). ATF2 contains equal amounts of positive and negative ´e ´and ´g ´residues, which stabilize both ATF2 homodimers and Jun/ATF2 heterodimers. Using mammalian two hybrid studies it was shown that the interaction between c-Jun and c-Fos can be efficiently blocked by the replacement of at least four ´e ´and/or ´g ´ residues by negatively charged aminoacids whereas this was not the case for c-Jun/ATF2 dimerization. By the replacement of glutamate at position e1 by lysine, the Fos-favouring mutant c-Jun-m0, by the introduction of ´e ´ and ´g ´glutamates in the Jun leucine zipper, the ATF2-favouring mutants c-Jun m0.1 and m.1 were generated.

The roles of preferential dimerization of c-Jun with c-Fos and ATF2 in oncogenic transformation was analyzed in an experimental system where chicken embryonic fibroblasts (CEF´s) were used as a model. CEF´s were retrovirally infected with Fos and ATF2 favoring mutants. The downregulation of endogenous avian c-Jun due to the overexpression of exogenous mutant Jun indicated that the mutant Jun proteins were the predominant Jun proteins present (van Dam *et al.*, 1998). A slight increase in doubling time of CEF proliferation in medium containing high concentrations of serum growth factors was observed by overxpression of Fos and ATF2 favouring mutants as similar as c-jun. This picture of proliferation on CEF cultures was changed when the analysis was performed under more restricted conditions, i.e in a semi-solid (soft agar) medium and medium containing low serum concentrations. Only Fos-favouring cells were able to form efficiently colonies in semi-solid medium whereas only ATF2 favouring cells were able to proliferate in low serum conditions. The fully transformed phenotype of cells were achieved by the coexpression of both mutants in the same cells, which indicated the existence of distinct and independent but complementary pathways: Jun/Fos activity causing anchorage independence via genes regulated through 7 bp Jun/Fos binding sites, and Jun/ATF2 activity triggering growth factor independence via genes regulated by 8 bp Jun/ATF2 sites as shown in figure 2. The contribution of both resulted in fully transformation (van Dam *et al.*, 1998)

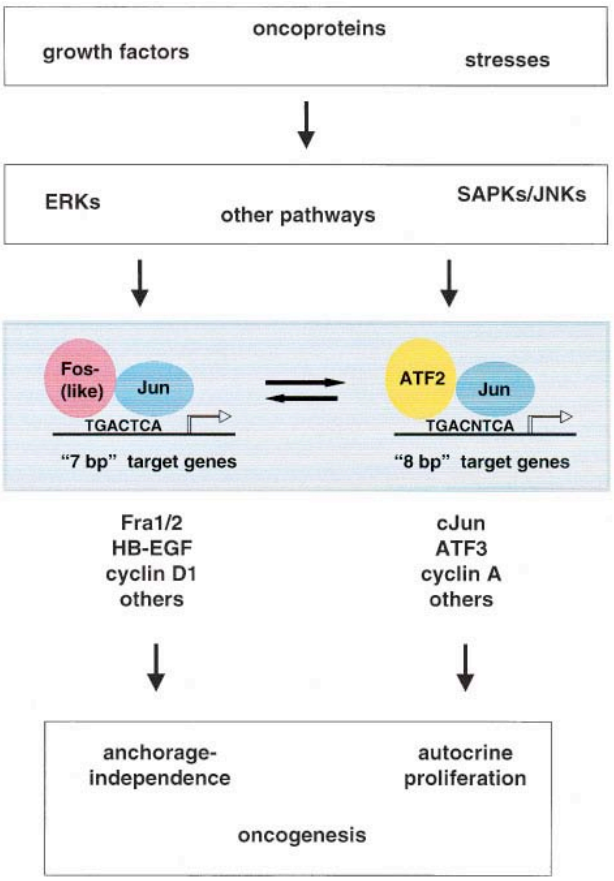


Figure 2: The model proposed for the role of Jun dimers in oncogenic transformation based on studies with avian mammalian cells (H van Dam and M. Castellazzi, 2001)

In order to get conclusive evidences concerning the nature of Fos- and ATF-like partners of c-Jun on transformation, another set of experiments were performed where the CEF´s were retrovirally infected only with v-Jun, ATF2 or Fra-2 and compared with the CEF´s which were infected with v-Jun+Fra2 and v-Jun+ATF2. ATF2 overexpression by itself was not able to induce transformation but potentiated v-Jun- and v-Jun-m1 induced proliferation in semi-solid medium, whereas slightly but significantly inhibited v-Jun mediated colony formation in agar (Huguier *et al.*, 1998). This resulted in the conclusion that ATF2 is actively involved in the Jun induced transformation pathway that triggers growth factor independence. Fra2 overexpression did not lead to v-Jun induced CEF colony formation in agar, but almost totally inhibited v-Jun induced proliferation in low serum (vam Dam H. and Castellazzi., 2001). These results indicate that there is a balance between v-Jun/ATF2

mediated and v-Jun/Fra2 mediated transformation in CEF´s and that v-Jun is limiting in case of its overexpression together with ATF2 or Fra2.

An ATF2/Fos designated molecule, where the leucine zipper of ATF2 was replaced by that of c-Fos was generated in order to confirm that v-jun induced autocrine proliferation mediated by enhanced ATF2 is due to increased levels of Jun/ATF2 heterodimers. The mutated ATF-2 molecule was not able to homodimerize but formed Jun/ATF2 heterodimers very efficiently. As wild type ATF2, ATF2/Fos by itself also could not induce transformation by itself, but was found to efficiently cooperate with v-Jun to allow CEFs to proliferate in low serum medium (Huguier *et al.*, 1998) which could explain a role of Jun/ATF2 heterodimers in CEF transformation.

It was shown that autocrine CEFs transformed by the ATF2 preferring mutant v-Jun-m1 formed efficiently primary fibrosarcomas in the chicken wing web whereas anchorage independent CEFs expressing the Fos-preferring mutant v-jun-m0 did not lead to primary fibrosarcoma formation. This indicated that primary tumor formation of CEFs is associated with the ability to proliferate in an autocrine fashion rather than in an anchorage independent manner. Even the contribution of Jun/Fra2 induced anchorage-independent proliferation to avian *in vivo* oncogenesis is not clear yet, based on the presumed role of Jun/Fos during the later stages of oncogenesis in rodents (Angel and Karin., 1991), it is speculated that enhanced levels of Jun/Fra2 dimers would affect cell motility and invasiveness (Bos *et al.*, 1999).

1.8 Motivation of the study

Our motivation in this project is;

1) to generate transgenic mice carrying the described Fos-or ATF-2 seeking Jun mutants using a knock-in approach in order to see the phenotypical consequences of preferential dimerization of c-Jun with c-Fos and ATF2.
2) to determine which AP-1 subunit dimer participate in crosstalk with GR and regulation by E1A.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General reagents and chemicals

Agarose	Serva, Heidelberg
Alkaline phosphatase	Boehringer, Mannheim
Ampicillin	Sigma, München
ATP	Sigma, München
Bacto-Pepton	GibcoBRL, Karlsruhe
Bacto Trypton	GibcoBRL, Karlsruhe
Bacto-Yeast	Difco Laboratories, Detroit
Bacto-Agar	Difco Laboratories, Detroit
Boehringer Block	Boehringer, Mannheim
Bromphenol blue	Serva, Heidelberg
Bowine Serum Albumin (BSA)	Serva, Heidelberg
Cell culture plates (15cm)	Greiner, Nürtingen
Cell culture plates for ES cells	Falcon
Calcium chloride	Roth, Karlsruhe
Calcium phosphate	Roth, Karlsruhe
Chloroform	Roth, Karlsruhe
Deoxy-ribonucleosidetriphosphates	Boehringer, Mannheim
Dexamethasone	Sigma, München
Dimethylsulfoxid(DMSO)	Fulka, Neu-Ulm
Dithiothreitol (DTT)	Boehringer, Mannheim
DNA Purification Kit , EASY PURE	Biozyme, Oldendorf
DNase I (RNase free)	Boehringer, Mannheim

Dulbecco´s Mod Eagle Medium without Sodium Pyruvat, with 4500mg/l Glucose, with Pyridoxin HCl	GibcoBRL, Karlsruhe
Dulbecco´s Mod Eagle Medium with 0.11g/l Sodium Pyruvate, with Pyridoxine	GibcoBRL, Karlsruhe
Electroporation cuvettes	Eppendorf, Heidelberg
Ethanol	Roth, Karlsruhe
Ethidium bromid	Sigma, München
Ethylendiamine tetraaceticacid (EDTA)	Roth, Karlsruhe
Fetal Calve Serum (FCS)	Bio Whittaker, Verviers, Belgium
G50 Sephadex	Roth, Karlsruhe
G418	GibcoBRL, Karlsruhe
Gelatine	Sigma, München
Glycerin	Roth, Karlsruhe
HEPES	Roth, Karlsruhe
Hybond American National Cam (Nylonmembrane)	Amersham Biotech, Freiburg
Hydrochloric acid (Hcl)	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
Klenow Fragment	Boehringer, Mannheim
Klenow Buffer (10x)	Boehringer, Mannheim
Ladder DNA („1 kb ladder“)	GibcoBRL, Karlsruhe PeqLab,
Leukemia Inhibiting Factor (LIF)	GibcoBRL, Karlsruhe
L-Glutamine	GibcoBRL, Karlsruhe
Lipofectamine Plus	GibcoBRL, Karlsruhe
Ligase Buffer (10x)	Boehringer, Mannheim
Magnesiumchloride solution (PCR)	Boehringer, Mannheim
β-Mercaptoethanol	Serva, Heidelberg
Na2HSO4	Roth, Karlsruhe
NaH2SO4	Roth, Karlsruhe
Non-essential aminoacids	GibcoBRL, Karlsruhe

Nuclease free water	Promega, Heidelberg
Nucleotide solution (PCR)	Boehringer, Mannheim
Oligonucleotides	Genset oligos, France
PCR Buffer (10x, magnesium free)	GibcoBRL, Karlsruhe
PCR reaction tubes	AGS, Heidelberg
Phenol	Roth, Karlsruhe
Preperation equipment for embryos	Fine Science Tools, Heidelberg
Sodium acetate	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Sodium chloride	Roth, Karlsruhe
Sodium dodecylsulphate (SDS)	Roth, Karlsruhe
Proteinase K	Merck, Darmstadt
TPA	GibcoBRL, Karlsruhe
Quiagen Plasmid preperation kit	Quiagen, Düsseldorf
Quiagen Typ 500 columns	Quiagen, Düsseldorf
Rediprime Random primer labelling kit	Amerham, Braunschweig
Restriction endonucleases and buffers	GibcoBRL, Karlsruhe, New England Biolabs, Schwallbach
Seriological pippets	Sarstedt, Nümbrecht
T3 DNA Polymerase	Boehringer, Mannheim
T4 DNA ligase	Boehringer, Mannheim
T7 DNA Polymerase	Boehringer, Mannheim
Taq DNA Polymerase with buffer	GibcoBRL, Karlsruhe
Tris-HCl, Tris-Base	Roth, Karlsruhe, Serva, Heidelberg
Trypsin	GibcoBRL, Karlsruhe
Whatmann 3MM paper	Bender&Hobein, Karlsruhe

2.1.2 Plasmids

pBluescript II KS +/-	Stratagene, Heidelberg
Cre expressing plasmid	obtained from K.Rajewski, Köln

Jun in pUC 19	obtained from A. Behrens in Wien
pGEM-LTNL	obtained from P. Mombaerts
pUC2.1 TA	Invitrogen, Netherlands

2.1.3 Bacteria

E.coli DH5α	Recombination defective, suppressive strain; *End*A1, *gry*A96, *hsd*R17 (rk-mk+), *lac*, *rec*A1, *rel*A1, *sup*E44, *thi*-1, F´[*pro* AB, *lac*qZDM15, Tn10]; Tn 10 ⇒ tetr

2.1.4 Cells

Embryonic stem cells	Hooper Edinburgh, obtained from Knobeloch
Mouse embryonic fibroblasts	prepared by myself
c-Jun -/- fibroblasts	obtained from A.Szabowski
c-Jun wild type fibroblasts	obtained from A.Szabowski
c-Jun wild type-ER fusion fibroblasts	obtained from A.Szabowski
c-Jun m0.1-ER fibroblasts	obtained from A.Szabowski
c-Jun m0-ER fibroblasts	obtained from A.Szabowski

2.1.5 Oligonucleotides and primers

Primers used to amplify 6kb genomic c-Jun

5´-AACTCTGTCCTCCGCCCCTATAATACATGTTGAAT-3

5´-GGAGTTGTCAGATTCAAAGTTGGAAGGAGACACCT-3´

Primers used to generate the m0 mutant construct

5´-AGCGGATCGCGCGCCTAAAGGAAAAAGTGAAAACCT-3´

5´- AGGTTTTCACTTTTTCCTTTAGGCGCGCGATCCGCA- 3´

Primers used to generate the m0.1 mutant construct

5´- CGCGCGCCTAAAGGAAGAAGTGAAAACTCTAGAAGC- 3´

5´- GCTTCTAGAGTTTTCACTTCTTCCTTTAGGCGCGCG- 3´

5´- CCAACATGCTCGAGGAAGAGGTGGCACAGCTTAAG- 3´

5´- CTTAAGCTGTGCCACCTCTTCCTCTTCCTCGAGCATGTTGG- 3´

Oligonucleotides to clone m0 targeting vector

5´-GGCCGCTTCGAATCTAGATTCGAAA-3

5´-CTAGTTTCGAATCTAGATTCGAACG-3

Primers used for Fra-1 PCR

5´-GTGAGACGCGAGCGGAAC-3

5´-CTGGGTCCTTCTTGTCTCCTTC-3´

2.1.6 Radiochemicals

α-^{32}P-dCTP	Amersham, Braunschweig

2.1.7 Solutions and media

1xTAE	40mM Tris, 20mMNaOAc; 1mM EDTA pH 8.0
Ethidium bromide solution	10mg/ml Ethidium bromide
50xTAE	2M Tris; 1M NaOAc; 50mMEDTA, pH 8.0
1xTE	10mM Tris pH 7.4; 1mM
EDTA;	pH 8.0
1xLB Medium	0.5% Yeast extract; 2%

Bacto-trypton,;	10mM NaCL; to be filled in a final volume of 1l and sterilized by autoclaving

2.1.8 Solutions for Southern Blotting

Hybridisation buffer	7% SDS; o.5M Na-Phosphate buffer; 1mM EDTA
Na-Phosphate buffer	684ml 1M Na2HPO4 316ml NaH2PO4
Wash solution I	40mM Na-Phosphate buffer; 1mM EDTA; 5% SDS
Wash solution II	40mM Na-Phosphate buffer; 1mM EDTA; 1% SDS

2.1.9 Solutions and media for ES-culture

Culturing medium of prrimary fibroblasts	A bottle of Dulbecco´s Mod Eagle medium with 0.11g/l sodium pyruvat, with pyridoxine; 5ml glutamin; 5ml pen/strep; 50ml FCS
Culturing medium of ES cells	A bottle of Dulbecco´s Mod Eagle medium without sodium pyruvat, with 4500mg/l glucose, with pyridoxin HCl; 5ml L-glutamine; 1ml β-mercaptoethanol; 5ml nonessential aminoacids; 5ml pen/strep; 500-1000 u/ml LIF; 75ml FCS
Selection medium G418	250µg G418 per ml ES medium

Electroporation buffer	PBS (-Ca/Mg); 5mM Hepes, pH 7.05
Freezing medium of ES cells and primary fibroblasts	20%(DMSO); 80% FCS
ES Lysis buffer	100mM Tris Hcl pH 8.5; 5mM EDTA; 0.2% gelatine, autoclave

2.2 METHODS

2.2.1 Analysis of nucleic acids

2.2.1.1 Spectrophotometric concentration measurement of nucleic acids

The concentration of the nucleic acids in the aqueous solutions was measured spectrophotometrically as an extinction at 260nm and 280nm wave lengths. OD260 =1 corresponds to 50µg/ml double stranded DNA, 40µg/ml of RNA or 20µg/ml single stranded oligonucleotides. The purity of DNA or RNA preparation was estimated by the calculation of the ratio OD260/OD280. Pure DNA solution should have an OD260/OD280 ratio of approximately 1,8 pure RNA solution- about 2,0.

2.2.1.2 Phenol/chloroform extraction of nucleic acids

To remove unwanted protein contaminants from nucleic acids, an equal volume of Tris-buffered phenol, chloroform and isoamylalcohol at a ratio of 25:24:1 was added and the mixture vortexed until the solution had a milky appearance. The two phases were separated by centrifugation at 15000rpm for five minutes which the upper aqueous nucleic acid containing phase was transferred to a new reaction tube and subjected to a further round of extraction with chloroform/isoamylalcohol (24:1).

2.2.1.3 Precipitation of nucleic acids from aqueous solutions

The precipitation of DNA was performed by adjusting the final salt concentration of the reaction mixture to 200nM with 3M Na-acetate (pH 4,8) and adding 2,5 volumes of 100% ethanol or 1 volume of 2-butanol (isopropanol) followed by 30 minutes of incubation at -20C. The precipitate was pelleted by centrifugation at 10000 rpm, at 12^0C

for 30 minutes after which the aqueous phase was discarded. The pellet was washed twice with 70% ethanol to remove excess salt and allowed to air dry for 10 minutes.

2.2.2 Preparation and isolation of Nucleic Acids

2.2.2.1 Small-scale plasmid DNA preparation (Miniprep)

Individual colonies were picked from a LB agar plate and used to inoculate 3 ml of LB medium (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl), containing 100 mg/ml ampicillin. The inoculated bacteria were the incubated with shaking (220rpm) o/n at 37C until a stationary phase had been reached upon which 1,5ml was removed and the bacteria pelleted by centrifugation at 5000rpm for 5 minutes. The pellet was resuspended in 100ul of solution I (50mM Glucose, 25mM Tris-Hcl pH 8.0, 10 mM EDTA and 300mg/ml RnaseA) and left at RT for 5 minutes before addition of 200 µl of solution II (0.2 M NaOH and 1.0% SDS). Once the resulting mixture appeared clear, it was neutralized with 150 μl of solution III (3 M Na-Acetate pH 5.2) and mixed by gentle inversion of the reaction tube. Following 15 minutes incubation on ice, the precipitated protein and chromosomal DNA was pelleted by centrifugation at 10000rpm for 10 minutes before the aqueous supernatant was removed. Extraction of the supernatant with P/C/I was followed by precipitation of the plasmid DNA with ethanol and the resulting DNA pellet resuspended in 50 µl of bi-dest H2O.

2.2.2.2 Large scale plasmid DNA preparation (Maxiprep)

Usually, a volume of 250 ml of LB (1x LB medium, 100 µg/µl ampicillin) medium supplemented with the relevant antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37^0C overnight until the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 4000rpm for 10 minutes at 4^0C and the pellet resuspended in 10 ml of buffer P1

(10 mM EDTA pH 8.0, 50mM Tris/HCl, 100µg/µl RNase A). Following 5-10 minutes incubation at RT, the cells were lysed by the addition of 10 ml of buffer P2 (200 mM NaOH, 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralized with 10 ml of cold buffer P3 (2.6 M KAc, pH 4.8) the entire contents gently inverted to aid mixing of the solutions. After an additional 10-20 minutes on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 1000 pm for 10 minutes at 4^0C in a fixed angle rotor. The retained supernatant was then added directly to a pre-equilibrated Quiagen-tip 500 column and the plasmid DNA was recoverd according to and using the manufacturers supplied buffers. The equilibration was performed with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0). The column was washed with 30 ml of buffer QC (1 M NaCl, 50mM MOPS, 15% ethanol, pH 7.0) and the DNA was eluted with 15 ml of buffer QF (1.25 M NaCl, 50mM MOPS, 15% ethanol, pH 8.2). The purified DNA was precipitated using 0.8-1.0 volumes of isopropanol. Then the elution was centrifuged at 13000 rpm for 30 minutes at 4^0C. The pellet was washed with 70% ethanol, air dried and dissolved in sterile water or 1xTE.

2.2.2.3 Isolation of genomic DNA from embryonic stem cells

The cells expanded into 24-well plates were washed twice with PBS without calcium and magnesium. Following this 500µl of ES lysis buffer was added onto the wells. The cells were then precipitated with 500µl isopropanol and fished. The DNA was resuspended in an appropriate volume of TE.

2.2.2.4 Extraction of Total RNA From Cells

Cells were pelleted in eppendorf tubes and homogenized in 1 ml RNA Pure reagent by vortexing till the pellet was dissolved. At this step homogenized samples can be stored at -20ºC. The extraction was performed When the starting material was less than $5x10^5$

cells, after the transfer of the aqueous phase to a new tube, 2 µl glycogen (20 µg) (Peq Lab) was added

2.2.2.5 Determination of RNA Concentration and Agarose Gel Electrophoresis of RNA Samples

RNA concentration was determined spectrophotometrically (Eppendorf spectrophotometer) by taking readings at 260 nm against a blank, ddH_2O. A solution of 50 µg/ml DNA or 40 µg/ml RNA in a 1 cm quartz cuvette will give an absorbance of 1 at 260 nm. The concentrations were calculated according to absorbance values and total volume of RNA solution. Protein or phenol contamination during the extraction procedure was assessed by A_{260}/A_{280} ratio with a value of 1.7 or greater as an indication of a good purity.

The quality of RNA samples and genomic DNA contamination were assessed by agarose gel electrophoresis. Aliquots of RNA samples (minimum 0.5 µg) were mixed with RNasin (4 U/sample) and 10x gel loading buffer and were loaded on 0.8% (w/v) agarose gels prepared in 1x TAE buffer and containing ethidium bromide to a final concentration of 0.3 µg/ml for visualization of nucleic acids. Gels were observed by illumination with UV light and photographs were taken using an Eagle Eye photocamera system (Stratagene).

2.2.2.6 Polymerase Chain Reaction (PCR)

All PCR reacrtions were performed in a total volume of 25-100 µl, in the presence of 200 µM dNTPs, 5-10 pmol of primer(s), 0,25 unit to 1 unit of Taq and 1x suppliers buffer and 1,5-2,0 mM $MgCl_2$ final concentrations. The reactions were carried out in a number of

commercially available PCR thermo cyclers, using specific cycling parameters depending on the application. Analysis of the PCR reaction was performed using gel electrophoresis.

2.2.2.7 First Strand cDNA Synthesis of Total RNA Samples

First strand complementary DNA (cDNA) was synthesized from total RNA by using Superscript™ II RNase H$^-$ reverse transcriptase (Invitrogen) as described by the manufacturer. One ng-5 μg total RNA was added in an Eppendorf tube together with 1 μl oligo $(dT)_{12-18}$ (500 μg/ml) (New England Biolabs) and 1 μl 10 mM dNTP mix (Peq Lab) (10 mM each dATP, dGTP, dCTP, dTTP at neutral pH). The volume was completed to 12 μl with sterile ddH_2O. The reaction mixture was heated to 65ºC for 5 min and quickly chilled on ice. Following brief centrifugation, 4 μl 5x first-strand buffer, 2 μl 0.1 M DTT (dithiothreitol) (Invitrogen) and 1 μl RNaseOUT recombinant ribonuclease inhibitor (Promega) (40 U/μl) were added and the samples were incubated at 42ºC for 2 min. Then, 1 μl (200 U) of Superscript II was added, mixed by pipetting up and down and samples were incubated at 42ºC for 50 min. Following heat inactivation at 70ºC for 15 min, cDNA samples were ready for amplification by PCR. The samples were diluted 5 fold (final volume 100 μl) with sterile ddH_2O before they were used as template for amplification.

2.2.2.8 Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction

The reaction mix was prepared by mixing the following per reaction:

5.7 μl ddH_2O
3 μl glycerol (Calbiochem, molecular biology grade)
2 μl 10x PCR buffer (-Mg) (Invitrogen)
0.6 μl $MgCl_2$ (50 mM, Invitrogen)
1.5 μl dNTPs (2.5 mM each) (Peq Lab)
0.1 μl [α-^{32}P] dCTP (3000 Ci/mmol; 10 μCi/μl, Amersham)
1 μl sense primer (10pmol//μl)
1 μl antisense primer (10pmol//μl)
0.1 μl Taq polymerase (0.5 U, Invitrogen)

The above mix was distributed as 15 µl/reaction into PCR tubes and 5 µl cDNA (from 100 µl final diluted volume) was added and mixed. Optimal PCR conditions (annealing temperature (Ta), number of cycles) were established for each primer pair . PCR was performed with MJ Research PTC 225 thermal cycler using the following programme:

94ºC 2 min
94ºC 45 sec
Determined Ta 45 sec
72ºC 45 sec for a total of optimised cycle number
8ºC soak

For determination of the appropriate cycle number, cDNA samples (for most of the primer pairs as a positive sample, cDNA synthesized from total mouse fibroblast RNA was used) were either left undiluted or diluted 3 and 9 fold, respectively and reactions were amplified with different cycle numbers with increments of 3 cycles. The radiolabeled PCR products were separated by 6% polyacrylamide gels. Following electrophoresis (200-250 Volts 2-3, h), gels were dried at 80ºC for 30 minutes using gel drier (Bio-Rad) and exposed using phosphoimager imaging plate (Fuji film FLA-3000). Quantifications were done using Aida software and the linear range was determined.

2.2.3 DNA Gel Electrophoresis

2.2.3.1 Agarose gel electrophoresis and DNA visualization

DNA fragments were fractionated by horizontal gel electrophoresis using standart buffers. Agarose, the amount of which was determined according to the required percentage, was completely dissolved in 1xTAE buffer. The agarose concentration varied between 0.8% and 2% (w/v) to maximize the resolution of fragments. 1.5µl of ethidiumbromide (10mg/ml) per 100 ml solution was added into the solution and the solution was poured and polymerized in a gel chamber. The DNA samples were supplemented with 1/10 volume of 10x sample buffer (10 mM EDTA pH 8.0, 10% glycerin, 0.02% bromophenol blue) and loaded to the gel. The gel was run in 1xTAE at

different voltages and times depending on the size and the fragment separation required. The DNA fragments were visualized under UV light.

2.2.3.2 Electrophoresis of RNA Through Gels Containing Formaldehyde

Agarose gel, 1.2%, was prepared by dissolving the corresponding amount of agarose in water and the solution was cooled to 60ºC in a water bath. Appropriate volumes of 5x MOPS buffer (final 1x) and formaldehyde (final concentration 0.5 M) were added under a fume hood. Samples were prepared as follows:

RNA (10 μg-30 μg)	up to 15 μl
5x MOPS buffer	6 μl
Formaldehyde	3 μl
Formamide	30 μl
10x gel loading buffer	6 μl
Final volume	**60 μl**

Samples were incubated at 55ºC for 15 min, chilled on ice and centrifuged briefly. Before loading, 1x MOPS gel running buffer was equilibrated at 4ºC and the gel was pre run at 130 V for 20 min. Samples were loaded and electrophoresis was performed in the cold room at 130 V (5 V/cm) for 1 h and then the voltage was increased to 160 V for additional 4 h till bromophenol blue migrated 7-8 cm. As a reference, one sample with ethidium bromide was run and visualized by UV after the run was complete.

2.2.3.3 Isolation and purification of DNA fragments from the agarose gel

The DNA band of choice was cut out from the gel, under long wave UV light with the aid of a scalpel. The purification was performed by using the DNA Purification Kit, EASY PURE, (Biozym) according the recommendations of the supplier.

2.2.4 Cloning

2.2.4.1 Restriction endonuclease digestion of DNA

Depending on the restriction enzyme used, 2-5 units (U) of the enzyme was added for each µg of DNA taken. The digestion was performed using the appropriate buffer and incubation temperatures as indicated by the supplier. Reactions were incubated for at least two hours at a final DNA concentration of 1-5mg/ml after which the reaction was terminated by either heat inactivation of the enzyme (65^0C for 10 minutes) or by phenol/chloroform extraction.

2.2.4.2 Dephosphorylation of 5´ DNA ends

The DNA fragment extracted and purified from the agarose gel was treated with 10µl of dephosphorylation buffer and filled with H2O to a final volume of 100µl. After the addition of 2µl of alkaline phosphotase (20u/µl) was the mix incubated 1 hour at 37^0C. Finally, the mix was run on a gel and the DNA fragment was eluted and purified.

2.2.4.3 Ligation of DNA fragments

All ligation reactions were performed in a total volume of 20 µl and incubated overnight at 16^0C, followed by heat inactivation of the ligase at 70^0C for 5 minutes before storing at -20^0C. For the ligation of the oligonucleotide adaptor, approximately 100-200 ng of DNA was ligated to a 3-4 molar excess of oligonucleotide adaptor, using 3 units of T4-DNA ligase and 2ul of 10x ligase buffer (50mM Tris pH 7.4, 10mM $MgCl_2$, 10mM DTT, 1mM spermidin, 1mM ATP). For the ligation of PCR fragments, after purification of the PCR fragment, the DNA was directly cloned via the T/A overhangs into the TA cloning vector pCR2.1 kit according to the suppliers protocol and using the kits components. For the ligation of the subcloned fragment, the cloned DNA was released from the vector

using appropriate restriction endonucleases purified by agarose gel electrophoresis and subsequently cloned into the new vector using compatible sites or through blunt end ligation.

2.2.4.4 Preparation of competent bacteria

One colony of *E.coli* DH5α was inoculated into a 100ml LB medium and incubated in a shaker for approximately 3 hours. Every half an hour the OD at 600nm was measured. By an OD value of 0,3-0,4 (the start of the logarithmic division phase), the cells were incubated on ice for 10 minutes and finally centrifuged by 4^0C at 800g for 10 minutes. The supernatant was removed. Then the pelllet was resuspended in 10ml of ice cold $CaCl_2$ and incubated on ice for 20 minutes. The cells were then centrifuged by 4^0C at 800g for 10 minutes, the supernatant was removed. The pellet then was resuspended in 20 ml of ice-cold $CaCl_2$ containing 15% glycerol. The suspension was then immediately aliquoted on ice (100μl), frozen in N2(fl) and stored at -80^0C.

2.2.4.5 Preparation of LB/Amp agar plates

5g of yeast extract, 10g of bacto-trypton, 10g of NaCl and 15g of bacto agar were put into a 1l bottle and the bottle was filled to 1l of volume. The mix was autoclaved. Before, pouring, the agar was cooled down to approximately 60^0C. Then, ampicillin (100μg/ml final conc.) was added onto he LB.

2.2.4.6 Transformation of DNA into competent bacteria

An aliquot of the competent DH5α bacteria was thawed slowly on ice. 10-100ng of supercoiled plasmid DNA was added to 100μl of competent cells and left on ice for a period of 30 minutes. Following this, the cells were heat-shocked at 42^0C for 90 seconds

before rapidly returning the tube to ice for 1-2 minutes. After addition of 800µl of LB medium, the bacteria were transferred to a shaker and incubated for 30 minutes at 37^0C. The cells were then pelleted lightly by a short centrifugation (3 min at 1000 x g) and 700 µl of the supernatant was removed before resuspending the cells in the remaining 100µl od supernatant. A volume of 50 µl was plated out on LB-agar plates supplemented with the correct antibiotic and the plates incubated 18-24 hours at 37^0C.

2.2.5 Preparation of radioactive labelled probes

0.5µg of DNA was resuspended in TE to a final volume of 45µl, denatured by heating to 95^0C for 5 minutes and was then rapidly cooled on ice. The entire solution was then added to a ReadyPrime reaction vial (redi Prime kit, Amersham Life Science) to which was added 5 µl of α^{32}P-dCTP. After incubation for 15-30 minutes at 37^0C, or one hour at room temperature unincorporated nucleotides were removed from the labelled DNA by sephadex column chromotography. The labelled DNA was eluted in a volume of 200 µl and denaturated at 95^0C for 2-3 minutes immediately before use.

2.2.6 Southern-Blot Analysis

Linearized genomic DNA was loaded onto an agarose gel and run in 1xTAE Buffer until the DNA had migrated sufficiently as determined by the visualization under UV-light. The gel was then removed and allowed to incubate for 30 minutes in a solution of 0,4M NaOH at RT with mild rocking. During this time a blotting chamber was set up. The gel was removed and carefully laid down on 3-4 pieces of 3MM Whatmann paper soaked in 0,4M NaOH. After ensuring no air bubbles under the gel were visible, a piece of Hybond N^+ membrane was placed on top of the gel followed by one piece of pre-wetted (in NaOH) Whatmann. A stack of Kimwipes was placed on top and weighted down with a suitable weight. Blotting was allowed to continue o/n upon which the membrane was

removed, the positions of the wells marked clearly before washing the membrane for 5-10 minutes in 5xSSC.

2.2.7 Flow Cytometry

Cells were trypsinized, pelleted by centrifugation and washed with FACS buffer once. Cells were then resuspended in FACS buffer and were used directly for FACS.

2.2.8 Immunofluorescence analysis

The medium of the cells were aspirated. The cells are washed twice with ice cold PBS and fixed for 8 minutes in ice cold acetone:methanol (1:1). The fixing solution was aspirated and the slides were air-dried at room temperature.

The coverslips are transferred into 24-wel plate dishes. To enhance wetting, the slides were treated with 0.02% Tween. The first antibody was added in a dilution of 1:250 diluted in DMEM+10% FCS to the slides. After one hour of incubation , the slides are washed 3 times with PBS 0.2% Tween. Then the second antibody diluted in 1:1000 DMEM+10%FCS, was added, and followed by another incubation of 1 hour. 5 µl DAPI was added for 5 minutes to counterstain the DNA. This was followed by the wash with PBS 0.02% Tween. The slides are mount and dried completely and analysed by microscopy.

2.2.9 Cell Culture Methods

2.2.9.1 Routine Culture of Cells and Storage

2.2.9.1.1 Media and Maintenance

All cells were maintained at 37ºC in an incubator C200 (Labotect GmbH) in 5% CO_2 and 95% humidity. Cells were grown on appropriate sized petri plates (Greiner Labortechnik). Below are the complete media used for different cell types:

Primary or established fibroblasts: DMEM (Phenol red Free), 10% fetal calf serum (Bio Whittaker), Penicillin (100 U/ml) / Streptomycin (100 µg/ml), Glutamine)
Starvation Medium: DMEM (Phenol red Free), **0.5%** fetal calf serum (Bio Whittaker) or 0.5% **CS** for NIH 3T3 cells, Penicillin (100 U/ml) / Streptomycin (100 µg/ml), Glutamax I (1x, 2 mM)

Transfection Medium: DMEM(Phenol red Free), 10% FCS (serum stripped) and Glutamax I (1x, 2 mM) without Pen/Strep.

Heat inactivated (at 56ºC for 30 min) serum was aliquoted to 50 ml, Pen /Strep and Glutamine (100x, 200 mM) stocks were aliquoted to 5 ml respectively and stored at -20ºC. For routine complete medium preparation, one aliquot of each were used per 500 ml of plain DMEM medium. All the media and trypsin were stored at 4ºC and prewarmed to 37ºC in a water bath prior to use.

For routine culture of fibroblasts, 15 cm petri plates were preferred. Fibroblasts were allowed to grow to 80-90% confluence for 3 days and then were splitted by trypsinization. For this procedure, 0.25% trypsin/0.5 mM EDTA solution was used. After the removal of medium, cells were washed once with PBS (-Ca^{2+}/Mg^{2+}, Invitrogen) and trypsin was added just enough volume to cover the cells. After incubation at 37ºC for 5 min, trypsin was diluted by the addition of complete medium and cells were transferred to appropriate falcon tubes to be pelleted by centrifugation at 1500 rpm for 5 min at RT.

The pellet was then reconstituted with fresh complete medium and re-plated at desired density.

2.2.9.1.2 Long Term Storage

Growing cells were trypsinized, pelleted and resuspended in complete medium at a density of 2-3 million cells/ml. In a cryo-vial, 1 ml cell suspension, 100 µl serum (10%) and 120 µl DMSO (10%) (Fluka Chemie) were mixed. Vials were placed in a cell freezing box (Nalgene™) and left at -80ºC O/N for gradual freezing. Frozen cell vials then were transferred to liquid nitrogen for long term storage. Repropagation was performed by placing the vial briefly at 37ºC and gradually thawing the cells by the addition of complete medium. The suspension was transferred to a Falcon tube, pelleted for the removal of DMSO and was resuspended in 10 ml complete medium to be plated in 10-cm tissue culture plates.

2.2.9.1.3 Starvation and Stimulation of Fibroblasts

Fibroblasts in this study were starved in 0.5% FCS containing DMEM medium for 24 hours. After starvation the fibroblasts were stimulated with the phorbol ester TPA (0,1 µl/ml) or 30J UVC. Tamoxifen induction was performed using 150 nm of 4-OH tamoxifen.

2.2.9.2 Embryonic Stem (ES) Cell Culture

2.2.9.2.1 Gelatinization of cell-culture plates

The petri-plates used for ES cells were layered and incubated with gelatine solution (0,2% in water) for few minutes. Then the gelatin was removed and the plates were used for further ES cell culture studies.

2.2.9.2.2 Splittage of ES cells

ES cells should be kept in a certain confluence in order to prevent their spontaneous differentiation which may lead to the loss of their pluoripotency. Therefore it is important to observe ES cells daily and split them 1:2 to 1:10 when necessary.

ES cells were washed with 1xPBS (-Ca^{2+}/-Mg^{2+}), treated with 1ml of 0.25% trypsin per 10cm plate an incubated approximately 5 minutes at 37^0C in incubator. The single cell suspension of ES cells is generated through intensive up and down pipetting of trypsinized ES cells. The single cell suspension was washed in 5 ml of ES medium, centrifuged by 1000 rpm for 3 minutes and plated to appropriate concentration.

2.2.9.2.3 Freezing of ES cells

After the observation of proper cell confluence, the cells were trypsinized, washed, centrifuged, resuspended in appropriate volumes of freezing medium (80%FCS/20%DMSO, ice-cold) and frozen by –80^0C.

2.2.9.2.4 Thawing of ES cells

The cells were thawed in water bath by 37^0C, washed in ES medium and finally plated onto gelatinized and feeder pre-treated and saturated plates.

2.2.9.2.5 Preparation and cultivation of embryonic feeder cells

Feeder cells were prepared from E13 or E14 embryos, which was determined according to the plug date of the female mouse. This mix population consists mostly of fibroblasts which would contain the same antibiotics resistance cassette as the targeting vector. The neomycine gene is the commonly used resistance gene which provides selection in G418 (neomycine) medium. Homozygote CD44 knock-out mice fulfills this requirement. The female mice that were were killed on the 13.-14. day after plug check by cervical dislocation. The uterus was removed and transferred into a plate containing 1xPBS (-Ca^{2+}/-Mg^{2+}) and the embryos were taken out from the uterus tissue. Head and internal organs of the embryos were removed on a petri plate. Equipment used was cleaned with PBS and 70% ethanol between processing of each embryo. Embryos were dissected in 10 ml 0.25% trypsin in 10-cm dish with fine scissors. The solution was pipetted up and down several times with 10 ml glass pipette and incubated at 37ºC for 30 minutes. Once complete, the cells were separated from leaving clumbs behind through 100 µm cell strainer (Falcon) and collected in a feeder medium containing falcon tube. Finally the cells were centrifuged for 3 minutes at 1000 rpm, resuspended in feeder medium and plated onto 20-cm plates (approx. 1embryo per plate). After the cells got confluent, they were trypsinized 2 or 3 times 1:5 and irradiated then in order to get them inactivated.

2.2.9.2.6 Inactivation of feeder cells

Feeder cells were trypsinized upon they grow to a confluence of 90-100% and collected in a falcon tube. The enzyme was inactivated by adding the same amount of feeder

medium. Then, the cells were irradiated with 2000 to 3000 rad in the gamma source, and were made unable to proliferate.

2.2.9.2.7 Freezing and Thawing of feeder cells

The feeder cells are frozen and thawed as same as ES cells by using feeder medium.

2.2.9.2.8 Linearisation of the targeting vector

25 µg of plasmid DNA was used for the linearization of the targeting vectors. Not I restriction enzyme is used for the linearization. The digestion is performed o/n in a total volume of 100 µl. The linearized targeting vectors are visualized by agarose gel electrophoresis, purified by phenol/chloroform extraction, alcohol precipitated, dried, taken up in 20 µl of TE and used for electroporation.

2.2.9.2.9 Electroporation of ES cells

After ES cells on a 5cm plate reached an appropriate confluence, they were trypsinized, washed twice with ice-cold-, and resuspended in 800 µl electroporation buffer and transferred to an ice-cold electroporation cuvette containing the linearized targeting vector. By using 400V and 250-500, ES cells were electroporated and plated onto 10cm plate.

2.2.9.2.10 Electroporation of ES cells

The neomycine selection was initiated after 24 hours of electroporation of the ES cells (250μg/μl G418, active substance). The cells were selected for 7-10 days. The cells which were not resistant for G418 began to die after approximately 5 days.

2.2.9.2.11 Pickage of neomycine resistant clones

After colonies achieved an appropriate size, they were picked, trypsinized in 96-well plates, and expanded both to 96- and 24- well plates. The clones in 96-well plates were frozen when they reached a proper colony number and the plates were wrapped with parafilm in order to prevent that they dry. The clones in 24-well plates were used for DNA isolation when they arrived a confluence of 90%.

2.2.10 Generation of mice-Preparation

2.2.10.1 Isolation of blastocysts

C57BL/6J mice were bred. Next morning the females were plug checked. Plugs are coagulated proteins of sperm fluid, which are visible after copulation for approximately 12 hours. Plug-positive mice were taken and killed on 3.5 dpc (*days post coitum*) by cervical dislocation. The skin was sterilized with 70% ethanol and the skin/peritoneal membrane was cut back. Intestines were layed aside and uterus horns were located. The fat on the wad ovary and fat along the fallopian tubes were cut away. Below the fork and below ovary was cut, horns were washed on PBS and the remaining fat was removed and the second ovary was cut away Holding the end of the horn with forceps, needle is inserted into tube, flushed in a plate with 2 ml M2-medium. 6-10 blastocysts/mouse flushed out and collected with a transfer needle.

2.2.10.2 Injection of blastocysts

First, the ES cells were prepared. They were tyripsinized a single cell suspension was performed. By pre-plating the ES cells were separated from feeder cells, fast centrifuged, resuspended and on ice for blastocyst injection maintained.

Microscopically 8-15 ES cells were injected in the blastocoel of a blastocyst. The injected blastocysts were transferred into M-16 medium and incubated in the incubator until transferred to foster mothers.

2.2.10.3 Production of foster mothers

The injected blastocysts were transferred into a 2.5 dpc (*days post coitum*) foster mother. Prior to injection, the foster mother was mated with a vasectomized male. The vasectomy was performed through the cauterization of the vas deferens. The mice are sterile however able to copulate. The copulation resulted in a hormonal cycle in the foster mother, which made the foster mothers pseudo-pregnant. Foster mothers are needed as hosts for the injected/ aggregated blastocyst.

2.2.10.4 Transfer of the blastocysts in the foster mother

The blastocysts were transferred into the uterus of the foster mother. The foster mother was anesthetized with Avertin. The anesthetized foster mother was placed lying ventrally, the ventral back part was cut, the uterus was found, six to eight blastocysts were transferred in the uterus. Then the uterus was pushed into the body and the body was sealed.

The isolation and injection of blastocysts, production of foster mothers, vasectomy, and the transfer of the blastocysts in the foster mother is performed by the technical assistans.

3. RESULTS

The result section in this work will be summarized in two parts. The first part will consist of the results concerning the generation of transgenic mice carrying c-Jun mutations by knock-in approach. Two different point mutations were attempted: one resulting in Jun which preferentially dimerizes with c-Fos the others which prefers ATF-2.

3.1 PART 1: GENERATION OF THE TRANSGENIC MICE

The roles of Jun/Fos (-like) and Jun/ATF-2(like) dimers on oncogenic transformation in CEFs has been reported by van Dam (van Dam *et al*., 1998). Similar roles of c-Jun-Fos (like) and c-jun-ATF-2 (like) dimers on oncogenesis of mammalian cells is provided by studies on Ras signalling and transformation by human adenoviruses (Wolthuis and Bos, 1999). It has been reported that three different Ras effector pathways are involved in the complete transformation of rodent fibroblasts. Raf effector pathway leads to morphological transformation (White *et al.*, 1995), whereas RalGEF effector pathway leads to colony formation on agar (D´Adamo *et al.*, 1997) These both partially transformed phenotypes induced by distinct Ras effectors are strikingly similar to the partially transformed phenotypes induced by Jun/Fra-2 and Jun/ATF- in avian fibroblasts. The activation of Raf signaling pathway leads morphological transformation due to anchorage independence, RalGEF pathway results in proliferation of the cells, and activation of PI-3K results in protection from apoptosis. These three independent pathways together result in tumorigenicity in fibroblasts. Taken that as starting point, the aim in this study was to assess the significance of c-Jun/c-Fos dimers and c-Jun/ATF-2 dimers in tumor development *in vivo*. After generation of these transgenic mice, fibroblasts could be established. The anticipation is that while stimulation of c-Jun/c-Fos transgenic fibroblasts with TPA would lead to anchorage independence, stimulation of c-Jun/ATF-2 transgenic fibroblasts would result in autocrine growth. In this *in vitro* system anchorage independence could be assayed by growth in agar and autocrine growth could

be tested by growing cells by serum reduced medium as described respectively. To analyse the role of these two dimer complexes in tumour formation *in vivo*, targeting vectors expressing c-Fos favouring c-Jun dimerisation (m0) mutant and ATF-2 favouring c.Jun dimerisation (m0.1) mutant were constructed and transfected into the embryonic stem (ES) cells to initiate the generation of transgenic mice by knock-in approach. It is reported that c-jun is capable of co-operating with c-Fos since osteosarcoma formation caused by *c-Fos* overexpression was enhanced in doubly transgenic mice overexpressing both Fos and Jun.

c-Fos knock-out mice are severely growth-retarded. They are much smaller and their body weight is 40-60% reduced. They have shorter limbs and shorter snout. Mutant mice lack teeth. The epiphyses and metaphyses of the long bones are much broader and the bone marrow spaces are calcified. The predominant phenotype is a form of osteopetrosis where the long bones are occupied by abundant bone and cartilage trabeculae, reducing the bone marrow cavity by 80%. Many other skeletal defects are present in *c-Fos* knock-out mice. *c-Fos* overexpression results in the development of osteosarcomas.

c-Jun is essential for normal mouse development. Embryos lacking *c-Jun* die at mid- to late-gestation and exhibit impaired hepatogenesis, altered fetal liver erythropoiesis and generalized oedema. In c-Fos favouring c-Jun transgenic mice, both predominant members of AP-1, *c-Jun* and *c-Fos* are still functional and able to heterodimerize. However the dimerization specificity of c-Jun is reduced only to c-Fos instead to additional counterparts. Because of this, I would expect to observe a phenotype, which is not as severe as in a c-Jun deficient mouse, at least not a lethal phenotype. The function of Fos is dependent on formation of heterodimers with Jun proteins and subsequent binding to AP-1 sites in the regulatory regions of specific target genes.Transgenic mice overexpressing c-Jun are normal. c-Fos overexpression results in osteosarcoma development. It is reported that Jun-Fos double transgenic mice develop osteosarcomas at a higher frequency than single Fos transgenic mice (Wang *et al.*, 1995). Therefore transgenic mice where the AP-1 function is based on preferential c-Jun/c-Fos

dimerization could develop osteosarcomas at a higher frequency than in a mice with basal c-Jun and c-Fos levels.

ATF-2 lacking mice are able to develop to term but die shortly after birth and display severe respiratory distress with lungs filled with meconium. These features are similar to those of a severe type of human meconium aspiration syndrome. ATF-2 plays an important role in the formation of the placenta and the relationship between placental anomalies and neonatal respiratory distress. It would be hard to speculate what kind of a phenotype would be observed in the transgenic mice carrying the ATF-2 favouring c-Jun mutant. The mice would be analyzed upon their generation and the direction of the analysis would be performed due to the observations in phenotype.

E14.1 and KPA ES cells were used initially to generate the transgenic mice by knock-in approach. It was reported that E14.1 ES cells grow very well (doubling time 24 hours) and can be heavily manipulated without loosing its capacity for germ-line transmission (R. M.Torres and R Kühn, 1997). Another subclone of E14.1 ES cell line, KPA was also used to generate the transgenic mice carrying c-Fos favouring c-Jun mutation and ATF-2 favouring c-Jun mutation. The other reason for using these cells was the availability of these ES cell strains in our institute.

The experimental procedure that will be followed in order to obtain the transgenic mice is summarized in figure 3.

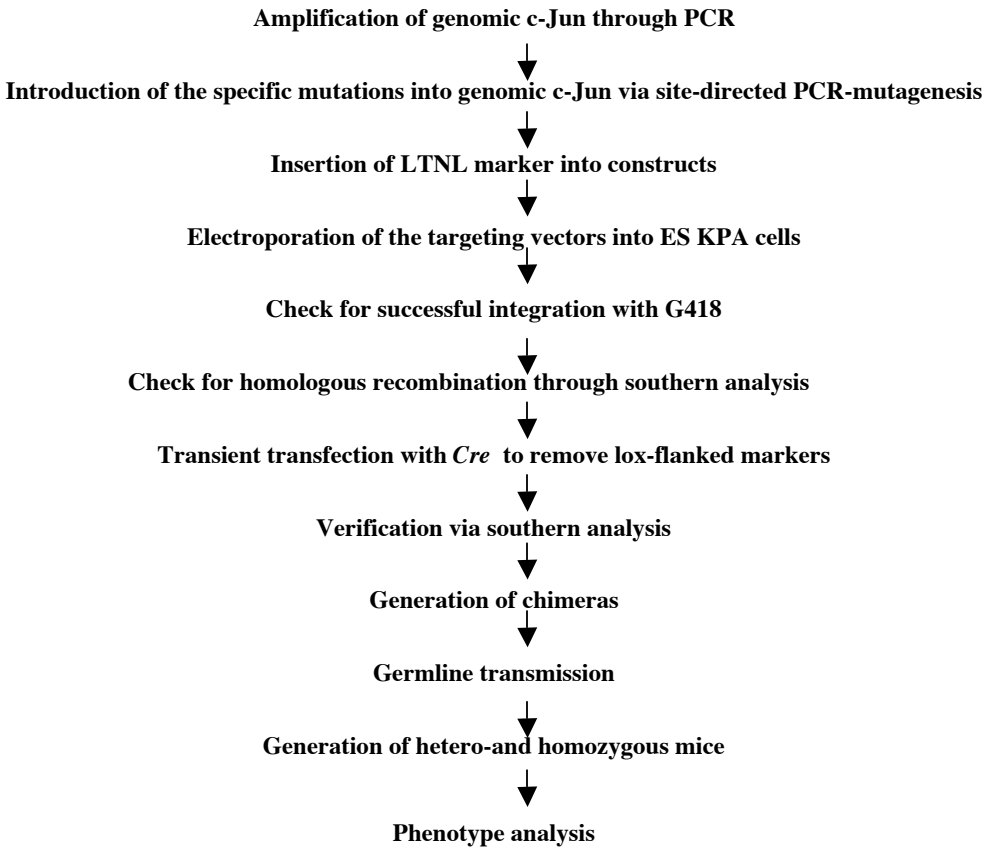


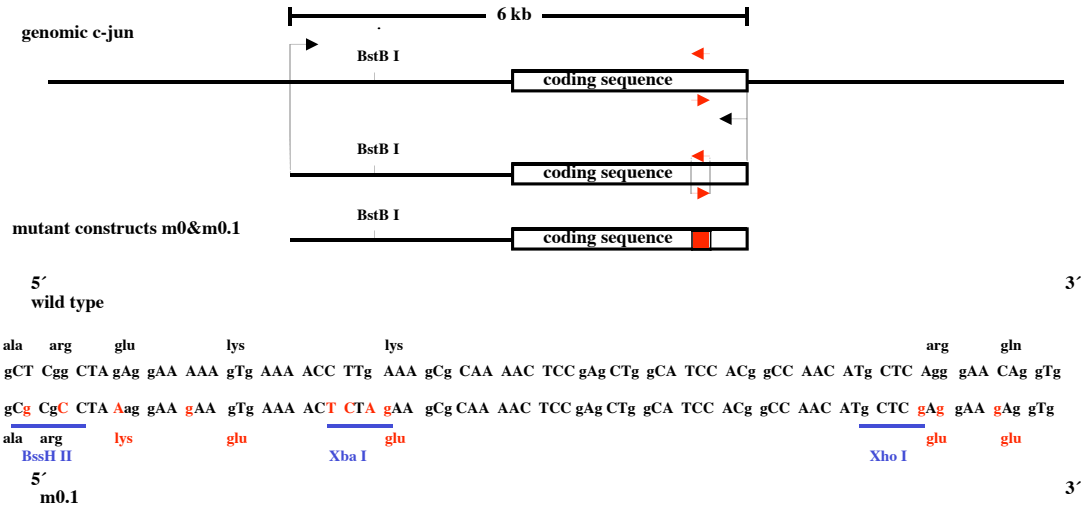
Figure 3: The experimental procedure in order to generate and investigate the knock-in mice carrying c-Jun with an altered dimerization specificity

3.1.1 Amplification of genomic c-Jun by Polymerase Chain Reaction (PCR)

My starting material was c-Fos favouring c-Jun vector and ATF-2 favouring c-Jun vector that had been constructed by Lila Komandou, a MIT graduate student who worked transiently at the ITG. By using two primers named c-Jun1 and c-Jun2 a 6 kb fragment of c-Jun had been amplified via Pfu DNA Polymerase. Magnesium chloride concentration was displayed in order to find the optimal amplification conditions. Both by using a buffer containing a magnesium concentration of 20mM or using a buffer without magnesium and adding a stock magnesium chloride of 25mM to have a final magnesium chloride concentration of 2.0mM the 6 kb genomic *c-Jun* fragment was amplified. The

PCR did not work by using 1.5mM final concentration of magnesium chloride. Figure 4 shows the amplification of the 6kb fragment of genomic c-Jun via PCR **(a)** and the detection by agarose gel electrophoresis of the successful amplification at different magnesium chloride concentrations **(b)**. By using different buffers and magnesium chloride conditions the reaction had been optimized and a fragment of 6 kb which is a quite large fragment for PCR, has been successfully amplified.

a)



b)

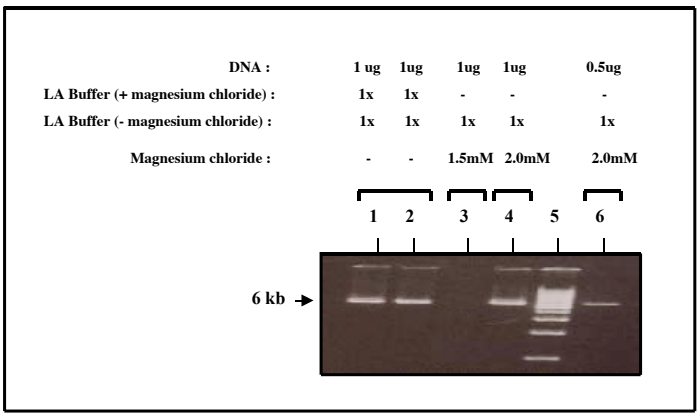


Figure 4: Amplification of genomic c-Jun **(a)** Schematic representation showing the amplification of genomic *c-Jun* by Pfu I DNA Polymerase using c-Jun1 and c-Jun 2 primers. c-Jun 1 anneals to a sequence in the promoter and c-Jun 2 anneals to a sequence in the coding sequence of genomic *c-Jun* and they lead to the amplification of a six kb fragment which is used for the generation of the c-fos and the ATF-2 favouring targeting vectors. The point mutations providing the generation of m0.1 ATF2 favouring mutant and the aminoacids changed (marked in red) and BssHII, XhoI and XbaI diagnostic restriction sites

(marked in blue) generated are shown here. BstB I restriction site in the promoter is the unique site which is used for the insertion of the marker casette. **(b)** Ethidium bromide stained 2 % agarose gel. Lane 1 and 2: PCR amplification product obtained by using 1 microgram DNA and LA buffer (plus magnesium cloride). Lane 3: PCR amplification by using 1 microgram of DNA, LA buffer (minus magnesium chloride) and1.5mM magnesium chloride. Lane 4: PCR product obtained by using 1 microgram DNA, LA Buffer (minus magnesium chloride) and 2.0mM magnesium chloride. Lane 5: DNA size marker (1 kb Ladder). Lane 6: PCR product obtained by using 0.5 microgram DNA and 2.0mM magnesium chloride. This data shows that the amplification works by a magnesium chloride concentration of 2.0mM and 1.5mM magnesium chloride concentration does not amplify the expected PCR product. The size of the expected six kb PCR products is shown on the side of the picture. This experiment has been performed by Lila Komandou.

3.1.2 Introduction of mutations into genomic c-Jun via site directed PCR-mutagenesis

3.1.2.1 Generation of a c-Fos favouring (m0) mutant

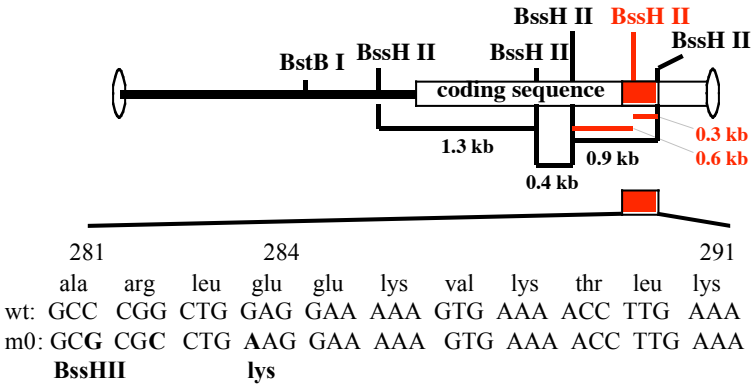
The 6 kb fragment was amplified in pCR2.1 TA cloning vector and used to introduce specific point mutations into the region coding for the leucine zipper. The subcloning of the 6 kb fragment into the 3.9 kb pCR2.1 vector resulted in the generation of a 9.9 kb fragment. To produce the c-Fos favouring c-Jun mutant (m0), a C was converted into a G in codon 281 and this generated a new BssH II restriction site additional to the BssH II sites that already existed in amplified c-Jun fragment, a G was converted into a C and a G was converted into an A in codon 284 by primer directed replication. Digestion of the wild type fragment cloned into pCR2.1 with BssH II resulted in the generation of 1.3 kb, 0.9 kb 0.4 kb 5.1 kb and 2.2 kb fragments. The diagnostic BssH II restriction site was introduced between two BssH II sites giving the 0.9 kb fragment by the restriction enzyme digestion. After the addition of the diagnostic restriction site this, 0.9 kb fragment was cleaved into 0.6 kb and 0.3 kb fragments as indicated with red arrows and white stars on gel picture.

The m0 mutant fragment served to generate the ATF-2 favouring c-Jun mutation. ATF-2 favouring c-Jun mutant had the XbaI and XhoI diagnostic restriction sites additional to the BssH II site which was present in Fos favouring c-Jun mutant. So the ATF-2

favouring mutant was also digested with the restriction enzyme BssH II and checked for the presence of the mutation.

The schematic representation of the mutations introduced into the coding sequence of the genomic *c-Jun* **(5a)** and the detection of the presence of mutations by restriction digestion analysis and agarose gel electrophoresis **(5b)** is shown in figure 5.

a)



b)

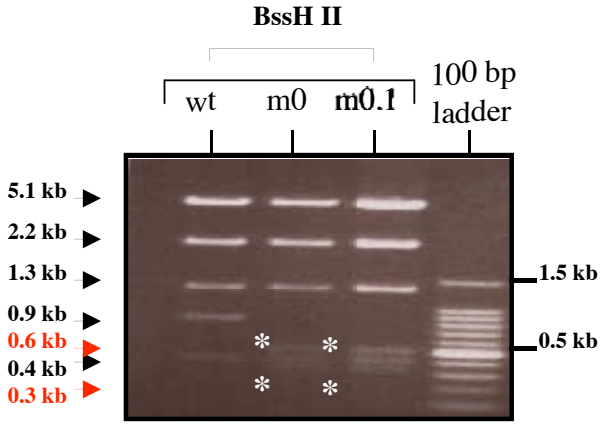


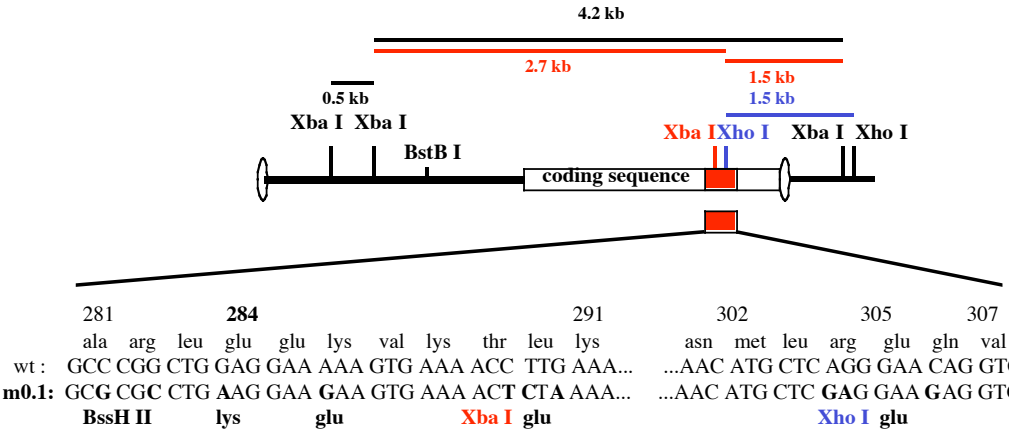
Figure 5: c-Fos favouring c-Jun mutations **(a)** Schematic representation of the mutations introduced into the *c-Jun* locus in order to generate the „m0„ c-fos favouring mutant and the distances between the BssH II sites before and after the introduction of the diagnostic BssH II restriction site(red labeled). The digestion of the wild type fragment in pCR2.1 cloning vector with the BssH II enzyme gives fragments of 1.3 kb, 0.4 kb 0.9 kb 5.1 kb and 2.2 kb fragments. The 0.9 kb fragment is cleaved into 0.6 and 0.3 kb fragments after the addition of the diagnostic Bssh II site. The coloured box in the scheme represents the sequence encoding the basic leucine zipper region of *c-Jun* where the mutations are introduced in. The point mutations which result in the conversion of wild type GCC sequence to mutated GCG sequence, generate a

dignostic BssH II restriction digestion site. The point mutation that leads the conversion of glutamine to lysine at position 284 is important in terms of the dimerization specificity of c-Jun with c-fos. **(b)** Ethidium bromide stained 0.8 % agarose gel. Lane 1 shows the digestion of the wild type c-Jun subcloned into the pCR2.1 vector with BssH II. The 0.9 kb fragments is cleaved to two smaller 0.6 kb and 0.3 kb fragments after the introduction of the diagnostic BssH II site. The 0.9 kb fragment seen in wt is not observable in m0 and m0.1 mutants since both mutants contain the BssH II mutation. The cleavage of 0.9 kb fragment to 0.6kb and 0.3 kb fragments (marked with white stars) are indicated with red arrows. This experiment has been performed by Lila Komandou.

3.1.2.2 Generation of an ATF-2 favouring (m0.1) mutant

Starting from the m0 mutant fragment, the ATF-2 favouring c-Jun (m0.1) mutant was constructed. To produce the ATF-2 favouring c-Jun (m0.1) mutant, a C was converted into a G in codon 281, a G was converted into a C in codon 282, a G was converted into an A in codon 284, an A was converted into a G in codon 286, a C was converted into a T in codon 289, a T was converted into a C, a G was converted into an A in codon 290, an A was converted into a G, a G was converted into an A in codon 304 and a C was converted into a G in codon 306. The alteration in codons 281, 289 and 304 created BssH II, Xba I and Xho I restriction sites respectively. The schematic representation of the mutations introduced to the coding sequence of *c-Jun* in order to generate the m0.1 mutant construct **(6a)** and the detection of the presence of the diagnostic restriction enzyme sites and agarose gel electrophoresis **(6b)** is shown in figure 6. The digestion of the wild type fragment in pUC2.1 vector with Xba I results in the generation of 5.2 kb, 4.2 kb and 0.5 kb fragments. The 4.2 kb fragment is cleaved into 2.7 kb and 1.5 kb fragments after the introduction of the diagnostic Xba I site which is shown with white arrows in the picture. The 6 kb fragment does not contain Xho I site, the digestion of the wild type fragment with Xho I linearized the m0 in pUC2.1 vector and resulted in the generation of the 9.9 kb fragment. The introduction of Xho I site in m0.1 ended with the generation of 1.5 kb and 8.4 kb fragments.

a)



b)

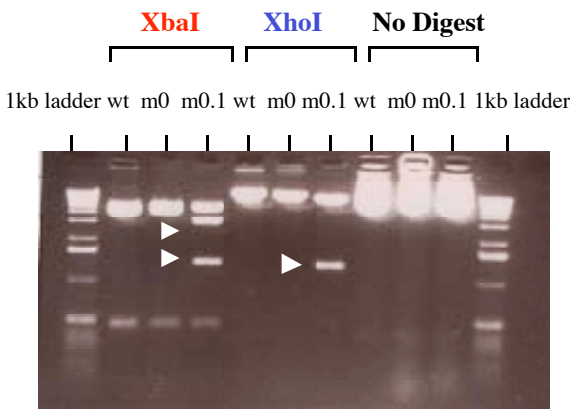


Figure 6: ATF-2 favouring c-Jun mutations **(a)** Schematic representation of the mutations introduced into the *c-Jun* locus in order to generate the ATF-2 favouring c-Jun mutant „m0.1„. The coloured box in the scheme represents the sequence encoding the basic leucine zipper region of *c-Jun* where the mutations are introduced in. The point mutations which result in the conversion of wild type GCC to mutated GCG, wild type ACC to mutated ACT and wild type AGG to mutated GAG generate the dignostic restriction digestion sites BssH II, Xba I and Xho I, respectively. The point mutations that lead the conversion of glutamine to lysine at the position 284, lysine to glutamine at the position 286, leucine to glutamine at the position 290 and arginine to glutamine at the position 305 are important in terms of the dimerization specificity of c-Jun with ATF-2. **(b)** Ethidium bromide stained 0.8 % agarose gel. Lanes 1 and 11 show the DNA size marker (1 kb ladder). Lanes 2, 3 and 4 show the digestion of the wild type, „m0„ and „m0.1„ mutants with Xba I respectively. Lanes 5, 6 and 7 show the digestion of the wild type, „m0 and „m0.1„ with Xho I respectively. The arrow indicates the bands which are observable in lanes 4 and 7 but not observable in lanes 3 and 6. This shows that the Xba I and Xho I dignostic sites are the restriction sites which are present in the „m0.1„mutant but absent in the „m0„ mutant as it is expected. Lanes 8, 9 and 10 show the undigested wild type c-jun, „m.0„ and „m0.1„. This experiment has been performed by Lila Komandou.

3.1.3 Introduction of a marker casette into wild type and mutant constructs

To create the targeting vector, the plasmids carrying the c-Jun mutant fragments were supplied with the marker cassette LTNL in which the HSV-Thymidine kinase (HSV-tk) and PGK-**N**eomycin (PGK-neo) markers were placed between two lox sites. The introduction of the marker casette was important in order to be able to select ES cells after transfection. After the transfection, the ES cells were cultivated in G418 selective medium. The clones in which the targeting vector containing the PGK-neo cassette is integrated into the genome, show G418 resistance. HSV-tk cassette makes the cells sensitive for ganciclovir, so the cells that contain this marker die in a selective medium containing ganciclovir. The presence of this cassette in LTNL is important to cultivate the cells that survive after the removal of the LTNL by Cre-mediated transient transfection.

The insertion of LTNL into the m0.1 mutant construct was performed by a blunt-end-ligation-based-cloning strategy. Here pGEM-LTNL was digested with Xba I and the LTNL was removed from the pGEM-backbone. The m0.1 mutant construct subcloned into pCR 2.1 was digested with BstB I. BstB I site was used for the insertion of LTNL. Both LTNL and linearized m0.1 fragment in pUC2.1 were filled in. After dNTP removal and alkaline phosphatase reactions of m0.1-pUC2.1 fragment, both m0.1-pUC2.1 and LTNL fragments were purified from agarose gel and ligated. So, the m0.1 (ATF-2 favouring c-JUN) targeting vector was generated. Figure **(7b)** shows the generation of the m0.1 targeting vector.

The insertion of LTNL into the m0 mutant construct was provided via a sticky-end-ligation based-cloning-strategy. Two oligonucleotides were annealed in order to generate a sequence where the Xba I site was placed between two BstB I sites with the 5´Nhe I and 3´Spe I overhangs. Then the bluescript vector was digested with Nhe I and Spe I restriction enzymes, in order to remove the Xba I site in the bluescript backbone. The oligonucleotides creating the relevant restriction enzyme sites and the Nhe I/Spe I digested bluescript were annealed and the bluescript vector containing the oligonucleotide (pBSK-0) was performed. pGEM-LTNL and pBSK-O were digested with

the Xba I restriction enzyme. The LTNL was removed from pGEM backbone and inserted into the pBSK-O construct via the Xba I site in the oligonucleotide. This new construct was called pBSK-O-LTNL. pBSK-O-LTNL and the m0 fragment in pUC2.1 (m0-pUC2.1) were digested with BstB I restriction enzyme in order to cut the LTNL from the pBSK-O-LTNL construct and insert it into the unique BstB I site in the m0-pUC2.1 and finally m0(c-Fos favouring c-Jun) targeting vector was generated. Figure **(7a)** shows the generation of the m0 targeting vector.

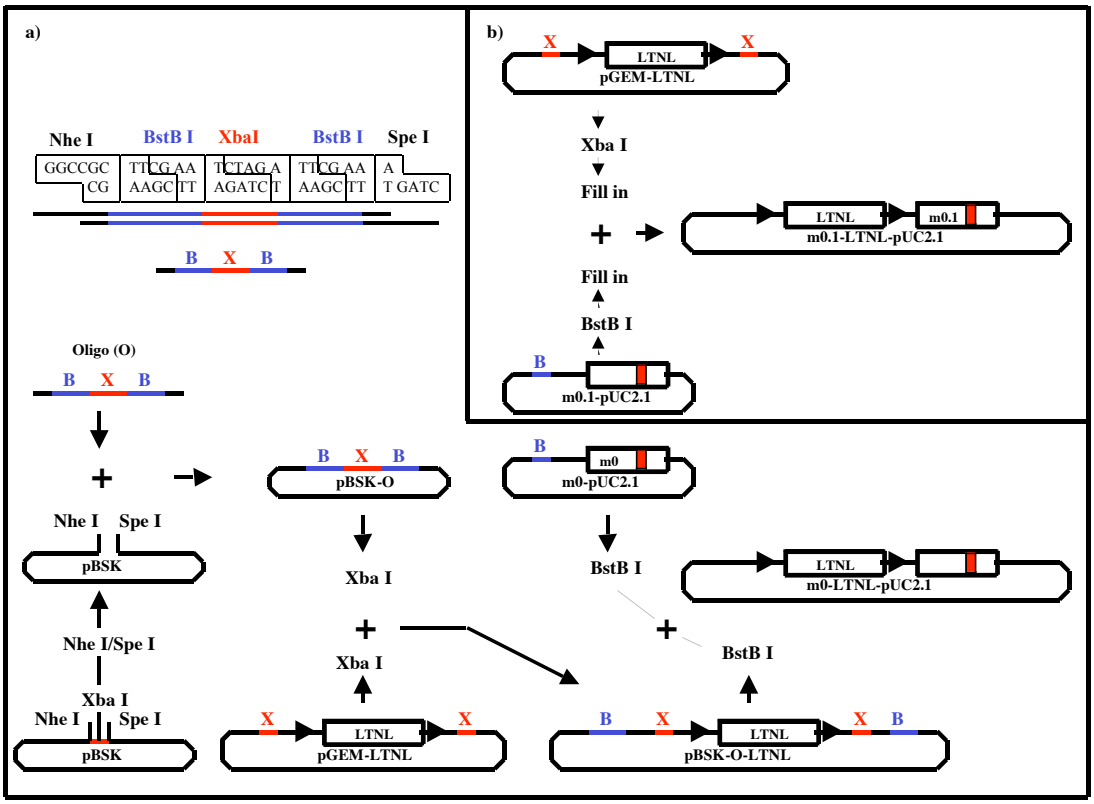


Figure 7: Schematic representation of the generation of the targeting vectors m0 and m0.1. **(a)** m0 (c-Fos favouring c-Jun) targeting vector was generated by a stick-end-ligation-based strategy. The oligonucleoteide (O) was ligated to the Nhe I/ Spe I digested pBSK and pBSK-O was generated. pBSK-O and pGEM-LTNL were digested with Xba I and the LTNL removed from pGEM was introduced into the pBSK-O and lead to the creation of pBSK-O-LTNL. Finally, the digestion of m0-pUC2.1 and pBSK-O-LTNL with BstB I and ligation resulted in the generation of the m0-LTNL targeting vector. **(b)** m0.1 (ATF-2 favouring c-Jun) targeting vector was made by a blunt-end-ligation-based strategy. pGEM-LTNL was digested with XbaI in order to cut LTNL. m0.1-LTNL-pUC2.1 constructs was digested with BstB I and both digest were filled in, ligated and the m0.1-LTNL targeting vector was generated.

3.1.4 Electroporation of embryonic stem (ES) cells

It was necessary to linearize the „m0„ and „m0.1„ targeting vectors in order to enable their integration into the genom in ES cells after the transfection. The targeting vectors „m0„ and „m0.1„ were linearized by digestion with the restriction enzyme Not I, and electroporated into E14.1, KPA and finally 129 Sv/Pas ES cells. Here the results obtained with 129Sv/Pas ES cells will be represented.

3.1.5 Selection of ES cells

The electroporated 129 Sv/Pas ES cells were kept under G418 selection. 256 clones derived from the electroporations with the „m0.1„ targeting vector and 210 clones derived from the electroporations with the „m0„ targeting vector were picked and expanded for further experiments.

3.1.6 Generation of an external (outside) probe

To detect recombinants southern blots were performed using an external probe. The use of an external probe was useful to discriminate between the selected cells that contain randomly integrated plasmid and the results generated if the specific recombination had occurred. By using external probe, we wanted to prevent the results that might come out due to random integrations of the targeting vectors into the genome. We contacted Axel Behrens from IMP and got the EcoR I fragment of *c-Jun* subcloned into the pUC19 plasmid. The sequence of 2.5 kb upstream of this fragment was known, however we had to do restriction mapping in order to find out the sequence of the 3.5 kb downstream and to use it for external probe preparation. The restriction mapping was performed and a 0.4 kb probe derived from this downstream 3.5 kb fragment was generated and used as the external probe. Fig 8 shows the restriction mapping and agarose gel purified fragment which is used as external probe.

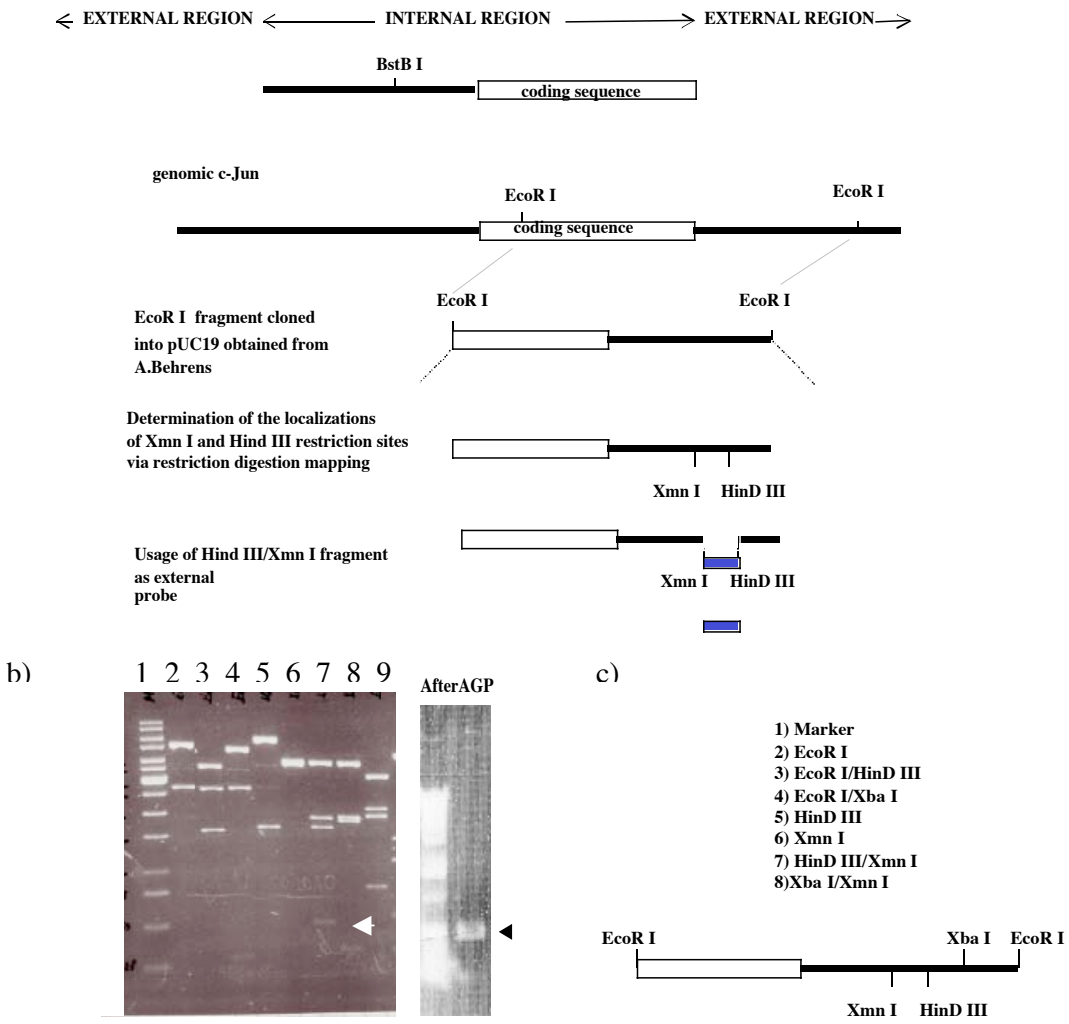
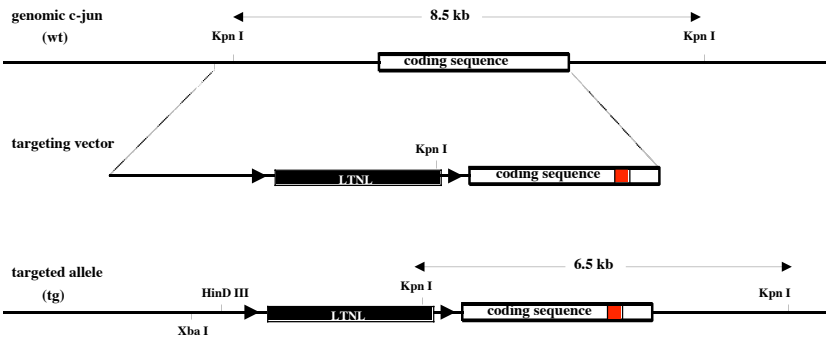


Figure 8: Schematic representation of the generation of the external probe in order to use for southern blot analysis. **(a)**. The restriction mapping was performed in order to map the downstream region which is not covering the targeting vector. The external probe was made by Hind III/ Xmn I digestion (white arrow) and used after agarose gel purification (AGP) **(b)**. The Xmn I, Hind III and Xba I sites were the relevant restriction sites for further analysis.

3.1.7 Southern-Blot Analysis

The southern blots were generated with the restriction enzyme Kpn I. The DNAs from 217 clones derived from the electroporations with the „m0.1„ and 120 clones derived from the electroporations with the „m0" targeting vectors were isolated and digested for southern analysis. The hybridization of the external probe with the wild type allele resulted in the generation of a 8.5 kb fragment. The homologous recombination between the targeting vector and the genome in ES cells decreased the size of 8.5 kb fragment in wild type allele to 6.5 kb in targeted allele. In six „m0" derived and two „m0.1" derived clones homologous recombination had occurred. The detection of the homologous recombination between the „m0„ and „m0.1" targeting vectors and the genomic c-Jun is shown schematically in figure **9a**. Figure **9b** represents the detection of the homologous recombination via an external probe by southern analysis.

a)



b)

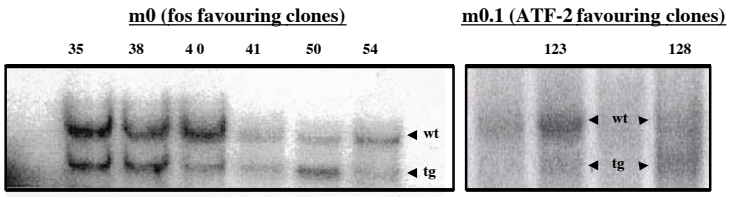


Figure 9: Gene targeting of c-Fos and ATF-2 favouring vectors **(a)** Targeting of the „m0.1„ and „m0„ targeting vectors to their cognate locus. Diagram representing the targeting vector (upper lane) and its method of integration into the genomic *c-Jun* (middle lane) locus. The targeting vector is represented schematically. The bold d line in 5´of the targeting vector corresponds for the promoter. The black arrowheads correspond to the 34 bp lox sites and the black box between these two sites corresponds for the LTNL selection marker. The white box in the downstream of the promoter and LTNL correspond for the coding sequence and the red colored square in this box corresponds for the sequence encoding the basic leucine zipper region where the point mutations are introduced. The digested DNAs of targeted alleles are hybridized via an external probe. The digestion of genomic *c-Jun* with Kpn I and hybridization with the external probe results in the generation of a large wild type fragment (middle lane). The homologous recombination between the genomic c-Jun and the targeting vector reduces the size of the mutated fragment

obtained after the Kpn I digestion and hybridization by southern analysis (lower lane). **(b)** Southern blot analysis of „m0.1„ and „m0„ targeted clones. Kpn I digested genomic DNA displays a single band of a large size (indicated as wild type). Homologous recombiation of a single copy of the „m0.1„ and „m0„ targeting vectors into their cognate locus results in production of an additional small mutated fragment (indicated as mutated).

3.1.8 Thawing of ES cell lines and transient transfection with *Cre*

"m0.1" clone numbers 123 and 128 and "m0" clone numbers 35,38,40,41,50 and 54 were identified as the clones which could be analyzed for further studies. The selection cassette had to be removed *in vitro* from the genom of the manipulated ES cells by the transient transfection of a *Cre* expression plasmid since the *tk*-gen in the HSV-tk/PGK-neo selection cassette is incompatible with the germline transmission of mutated ES cells. Another reason to remove the marker cassette LTNL was that it would potentially disturb expression of c-Jun. To remove the marker cassette, I transfected a *Cre* expression plasmid. The *Cre* (causes recombination)-protein is an enzyme of bacteriophage P1. This recombinase specifically removes DNA sequences which are flanking between the loxP-(locus of crossover P1) elements in same orientations. *Cre* recognizes the lox sites and causes site directed recombination eliminating the region between the lox sites.

The 96 well plates containing the "m0.1" clone numbers 123 and 128 and "m0." clone numbers 35,38,40,41,50 and 54 were thawn and grown to confluency. Then they were used for *Cre*- transient transfection. 20 microgram of *Cre* expressing plasmid was electroporated into positive cell lines. The cell lines were selected with ganciclovir. HSV-tk cassette makes the cells sensitive for ganciclovir, so the cells that contain this marker die in a selective medium containing ganciclovir. Only cells which lost the LTNL marker cassette would survive in a medium containing ganciclovir. Cells were cultivated in ganciclovir medium and clones for each cell line were picked for further analyses. They were digested with Kpn I in order to detect whether the LTNL cassette was removed or not by southern blot analysis. The digestion of the targeted allele with the restriction enzyme Kpn I resulted in the generation of a 6.5 kb fragment. The 8.5 kb fragment size of endogeneous allele was decreased to 6.5 kb because of the insertion of the Kpn I

restriction enzyme site in the LTNL cassette into the genome of the transfected ES cell. This Kpn I site disappeared after the *Cre* transient transfection due to the removal of the LTNL cassette and increased the size of targeted allele from 6,5 kb to 8.5 kb. This alteration in the size of targeted allele indicated that the removal of the LTNL was successful.

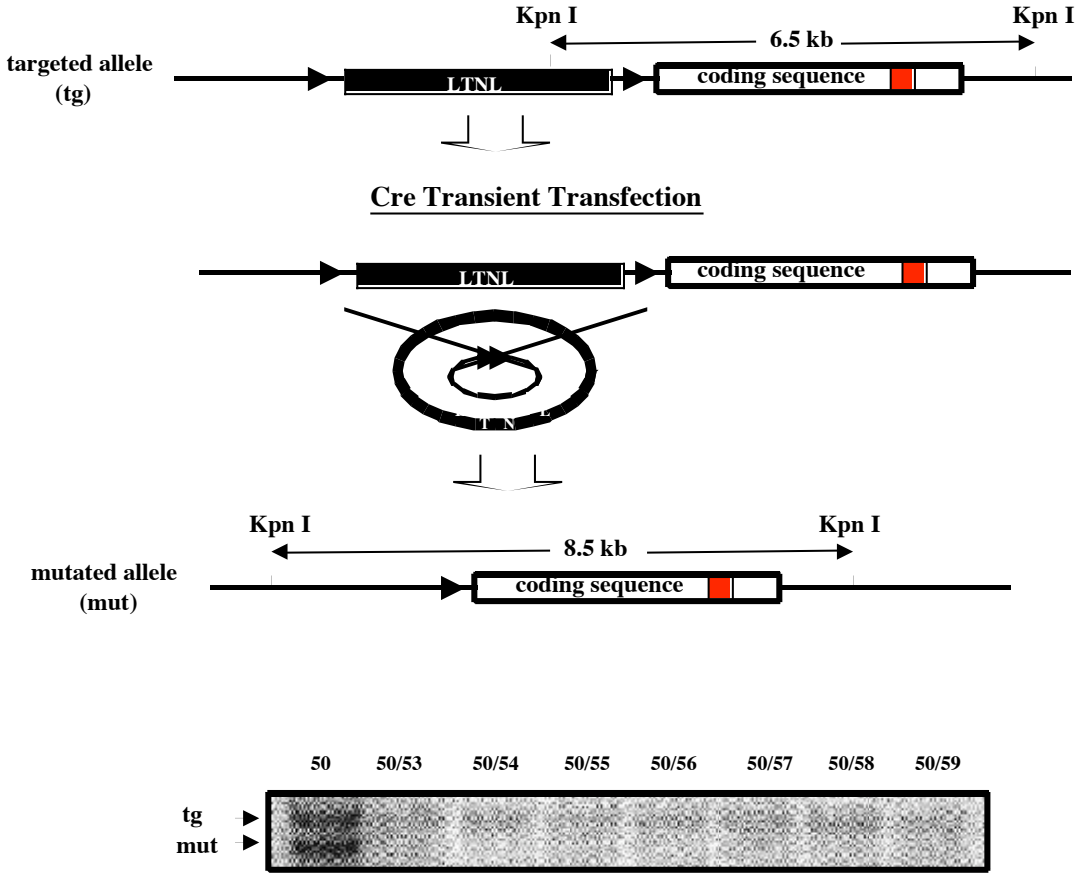


Figure 10: Removal of the LTNL marker cassette. Schematic representation of *Cre* transient transfection and the products of *Cre* transient transfection. Through the *Cre* transient transfection, the LTNL is recognized from the both lox sites and removed. The presence of the LTNL in the targeted allele between two lox sites is shown in the upper part of the figure. *Cre* transient transfection results in the removal of the LTNL from the promoter and the remaining of a single lox site as shown in the lower part of the figure. The removal of LTNL leads to the increase of 6.5kb fragment that can be obtained via Kpn I digestion by southern analysis to 8.5kb fragment after LTNL removal.

3.1.9 Verification of the presence of mutations in the basic leucine zipper domains

The targeting vectors were electroporated into ES cells. The insertion of the marker casette and the occurrence of the homologous recombination between the targeting vector and the genomic c-Jun was checked by southern analysis. The next step of the study was to check the presence of the mutations at the leucine zipper domain. It was important to know whether the mutations at the basic leucine zipper domain was maintained during the manipulation of the ES cells. In order to get rid of the risk that the mutations at the basic leucine zipper domain might be lost, a southern blot strategy was designed. The presence of Xba I diagnostic site in "m0.1" enabled the check for the presence of mutations in basic leucine zipper region by southern blot analysis. The analysis of "m0.1" clones for the maintenance of the mutations by southern is shown in figure 11. The maintenance of the mutations in basic leucine zipper region in "m0.1" clones was confirmed by a PCR based genotyping.

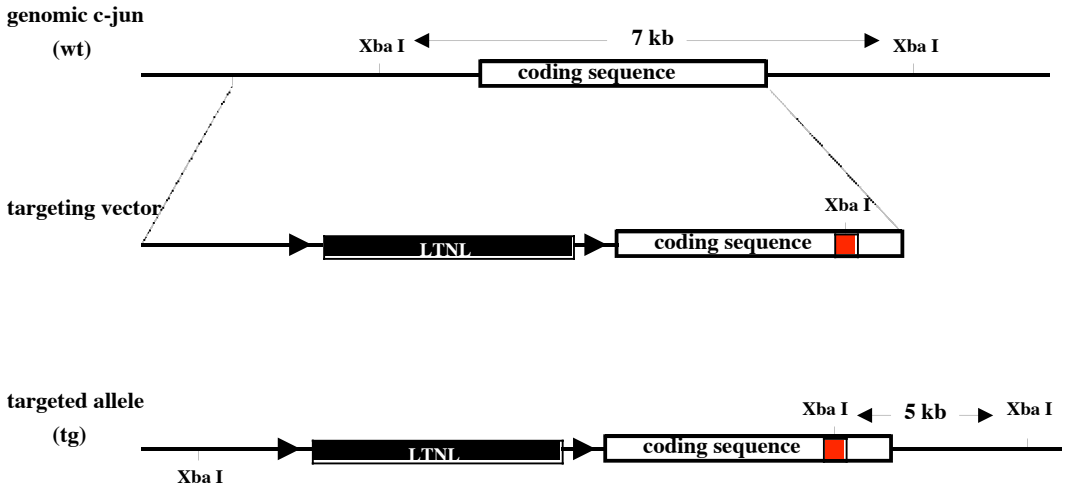


Figure 11: Detection of the presence of mutations in bZIP after genomic integration. Targeting of the „m0.1„ targeting vector to its cognate locus and the detection of the presence of the mutations in basic leucine ziper region in the targeted allele. Diagram representing the „m0.1„ targeting vector (upper lane) and its method of integration into the genomic c-Jun (middle lane) locus. The targeting vector is represented schematically. The bold d line in 5´of the targeting vector corresponds for the promoter. The triangels correspond for the 34 bp lox sites and the black box between these two sites corresponds for the LTNL selection marker. The white box in the downstream of the promoter and LTNL corresponds for the coding sequence and the coloured square in this box corresponds for the sequence encoding the basic leucine zipper region where the point mutations are introduced in. Xba I restriction enzyme digestion is used for southern blot analysis. The digested DNAs of targeted alleles are hybridized via an external probe covering a region in downstream of the coding sequence of c-Jun which does not overlap with the targeting vector. The digestion of genomic c-Jun with Xba I and hybridization with the external probe results in the generation of 7 kb wild type fragment (middle lane). The homologous recombination between the genomic c-Jun and the targeting vector introduces a diagnostic Xba I site into the coding sequence of the targeted allele, which reduces the size of the mutated fragment obtained to 5kb after the Xba I digestion and hybridization by southern analysis (lower lane). ladder).

3.1.10 Genotyping by polymerase chain reaction

The genotyping was designed to indicate the presence of the Jun mutations but should also help later in defining the genotype of transgenic mice. The principle of the strategy

was as follows. A 0.6 kb fragment containing the basic leucine zipper region with mutations was amplified by PCR. According to the digestion results of this amplified region with the diagnostic enzymes, the presence of the mutated alleles were determined. Figure 12 shows the PCR based genotyping strategy with the "m0.1" targeted allele.

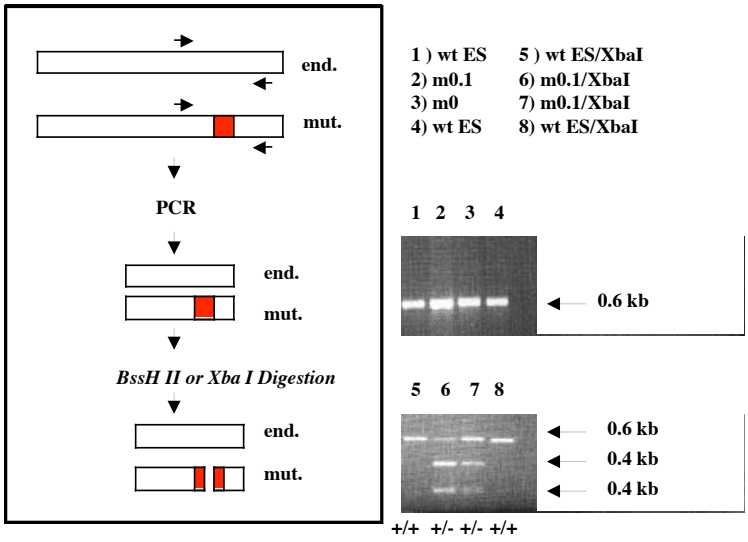


Figure 12: Genotyping by PCR**.** Schematic representation of the PCR based genotyping strategy. A 6kb fragment containing the basic leucine zipper mutation domain is amplified. The digestion of this fragment with the diagnostic XbaI in "m0.1" heterozygote cells, results in the generation of the 0.6 kb endogenous fragment and the 0.4 kb and 0.2kb fragments indicating the presence of the diagnostik restriction site in the targeted allele.

3.1.11 Blastocyst injection

The clones identified to carry the proper mutations and to have lost the LTNL casette were injected into blastocytes. Because of the reason that the blastocytes (C57BL/6J; black coat colour) and KPA (OLA/129; beige/brown coat colour) and 129 ES cell lines are not stemming from the same mouse line, a chimaeric animal with a different coat color will be generated upon the integration of the embryonic stem cells (Thomas and Kühn, 1997). The degree of the chimaerism, indicates how many ES cells contributed to

the transgenic animal and can be estimated by the coat color. Figure 13 shows the chimeras derived from KPA ES cell lines studied.

Figure 13: Chimaeric animals obtained from "m0.1" KPA derived cells. The mice indicated with white arrows have lighter coat coloures, so are higher chimaerics.

3.1.12 Current status of the work

Unfortunately, the chimaeric animals derived from KPA and E14.1 ES cells lines did not give transgenic offsprings, the germ line transmission with these ES cells were not successful. Using 129Sv/Pas ES cell lines the whole study was repeated. The "m0" chimaeras are made. "m0.1" clones are ready for blastocyst injection. The studies are ongoing.

3.2 PART 2: DETERMINATION OF AP-1 SUBUNIT DIMER PARTICIPATING IN CROSSTALK WITH GR

AP-1 activity is a function of a number of distinct dimeric protein complexes consisting of members of the Jun and Fos families, as well as specific members of the ATF/CREB families e.g *c-Jun, Jun B, Jun D, c-Fos, Fos B, Fra-1, Fra-2* and ATF-2. Depending on the composition of the dimer, different sequence elements are preferentially recognized. Jun/Fos dimers preferentially bind to heptameric AP-1 binding sites while Jun/ATF dimers bind octameric AP-1 binding sites.

MAPK pathways regulate both the amounts and transactivating capacities of the Jun, Fos and ATF-2 proteins in a stimulus-specific manner: treatment of fibroblasts with PDGF, serum or phorbol esters predominantly activates the Extracellular-Regulated Kinase (ERK) members of the MAPK family, which leads to strong stimulation of Jun/Fos activity via *de novo* synthesis of c-Jun/c-Fos, Jun B/c-Fos, and Jun B/Fos B. Treatment of the same cells with stress-inducing stimuli like ultraviolet light or alkylating agents predominantly activate the Jun N-terminal Kinase (JNK)/Stress Activated Protein Kinase (SAPK) members of the MAPK family which preferentially enhance Jun/ATF-activity via phosphorylation of c-Jun-Ser63/73 and ATF-Thr69/71 located in the respective transactivation domains. The Jun activity is enhanced through the *de novo* synthesis Jun/Fos, Jun/ATF and ATF/ATF

It is reported that GR inhibits AP-1 target gene activity by the inhibition of signal transduction. The inhibition of the phosphorylation of c-Jun amino terminal serine 63 and 73 residues by JNK is proposed to be the model for the repression of AP-1 activity by GR. There are also reports where it is shown by indirect evidences that GR repress AP-1 activated transcription by tethering to the bound AP-1 factor (Konig 1992). Through chromatin immunoprecipitation assays using anti-GR polyclonal antibodies it has been also shown directly that GR associated with the AP-1 bound col3A (collagenase 3A) element *in vivo* in a hormone dependent manner. TIF2 and GRIP1 were the members of p160 coactivators which were found to be associated as TIF2/GRIP1 with GR at the

col3A element with GR. GRIP 1 was found to be a coactivator which potentiating GR-dependent transcriptional repression (Rogatsky *et al.*, 2001).

Taken together, by knowing that GR inhibited AP-1 activity by direct protein-protein interaction, it was decided to find out whether c-Jun/ATF-2 subunit or c-Jun/c-Fos subunit of AP-1 was involved in crosstalk with GR. An experimental setting was designed to test whether c-Jun/c-Fos complex or c-Jun/ATF-2 complex was involved in the repression of AP-1 target gene activity by GR. In order to distinguish between the functions of Jun/Fos and Jun/ATF on the repression of AP-1 target gene activity, I made use of genetically manipulated fibroblasts. *c-Jun* -/- cells were stably transfected with constructs expressing c-Jun-ER dimerization mutants. These mutant constructs consist of an EcoRI fragment of *c-Jun* encoding the amino terminus of the protein including the basic leucine zipper domain and a sequence encoding the estrogen receptor ligand binding domain in the carboxyl terminus. The mutations propogating the proteins to dimerize with c-Fos or ATF-2 were inserted into the basic leucine zipper of c-Jun sequence. The construct without any mutations in wild type c-*Jun* basic leucine zipper domain fused to estrogen receptor ligand binding domain was also generated and called wt-ER fusion protein. The estrogen receptor ligand binding domain was important for the translocation of the fusion cells from the cytoplasm to the nucleus since the fusion proteins were cytoplasmic upon their activation with tamoxifen via the estrogen receptor ligand binding domain. The constructs were under the control of the ubiquitin promoter. The stably transfected fusion protein expressing cells were made by the cotransfection with a hygromycin resistance vector. These cells were made by and obtained from Dr. Axel Szabowski (DKFZ).

I used the phorbol ester TPA in order to activate the ERK members of MAPK family, to observe the regulation in transcriptional activation on heptameric TRE binding sites in *c-Jun* -/-, c-Jun/c-Fos (m0-ER) cells. UVC light was used in order to activate the JNK/SAPK members of MAPK family, to observe the regulation in transcriptional activation on octameric TRE binding sites in *c-Jun* -/-, c-Jun/ATF-2 (m0.1-ER) cells. Reporter assays were performed to see the transcriptional activation on heptameric

collagenase-TRE reporter in m0-ER cells upon TPA stimulation. The increase in transcriptional activation on octameric jun-TRE reporter in m0.1-ER cells was tested upon UVC stimulation. As control I used c-Jun deficient fibroblasts stably transfected with the wild type c-Jun expression vector (wt-ER). By using the mutant fibroblasts, we wanted to determine the composition of the AP-1 which might be involved in the crosstalk with GR.

	wt	**c-jun -/-**	**c-jun -/- c-Jun-wt ER**	**c-jun -/- c-jun-m0 ER**	**c-jun -/- c-jun-m0.1 ER**
			-TAM +TAM	**-TAM +TAM**	**-TAM +TAM**
	- TPA UV	**- TPA UV**	**- TPA UV - TPA UV**	**- TPA UV - TPA UV**	**- TPA UV - TPA UV**
collagenase TRE-LUC	- ++ +	- - -	- - - - ++ +	- - - - ++ +	- - - - - -
jun TRE-LUC	- + ++	- - -	- - - - + ++	- - - - - -	- - - - + ++

TAM: Tamoxifen

Figure 14: The experimental setting for reporter assays. The transcriptional regulation mediated by distinct c-Jun dimers on reporter constructs using *c-Jun* -/- c-Jun-wt-ER, c-Jun -/- c-Jun-m0-ER and *c-Jun* -/- c-Jun-m0.1-ER fibroblasts were to be addressed

3.2.1 Optimization of transfection conditions in genetically manipulated fibroblasts which express the fusion proteins

In order to test the AP-1 reporters, it was planned to transfect the fibroblast cells transiently with the reporter constructs, starve the cells for 24 hours and finally induce the cells by the phorbol ester TPA and by UVC. Before initiating these experiments it was important to check whether the cells were mycoplasma-contaminated or not since it is known that mycoplasma-contamination may decrease the transfection efficiency of the cells. A PCR based mycoplasma test had been performed, in order to find out whether the

cells were contaminated and it came out that the fibroblasts were contaminated as shown in figure 15

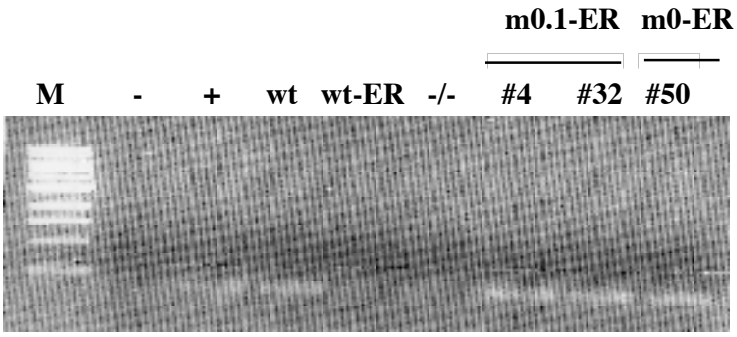


Figure 15: Detection of mycoplasmic contamination by a PCR based method. (-) indicates the negative control, (+) the positive control for mycoplasma contamination. The wt fibroblasts, #4 and #32 m0.1-ER fibroblasts and #50 m0-ER fibroblasts are positive for mycoplasma contamination as the PCR products were amplified due to the contamination as indicated by the arrow

The cells were cleaned with plasmocin, a drug destroying the cell wall of the bacteria. The next step was to check the transfection efficiency in these fibroblast cells. The cells were checked for their transfection efficiency mediated by both calcium-phosphate and lipofectamine transfections in order to find out the best method that could be used for transfections. Cell number, DNA amount and two transfection methods were used as parameters to optimize the transfection. Cells were counted and 400.000 and 800.000 cells were transfected with 2,5 µg and 5 µg of green fluorescence protein (GFP) expressing plasmid DNA by calcium-phosphate and lipofectamine methods. The transfection efficiency was measured by fluorescence-associated cell sorting (FACS) analysis which would detect green fluorescence protein expressing cells. The transient transfection of the fibroblast cells by the calcium-phosphate method was very inefficient, it was not possible to obtain any transfection efficiency in *c-Jun* -/- cells. The transfection efficiency of *c-Jun* -/- c-Jun-wt-ER cells by calcium-phosphate precipitation was also very low. By calcium-phosphate precipitation studies it was observed that less number of

cells transfected with higher amount of DNA increased the transfection efficiency as shown in figure 16.

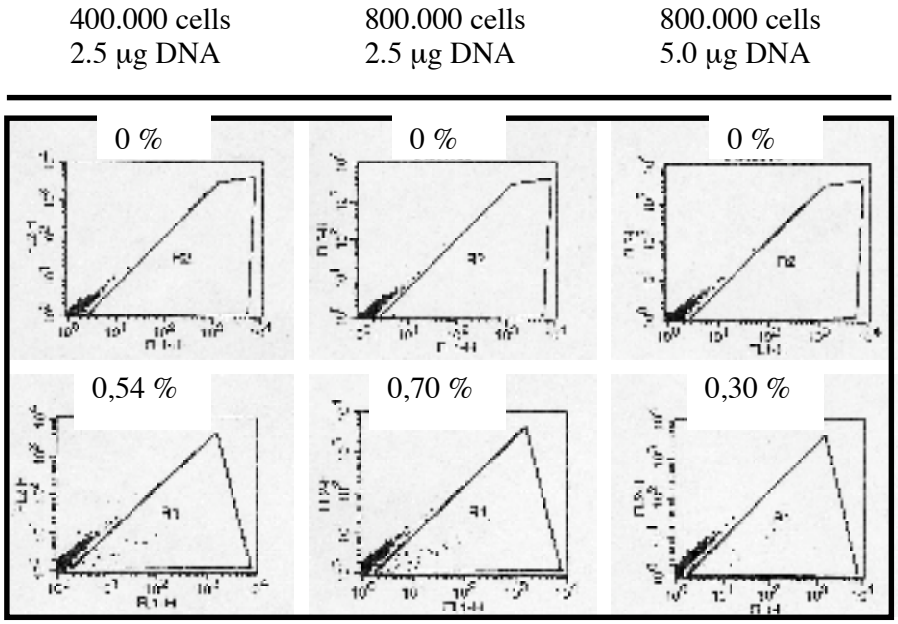


Figure 16: FACS analysis of fibroblasts transfected by calcium-phosphate precipitation. Cells were transfected with GFP expressing construct. Transfection efficiency was determined by percentage of the cell populations in the gate due to the shift in FL-1 channel upon expression of GFP. Even under the best conditions transfection efficiency was below 1%.

In order to work with higher transfection efficiencies, the lipofectamine plus method was used. The *c-Jun* -/- fibroblasts were transfectable by the lipofectamine plus method but the transfection efficiency was again not very high. In *c-Jun* lacking fibroblasts stably transfected with the wild type c-Jun expressing vector (c-Jun -/-, c-Jun-wt-ER) it was obvious that the transfection of 400.000 and 800.000 cells with 2,5 µg and 5 µg of DNA by lipofectamine plus method end up with much higher transfection efficiency than the transfection of 400.000 and 800.000 cells with 2,5 µg and 5 µg of DNA by calcium-

phosphate method. In optimal conditions, the transfection efficiency by lipofectamine plus was approximately six fold higher than that by the calcium-phosphate method. The result of FACS analysis by lipofectamine plus transfection is shown in figure 17.

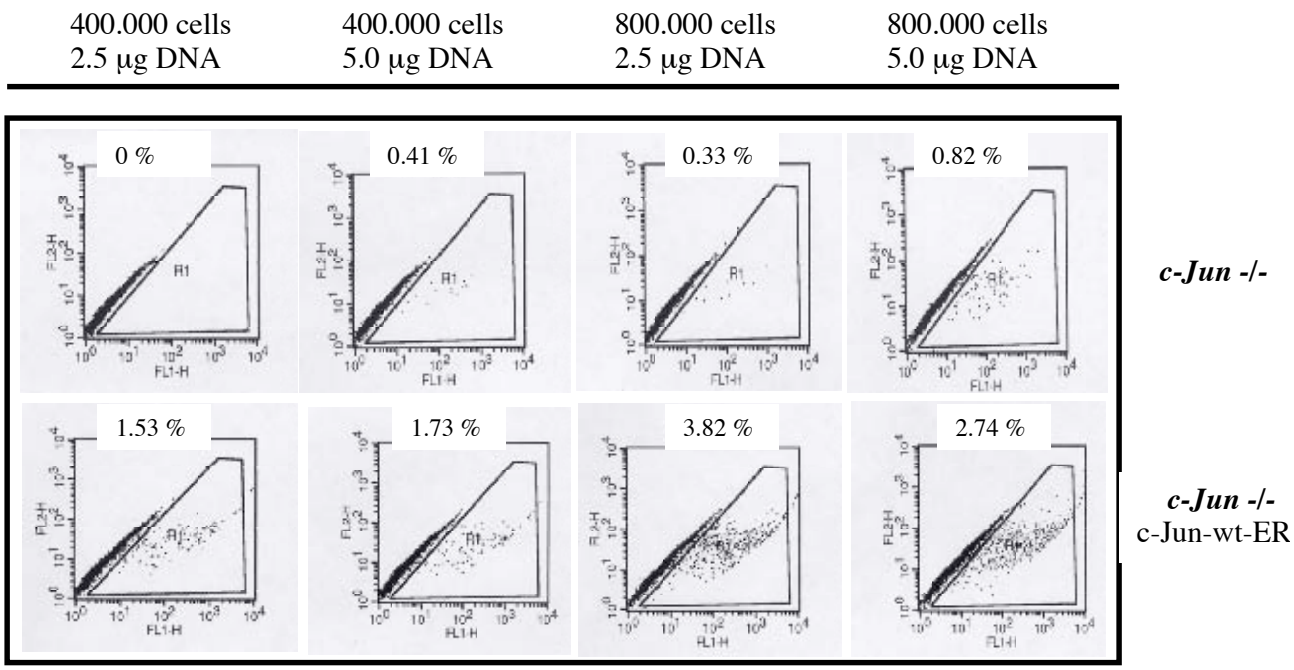


Figure 17: FACS analysis of fibroblasts transfected by lipofectamine-plus method. Cells were transfected with GFP expressing construct. Transfection efficiency was determined by percentage of the cell populations in the gate due to the shift in FL-1 channel upon expression of GFP. Transfection of c-Jun -/- fibroblasts was slightly increased to 0.8% whwn 800.000 cells were transfected with 5ug DNA (compare with figure 16 right top plot in figure 16). In addition transfected c-Jun -/- c-Jun wt-ER cell population increased to 3.82% (compare with 0.7% middle bottom plot in figure 16).Lipofectamine plus method in comparison to calcium phosphate precipitation resulted in slightly better transfection efficiency.

3.2.2 Translocation of cytoplasmic wild type c-,Jun, c-Fos favoring c-,Jun and ATF-2 favouring c-,Jun fusion proteins into the nucleus in genetically manipulated fibroblast cells

Before initiating the reporter assays, it was important to know that the fusion proteins were translocated into the nucleus. The transcriptional activation on reporter constructs would only be observed after the translocation of the fusion proteins into the nucleus via their estrogen receptor ligand binding domain upon tamoxifen induction and binding in the nucleus to the reporter constructs. The principal idea in these assays was to induce the genetically reconstituted cells with the phorbol ester TPA and UVC and study the transcriptional regulation on the reporter constructs mediated by the fusion proteins. The fusion proteins are localized in the cytoplasm of the cells and the translocation of these proteins into the nucleus happens through the stimulation of these cells with tamoxifen which is an estrogen analog. The structure of the fusion proteins in reconstituted cells and the presence of the estrogen receptor ligand binding domain in fusion proteins, end up with the nuclear translocation of the fusion proteins upon tamoxifen treatment

Immunofluorescent staining of the cells before and after tamoxifen induction was used in order to see the nuclear localization of the fusion proteins into the nucleus. Figure 18 shows the results obtained by immunofluorescent staining, Immunofluorescent staining of the cells resulted in the conclusion that fusion proteins in reconstituted cells were translocated into the nucleus upon tamoxifen treatment.

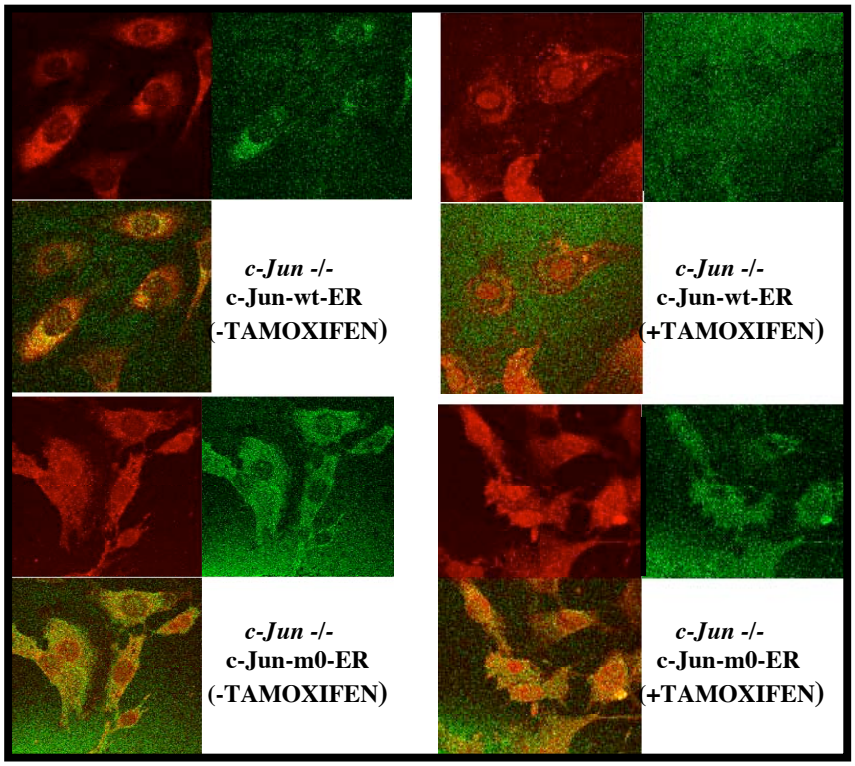


Figure 18: Detection of nuclear translocation of fusion proteins by immunofluorescent staining. *c-Jun* -/- c-Jun-wt-ER and *c-Jun* -/- c-Jun-m0-ER cells were used for immunofluorescent based detection of the fusion proteins in the nucleus upon tamoxifen treatment. The localization of the proteins before tamoxifen induction is both in *c-Jun* -/- c-Jun-wt-ER and *c-Jun* -/- c-Jun m0-ER cells cytoplasmic. The treatment of the cells with tamoxifen results in the translocation of the fusion proteins into the nucleus. The nuclear localization of the fusion proteins upon tamoxifen treatment is observable by immunofluorescent staining.

3.2.3 Transcriptional regulation on collagenase-TRE reporter in reconstituted fibroblasts

Collagenase-TRE –517/63 was used initially in order to study the transcriptional regulation in *c-Jun* -/- c-Jun wt-ER, *c-Jun* -/- c-Jun m0-ER and *c-Jun* -/- c-Jun m0.1-ER cells on reporter constructs. Wild type *c-Jun* fibroblast cells were used in order to compare the transcriptional activity of the reconstituted cells with the wild type

fibroblasts. *c-Jun* -/- cells were used as the negative control of the experiment. *c-Jun* -/- cells, *c-Jun wt* cells, *c-Jun* -/- c-Jun wt-ER, *c-Jun* -/- c-Jun m0-ER and *c-Jun* -/- c-Jun m0.1-ER cells were transiently transfected with the reporter construct –517/63 collagenase-TRE. Even the reporter assay on collagenase-TRE construct was performed several times, I was not able to obtain a consistent and conclusive result. There was a high basal level of transcription on this reporter in genetically manipulated fibroblast cells even in unstimulated case, and neither TPA nor UVC stimulation ended with a conclusive results.

Another experimental set-up was designed in order to obtain conclusive results from the reporter assay on the same collagenase reporter. Instead of using the stably transfected reconstituted cells, the *c-Jun* -/- cells were transiently transfected with the constructs expressing the fusion proteins; so the cells were transiently transfected with ubi-c-Jun-wt-ER, ubi-c-Jun-m0-ER and ubi-c-Jun m0.1-ER. The genetically manipulated fibroblasts were cotransfected with the reporter construct collagenase-TRE –517/63. After transient transfection, the cells were serum-starved for 24 hours and then stimulated by TPA and UVC. In both experimental settings phenol/red free medium and chuckhall stripped serum was used in order to remove the residual steroids which might activate estrogen receptor. The results obtained by reporter assays based on the transient transfection of the *c-Jun* -/- cells and cotransfection with collagenase-TRE reporter also did not give conclusive results

Since the studies on reporters did not result in a conclusive outcome, it was decided to study the transcriptional regulation on endogenous targets of c-Jun using the genetically reconstituted cells we have.

3.2.4 Transcriptional regulation on endogeneous target genes in reconstituted fibroblasts

Fra-1 was the target gene with which the studies on endogenous targets of *c-Jun* in reconstituted fibroblast cells was initiated. *Fra-1* contains a heptameric AP-1 binding element. As in reporter assays, the cells were serum starved for 24 hours and the translocation of the fusion proteins into the nucleus was mediated by the addition of tamoxifen. The cells were treated with the phorbol ester TPA or UVC, harvested after 6 hours and total RNA was isolated. RNA was quantified by RT-PCR in order to see the transcriptional regulation on *Fra-1*. In *c-Jun* -/- fibroblasts no transcription was observable. In c-Jun wild type cells, transcriptional activation of *Fra-1* was seen upon TPA or UVC stimulation. This was showing that *Fra-1* is a target of *c-Jun*. In *c-Jun* -/- c-Jun wild type, in the absence of tamoxifen, a band was seen upon TPA stimulation. This band was also seen after tamoxifen addition upon TPA stimulation. It was thought that this might be due to the leakiness of c-Jun expressing fibroblasts. In *c-jun* -/- c-Jun m0-ER cells, expressing c-Fos favouring c-Jun mutant, no transcriptional activity was seen in the absence of tamoxifen. Addition of tamoxifen and stimulation of c-Fos favouring c-jun fibroblast cells with the phorbol ester TPA resulted in an increased transcriptional activity since Fra-1 had the heptameric AP-1 binding site. In ATF-2 favouring c-Jun fibroblasts no transcriptional activity was seen since the octameric cis-acting element for the binding of c-Jun/ATF-2 dimer was not present in the promoter of *Fra-1*. These results were relevant and conclusive. The results obtained are shown in figure 19.

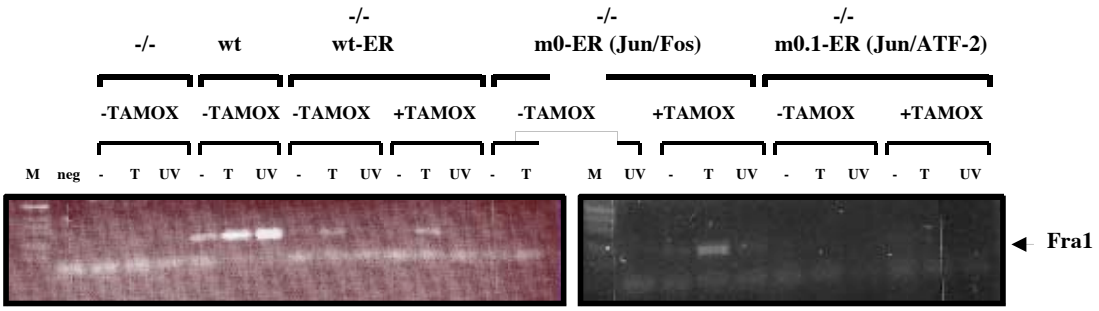


Figure 19: Analysis of transcriptional regulation on the endogeneous target *Fra-1* by RT-PCR based method. The cells were serum starved for 24 hours tamoxifen induced, TPA and UVC stimulated after 24 hours of serum starvation. Total RNAs were extracted and c-DNAs were synthesized. These c-DNAs were used to determine the transcriptional regulation on *Fra-1*

Another PCR strategy was designed in order to check the transcriptional regulation on *c-Jun*. The reason for this PCR was to see the regulation on octameric AP-1 binding site of endogeneous *c-Jun*. The *c-Jun* -/- cells used for the generation of genetically reconstituted fibroblasts were inactivated by the targeting of the neomycin selection cassette into the genomic locus of *c-Jun*. By using a PCR strategy where one of the primers annealed with the neomycin cassette and the other primer with a region 5´ of neomycin cassette in the coding sequence of *c-Jun*, the transcriptional activation on endogeneous c-Jun was studied. The results obtained from cold PCRs did not allow a quantification and it was also not possible to obtain a conclusive result concerning the transcriptional regulation of endogeneous c-Jun. Semiquantitative PCRs were performed in order to obtain conclusive results due to the sensitivity of the semiquantitative PCR. In semiquantitative PCR, radioactive labeled dCTP is used additional to the dNTPs used for the amplification by cold PCR. The expectation was to see an increased transcriptional activation upon tamoxifen addition and UVC stimulation in c-Jun -/- cells expressing wild type c-Jun and in ATF-2 favouring c-Jun mutant expressing cells. The increase in transcriptional activation upon tamoxifen addition and UVC stimulation could be shown (indicated in number 12) Figure 20 shows the results obtained by semiquantitative PCR. A basal level of transcription was observable as it is shown in the results of PCR in *c-Jun*

-/- cells. As expected no amplification was seen in wild type fibroblasts since they did not contain the neomycin cassette. In *c-Jun* -/-, c-Jun-wt-ER cells, in the absence of tamoxifen in unstimulated situation a high basal transcriptional level was obtained. The transcriptional level upon UVC stimulation was as much as in c-Jun -/- cells. The addition of tamoxifen and stimulation with the phorbol ester TPA resulted in two fold increase in transcription. This increase was observed as four fold upon UVC stimulation of *c-Jun* -/-, c-Jun-wt-ER fibroblast cells. This was due to the binding of Jun/ATF proteins to the octameric AP-1 binding site on c-Jun promoter. In *c-Jun* -/-, c-Jun-m0-ER cells, there was again a high basal transcriptional level in unstimulated situation. UVC stimulation resulted in a two fold increase in transcription. TPA mediated transcription was lost. Upon tamoxifen induction, in unstimulated and TPA stimulated fibroblasts almost no transcription was observable however UVC irradiation resulted in a four fold increase in transcriptional activation. The results obtained by c-Jun -/- c-Jun-m0-1-ER fibroblasts were not explainable. The basal transcriptional level was too high and no increase in transcription was seen.

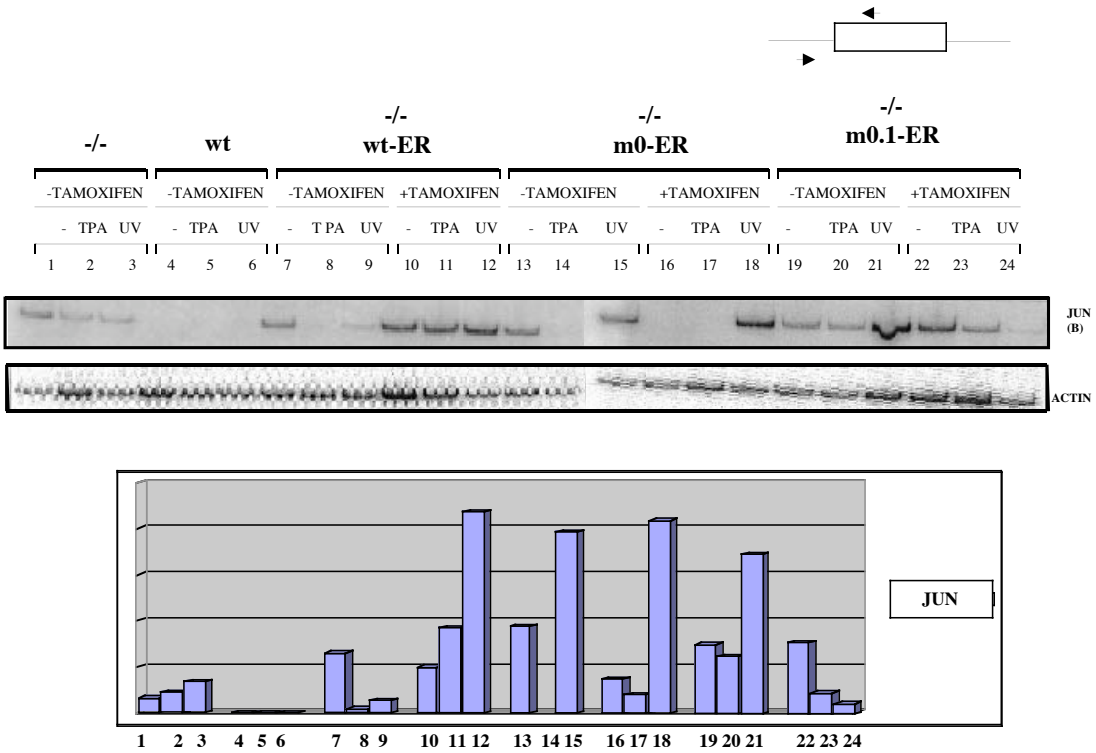


Figure 20: Analysis of transcriptional regulation on the endogeneous Jun promoter by semiquantitative PCR. The cells were grown, serum starved for 24 hours and induced with tamoxifen for nuclear translocation. They were stimulated with TPA and UVC for 6 hours and harvested for total RNA extraction and cDNA synthesis. The c-DNAs were used for semiquantitative PCRs. The actin PCR was used for equalization of the amount of PCR products. The fragments of 1.5 kb amplified by Jun and neo primers were quantified by phoshoimager

3.2.5 Current status of the work

Neither by reporter assays nor by the assays on endogeneous targets, no conclusive result was obtained. The next step will be the generation of kinetic studies to find out the optimal time point where the transcripts are present in the nucleus, to design and optimize new PCR conditions and to repeat the experiments on endogeneous targets.

4. DISCUSSION

4.1 PART 1: WORK ON TRANSGENIC MICE

Introduction of site-specific modifications into the mouse genome by homologous recombination is called gene targeting and is generally used for the production of mutant animals to study gene functions *in vivo*. Homologous recombination of foreign DNA with endogeneous genomic sequences is a relatively infrequent event in mammalian cells, compared to its random integration. The utilization of pluripotent murine embryonic stem cell (ES) lines in gene targeting came out to be an efficient method to study gene function *in vivo* since genetically modified ES cells can contribute, even after extensive *in vitro* manipulations, to all cell lineages including germ cells of the resulting chimaeric animal when they are introduced into a preimplantation embryo. The breeding of germline chimaeras which transmit an ES-cell derived mutant chromosome(s) to their progeny, allows the establishment of an animal heterozygous for the genetic alteration. Further breeding of these heterozygote animals results in the generation of the mutant homozygous mice (Brinster *et al*., 1994).

There are several gene targeting strategies as simple gene activation with a replacement type vector, introduction of point mutations by "hit and run" method, introduction of a point mutation by double replacement method or coelectroporation of a targeting vector with a point mutation and a second targeting vector with the marker in the target sequence, which might be used for genetic manipulations in ES cells (Torres and Kühn., 1997). The use of Cre/loxP recombinase system is the latest addition in the technical improvements in gene targeting. This is based on the recognition of 34 bp sequences refererred to as loxP (locus of crossover in P1) and site-specific recombination mediated by the bacteriophage Cre recombinase (Sternberg *et al.*, 1981). Cre/lox system allows, apart from the introduction of subtle mutations, for a number of other genotypic options in ES cells by the strategic incorporation of loxP sites into the targeting vector. It is

reported to be a technically easier and more reproducible system compared to the other two-step targeting methods.

The construction of the targeting vector is a very important aspect of gene targeting and to know how much genomic DNA is available, how well the locus is characterized and what convenient restriction sites exist, is important to initiate the studies for gene targeting. The DNA sequence of the *c-jun* promoter and of the coding sequence are well documented. The locus of *c-Jun* is well-characterized. A BstB I restriction site in the promoter is a unique site to insert the LTNL marker containing the HSV-tk and PGK-neo cassettes. Appropriate restriction sites were available to construct the both targeting vectors by blunt-end- or sticky-end-ligation-based cloning strategies as explained in the result section in detail.

It has been reported that the targeting vector used should be from the same source as that of the ES cell to avoid a reduced targeting frequency due to the sequence polymorphism (te Riele *et al*., 1992). The source of the genomic DNA utilized here was therefore that of the targeted KPA ES cells, so the reduction of targeting frequency caused by the difference in sequence polymorphism between the targeting vector and targeted ES cell DNA was avoided. However it was also reported that the usage of nonisogenic DNA results in similar targeting frequencies as obtained with isogenic DNA. The targeting vectors in this study were also transfected into the E14.1 and 129 Sv/Pas ES cell lines. The use of isogenic DNA is ideal to avoid the reduction of targeting frequency.

The length of the region of homology is also important. It is believed that the longer the region of homology the higher the frequency of homologous recombination (Deng *et. al*., 1992). The homologous region in our constructs is about six kb. All targeting constructs possess a short and long arm of homology. Lengths of at least 0,5kb for the short arm and 4kb for the long arm are considered generally. However these lengths are very general and not definitive.

The selection of the marker cassette is also important. The use of the bacterial aminoglycosidase phosphotransferase (neo) gene controlled by the HSV-tk promoter is reported to provide efficient selection. The use of stronger expression cassettes does not necessarily equal better selection since a weak neo gene like MC1neopA confers G418 resistance when integrated at a targeted genomic locus (Soriano *et al.*, 1991). The background of random integrants may be increased by the use of a more potent neo expression and thereby reduce the frequency at which homologous recombinants are isolated. For this reason we used aminoglycosidase phosphotransferase controlled by the HSV-tk promoter in order to construct our targeting vector.

The presence of a unique restriction site linearizing the targeting vector is important since homologous recombination occurs more frequently using linear DNA (Hasty *et al.*, 1992). Not I was the unique restriction site linearizing our targeting vectors.

The strategy how our targeting vectors are generated, how homologous recombination occurred, how LTNL was removed is summarized in figure 21.

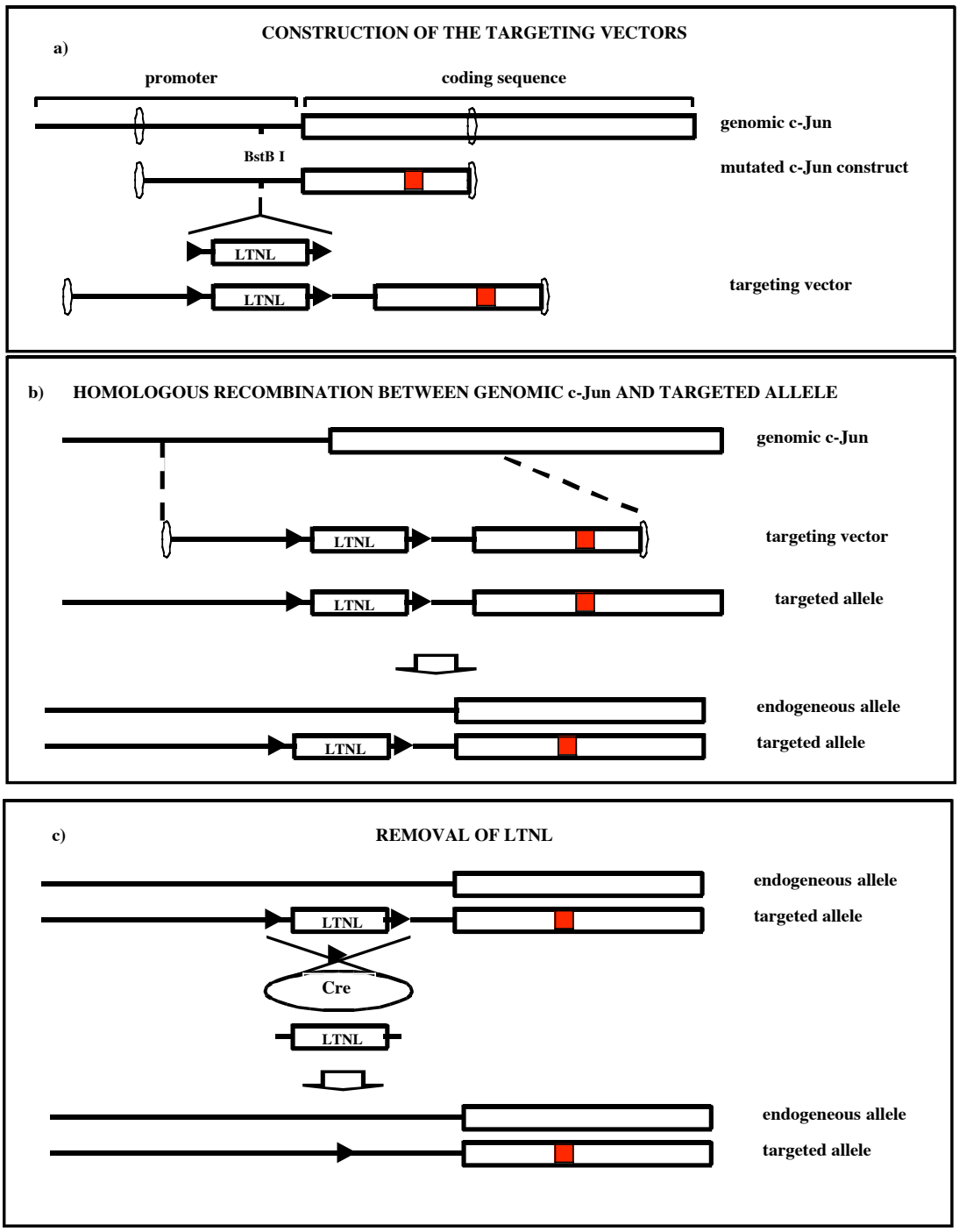


Figure 21: The construction of the targeting vectors (a), replacement between the genomic c-Jun and the targeting vector (b), and the removal of LTNL by Cre recombinase system (c)

The usage of the ES cells demonstrating the ability to colonize the germline at a high frequency is an important aspect for a successful gene targeting experiment. D3, E14, AB1, R1 are some of the ES cell lines which are frequently used. The successful transmission of E14 ES cell lines into the germ line was shown in a study to generate HPRT-deficient mice (Hooper *et al.*, 1987). The mistreatment of the ES cells may easily lead to differentiation from their pluripotent developmental state and result in inability to participate in development and, therefore, no chimaerism and/or germline transmission would occur. It is very important to keep the cells in their pluripotent states with respect to embryonic development. The culturing of ES cells requires great care. The ES cells should be split very frequently (every 2-3 days) so that the individual colonies do not get too large, and by trypsinizing it should be kept in mind that the cell clumps are fully resuspended so that no large aggregates are transferred to new plates. The large colonies would easily differentiate and loose their pluripotency. The confluence of the ES cells should not exceed 50% because of the possibility that they may completely differentiate into endoderm-like cells within a few days if they get too confluent (Torres and Kühn., 1997).

The ES cells should be mycoplasma-free since mycoplasmic contamination may disturb the generation of the chimaeras or the contribution of the ES cells to the germline (Rottem *et al.,* 1993). However there are also cases reported where mycoplasma-contaminated ES cells demonstrated the ability to efficiently colonize the germline. HPRT-deficient (Lesch-Nyhan) mouse embryos were derived from the germline colonization by E14.1 cells, which are known to be mycoplasma contaminated (Hooper *et al*., 1987). In our study the cells were regularly checked for their presence for mycoplasma contamination. The KPA ES cells were mycoplasma-free while the E14.1 cells were mycoplasma-contaminated.

The first ES cell lines we used for gene targeting were KPA and E14.1. KPA was a subclone of E14.1 and was obtained from K.Knobeloch in Berlin. The E14.1 ES cell line was derived from the 129/Ola mouse strain and was obtained from K. Rajewsky in Cologne. The capacity of E14.1 ES cells to transmit to germline is known to be very high

despite their contamination with mycoplasma which is illustrated by the fact that these cells were used in the successful generation of many transgenic mice. The targeting of E-cadherin gene resulting in defective preimplantation development (Rietmacher D. *et al.*, 1995) or disruption of the NF-IL6 gene showing its essential role in bacteria killing and tumor cytotoxicity by macrophages (Tanaka T. *et al.*, 1995) are some of the studies which were performed by using E14.1 ES cell line. The reported robust growth of E14.1 and KPA ES cells and their ability to transmit to germline despite extensive manipulations led to the usage of these cells in this study. Experience here dis not substantiate the reported observations.

Despite the fact, that we worked with optimal ES cell culture conditions, we unfortunately were not able to get germline transmission. This might depend on the usage of germline incompetent cells which were not distinguishable from the germline competent cells by morphology, or the loss of the pluripotency of the cells by passaging. We obtained chimaeric mice with low degrees of chimaerism and these chimaeras did not give rise to germline transmission. The reason for this might be that a typical ES cell population as used for transfection probably consisted of germline-competent cells, and also of a varying fraction of cells, which for unknown reasons still were able to form somatic but not germ cells in chimaeras. Another reason might be the loss of pluripotency in ES cells depending on the increase in passage numbers. It is not possible to distinguish germline-competent ES cell lines by morphology, karyotype, *in vitro* differentiation or any other *in vitro* test from the germ-line incompetent cells (Torres and Kühn, 1997). Therefore this may result in the unfortunate situation that the majority of the gene-targeted clones were unable to contribute to germ cells. Using 129Sv/Pas ES cell lines the whole study was repeated in order to obtain germ-line competent cells by gene targeting. These studies are currently in progress.

It would be important to perform phenotypical analyses upon generating the c-fos favouring c-Jun and ATF-2 favouring c-Jun mutant transgenic mice. The phenotypes of c-Jun, c-Fos and ATF-2 knock out mice and the expectations in the transgenic mutant mice were explained in result part I in detail.

4.2 PART 2: WORK ON THE DETERMINATION OF AP-1 SUBUNIT DIMER PARTICIPATING IN CROSSTALK WITH GR

It has been reported that distinct dimeric protein complexes of AP-1 consisting of members of the Jun and Fos families, as well as specific members of the ATF/CREB families bind preferentially to different sequences depending on the composition of the dimers elements. c-Jun/c-Jun homodimers and c-Jun/c-Fos heterodimers prefer heptameric AP-1 binding sites while ATF-2 homodimers and c-Jun/ATF-2 heterodimers prefer octameric AP-1 sites to bind (Hai and Curran, 1991). As an example the binding of E1A to different sequence elements may be given. The interaction of E1A with the AP-1 family happens in a complicated manner. An E1A/AP-1 interaction represses collagenase and stromelysin promoters through the TRE by inhibition of transactivation mediated by c-Jun/c-Jun or c-Jun/c-Fos (Offringa *et al.*, 1990) whereas this interaction results in an increase in transcriptional activation on *c-Jun* promoter which is also controlled by AP-1 binding sites (Stein *et al.*, 1992). This suggests that E1A can distinguish between different members of the AP-1 family. E1A represses transcription of the collagenase gene via the phorbol ester responsive element collTRE. The transcription of collagenase is reduced in E1A expressing cells. E1A represses the collagenase gene by decreasing specifically the activity of the transcription factor AP-1. The inhibition of AP-1 is a direct effect of E1A rather than a secondary effect of transformation. E1a mediated repression of AP-1 activity is much in common with that by glucocorticoid hormones in respect that both involve the reduction of AP-1 activity without inhibition of its synthesis or DNA binding. An important class of genes repressed by steroid hormones are the genes under the positive control of the transcription factor AP-1 (Ponta *et al.*, 1992)

Gene regulation requires the coordinated integration of distinct signaling pathways. Both the amounts and transactivating capacities of the Jun, Fos and ATF-2 proteins are regulated by MAPK pathway in a stimulus-dependent manner. Jun/Fos dimers predominantly bind to the heptameric AP-1 binding sites upon the activation of the ERK members of MAPK pathway by a phorbol ester, serum or PDGF mediated stimulation in fibroblasts. Stimulation through alkylating agents or UV light predominantly activates the

Jun N-terminal Kinase (JNK)/Stress Activated Protein Kinase (SAPK) members of the MAPK family which preferentially enhance Jun/ATF-activity via phosphorylation of c-Jun-Ser63/73 and ATF-Thr69/71 located in the respective transactivation domains.

The steroid hormone receptors are members of a large family of structurally related proteins that function as ligand-activated transcription factors controlling genes involved in many biological functions. GR inhibits AP-1 target gene activity by the inhibition of signal transduction via the inhibition of the phosphorylation of c-Jun amino terminal serine 63 and 73 residues by JNK (Smeal *et al.*, 1991). However it is also reported that there is a direct interaction between the GR and AP-1 (Rogatsky *et al.*, 2001)

Taken all these data together the aim was to test whether c-Jun/c-Fos complex or c-Jun/ATF-2 complex was involved in the repression on AP-1 target gene activity by GR mediated AP-1 repression.

Each reporter assay resulted in very high basal levels of transcription by using both c-Fos favouring and ATF-2 favouring fibroblast cells. This high basal level could not be increased upon UVC or TPA stimulation in ATF-2 or c-Fos favouring c-Jun expressing cells respectively. Despite in absence of tamoxifen, which was used to induce the translocation of the cytoplasmic fusion proteins into nucleus, this high basal level of transcription was present, indicating that this was not due to the effect of distinct AP-1 dimers. No conclusive results based on reporter assays were obtained. The study has been repeated with the analysis of endogenous targets. The transcription on a heptameric AP-1 binding site containing *Fra-1* promoter was induced in c-Fos favouring c-Jun expressing fibroblasts upon their stimulation with the phorbol ester TPA.

To obtain conclusive results on reporter assays, it would be useful to obtain the optimal working conditions with the genetically manipulated fibroblast cells. A kinetics should be done in order to obtain the time point of transcriptional activation on heptameric AP-1 binding site upon the induction with the phorbol ester TPA in c-Fos expressing c-Jun fibroblasts and to obtain the time point of transcriptional activation on octameric AP-1

binding site upon the induction with UVC in ATF-2 favouring c-Jun expressing cells. Retroviral vector mediated gene delivery to these genetically manipulated cells could increase transfection efficiency significantly. Additional to that, the test on the endogeneous targets might be repeated on the genes containing the octameric AP-1 on their promoters. Finally similar experiments should be repeated by adding dexamethasone in order to test the inhibition of the transcription on heptameric or octameric reporter constructs to find out the subunit of AP-1 involved in the repression of AP-1 target genes by GR.

5. REFERENCES

Agarwal SK, Guru SC, Heppner C, Erdos MR, Collins RM, Park SY, Saggar S, Chandrasekharappa SC, Collins FS, Spiegel AM, Marx SJ, Burns AL (1999). Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. *Cell.*, **96(1),**143-52

Angel P, Baumann I, Stein B, Delius H, Rahmsdorf HJ, Herrlich P. (1987). 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. *Mol Cell Biol.*, **7(6),** 2256-66

Angel P, Karin M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta*., **1072(2-3),** 129-57. Review

Arany Z, Sellers WR, Livingston DM, Eckner R. (1994). E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. *Cell,* **77(6),** 799-800

Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. (1995). Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science.,* **270(5234),** 286-90

Bannister AJ and Kouzarides T. (1996). The CBP co-activator is a histone acetyltransferase**.** *Nature,* **384(6610),** 641-3

Barker PE, Rabin M, Watson M, Breg WR, Ruddle FH, Verma IM (1984).Human c-fos oncogene mapped within chromosomal region 14q21----q31. *Proc Natl Acad Sci,* **81(18),** 5826-30

Barnes PJ and Karin M. (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases**.** *N Engl J Med***, 336(15),** 1066-71

Barnes PJ. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Lond)., **94(6),** 557-72. Review

Beato M, Herrlich P, Schutz G. (1995). Steroid hormone receptors: many actors in search of a plot. *Cell,* **83(6),** 851-7. Review

Benbrook DM and Jones NC. (1990). Heterodimer formation between CREB and JUN proteins. *Oncogene*, **5(3)**, 295-302

Berger SL. (1999). Gene activation by histone and factor acetyltransferases. *Curr Opin Cell Biol***., 11(3),** 336-41. Review

Bos TJ, Margiotta P, Bush L, Wasilenko W. (1999). Enhanced cell motility and invasion of chicken embryo fibroblasts in response to Jun over-expression. *Int J Cancer.*, **81(3),** 404-10

Brinster RL and Avarbock MR. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci*., **91(24)**, 11303-7

Caelles C, Gonzalez-Sancho JM, Munoz A. (1997). Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev***.**, **11(24),** 3351-64

Carrasco D and Bravo R. (1995). Tissue-specific expression of the fos-related transcription factor fra-2 during mouse development. *Oncogene,* **10(6),** 1069-79

Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wozney JM. (1986). Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. *EMBO J*., **5(8),** 1885-90

Chatton B, Bocco JL, Goetz J, Gaire M, Lutz Y, Kedinger C. (1994). Jun and Fos heterodimerize with ATFa, a member of the ATF/CREB family and modulate its transcriptional activity. *Oncogene*, **9(2),** 375-85

Chen JY, Penco S, Ostrowski J, Balaguer P, Pons M, Starrett JE, Reczek P, Chambon P, Gronemeyer H. (1995). RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *EMBO J*. **14(6)**, 1187-97

Chiba H, Muramatsu M, Nomoto A, Kato H. (1994). Two human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucleic Acids Res.*, **22(10),** 1815-20

Chida K, Nagamori S, Kuroki T. (1999). Nuclear translocation of Fos is stimulated by interaction with Jun through the leucine zipper. *Cell Mol Life Sci.,* **55(2)**, 297-302

Cohen DR, Ferreira PC, Gentz R, Franza BR Jr, Curran T. (1989). The product of a fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. *Genes Dev.*, **3(2),** 173-84

Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schutz G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev***, 13,** 1608-21

Curran T, Franza BR Jr. (1988). Fos and Jun: the AP-1 connection. *Cell*, **55(3),** 395-7. Review.

D'Adamo DR, Novick S, Kahn JM, Leonardi P, Pellicer A.(1997). rsc: a novel oncogene with structural and functional homology with the gene family of exchange factors for Ral. *Oncogene*, **14(11),** 1295-305

Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J. (1991). Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. *J Biol Chem.*, **266(5)**, 3107-12

De Bosscher K, Vanden Berghe W, Vermeulen L, Plaisance S, Boone E, Haegeman G. (2000). Glucocorticoids repress NF-kappaB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc Natl Acad Sci*., **97(8),** 3919-24

De Cesare D, Vallone D, Caracciolo A, Sassone-Corsi P, Nerlov C, Verde P. (1995). Heterodimerization of c-Jun with ATF-2 and c-Fos is required for positive and negative regulation of the human urokinase enhancer. *Oncogene*, **11(2)**, 365-76

Deng C and Capecchi MR. (1992) Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol Cell Biol.,* **12(8),** 3365-71

Devary Y, Gottlieb RA, Smeal T, Karin M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell,* **71(7),** 1081-91

Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR. (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science*. **249(4974),** 1266-72

Dumont A, Hehner SP, Schmitz ML, Gustafsson JA, Liden J, Okret S, van der Saag PT, Wissink S, van der Burg B, Herrlich P, Haegeman G, De Bosscher K, Fiers W. (1998). Cross-talk between steroids and NF-kappa B: what language. *Trends Biochem Sci.,* **23(7),** 233-5. Review

Eckner R, Ewen ME, Newsome D, Gerdes M, DeCaprio JA, Lawrence JB, Livingston DM. (1994). Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.*, **8(8),** 869-84

Eferl R, Sibilia M, Hilberg F, Fuchsbichler A, Kufferath I, Guertl B, Zenz R, Wagner EF, Zatloukal K. (1999). Functions of c-Jun in liver and heart development. *J Cell Biol.,* **145(5),** 1049-61

Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, Graupner G, Lu XP, Pfahl M. (1994). A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature*. **372(6501)**, 107-11

Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB, Yamamoto KR. (1988). The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature,* **334(6182),** 543-6

Fryer CJ, Archer TK. (1998). Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature*, **393(6680),** 88-91

Glass CK and Rosenfeld MG. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.*, **14(2),** 121-41. Review

Glover JN and Harrison SC. (1995). Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. *Nature,* **373(6511)**, 257-61

Gonzalez MV, Jimenez B, Berciano MT, Gonzalez-Sancho JM, Caelles C, Lafarga M, Munoz A. (2000). Glucocorticoids antagonize AP-1 by inhibiting the Activation/phosphorylation of JNK without affecting its subcellular distribution. *J Cell Biol.*, **150(5),** 1199-208

Grigoriadis AE, Schellander K, Wang ZQ, Wagner EF. (1993). Osteoblasts are target cells for transformation in c-fos transgenic mice. *J Cell Biol.,* **122(3),** 685-701

Gruda MC, van Amsterdam J, Rizzo CA, Durham SK, Lira S, Bravo R. (1996). Expression of FosB during mouse development: normal development of FosB knockout mice. *Oncogene.* **12(10),** 2177-85

Hai T and Curran T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci.,* **88(9),** 3720-4

Hai TW, Liu F, Coukos WJ, Green MR. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.*, **3(12B),** 2083-90

Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron*, **14(5),** 927-39

Hasty P, Rivera-Perez J, Bradley A. (1992). The role and fate of DNA ends for homologous recombination in embryonic stem cells. *Mol Cell Biol*., **12(6),** 2464-74

Heck S, Bender K, Kullmann M, Gottlicher M, Herrlich P, Cato AC. (1997). I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. *EMBO J.*, **16(15),** 4698-707

Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato AC. (1994). A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J.*, **13(17),** 4087-95

Heery DM, Kalkhoven E, Hoare S, Parker MG. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature*, **387(6634),** 733-6

Herrlich P. (2001) Cross-talk between glucocorticoid receptor and AP-1. *Oncogene*, **20(19),** 2465-75. Review.

Hilberg F, Aguzzi A, Howells N, Wagner EF. (1993). c-jun is essential for normal mouse development and hepatogenesis. *Nature,* **365(6442),** 179-81

Hoeffler JP. (1992) Structure/function relationships of CREB/ATF proteins. *J Invest Dermatol.,* **98(6 Suppl)**, 21S-28S

Hong H, Kohli K, Garabedian MJ, Stallcup MR. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol Cell Biol.*, **17(5),** 2735-44

Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR. (1996). GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc Natl Acad Sci.*, **93(10),** 4948-52

Hooper M, Hardy K, Handyside A, Hunter S, Monk M. (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature,* **326(6110),** 292-5

Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L. (1996). Nuclear receptor coactivators and corepressors. *Mol Endocrinol,*. **10(10),** 1167-77 Review.

Hu X and Lazar MA. (1999). The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature*, **402(6757),** 93-6

Huguier S, Baguet J, Perez S, van Dam H, Castellazzi M. (1998). Transcription factor ATF2 cooperates with v-Jun to promote growth factor-independent proliferation in vitro and tumor formation in vivo. *Mol Cell Biol.,* **18(12),** 7020-9

Ivashkiv LB, Liou HC, Kara CJ, Lamph WW, Verma IM, Glimcher LH. (1990). mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-O-tetradecanoylphorbol-13-acetate, response element. *Mol Cell Biol.*, **10(4),** 1609-21

Jantzen K, Fritton HP, Igo-Kemenes T, Espel E, Janich S, Cato AC, Mugele K, Beato M. (1987). Partial overlapping of binding sequences for steroid hormone receptors and DNaseI hypersensitive sites in the rabbit uteroglobin gene region. *Nucleic Acids Res.*, **15(11),** 4535-52

Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H, Herrlich P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell,* **62(6),** 1189-204

Kallunki T, Deng T, Hibi M, Karin M. (1996). c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell*, **87(5),** 929-39

Kang KI, Meng X, Devin-Leclerc J, Bouhouche I, Chadli A, Cadepond F, Baulieu EE, Catelli MG. (1999). The molecular chaperone Hsp90 can negatively regulate the activity of a glucocorticosteroid-dependent promoter. *Proc Natl Acad Sci*, **96(4),** 1439-44

Karin M, Haslinger A, Holtgreve H, Richards RI, Krauter P, Westphal HM, Beato M. (1984). Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. *Nature*, **308(5959),** 513-9

Karin M, Liu Z, Zandi E. (1997). AP-1 function and regulation. *Curr Opin Cell Biol.,* **9(2),** 240-6

Kerppola TK, Luk D, Curran T. (1993). Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity in vitro. *Mol Cell Biol.* **13(6),** 3782-91

Konig H, Ponta H, Rahmsdorf HJ, Herrlich P. (1992). Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation in vivo. *EMBO J*., **11(6),** 2241-6

Lamb NJ, Fernandez A, Tourkine N, Jeanteur P, Blanchard JM. (1990). Demonstration in living cells of an intragenic negative regulatory element within the rodent c-fos gene. *Cell,* **61(3),**485-96

Lazo PS, Dorfman K, Noguchi T, Mattei MG, Bravo R (1992). Structure and mapping of the fosB gene. FosB downregulates the activity of the fosB promoter. *Nucleic Acids Res.*, **20(2),** 343-50

Lee SK, Kim HJ, Kim JW, Lee JW. (1999). Steroid receptor coactivator-1 and its family members differentially regulate transactivation by the tumor suppressor protein p53. *Mol Endocrinol*., **13(11),** 1924-33

Lee W, Mitchell P, Tjian R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell.*, **49(6),** 741-52

Lefstin JA and Yamamoto KR. (1998) Allosteric effects of DNA on transcriptional regulators. *Nature*, **392(6679)**, 885-8. Review

Li B, Tournier C, Davis RJ, Flavell RA. (1999). Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. *EMBO J.*, **18(2)**, 420-32

Lucibello FC, Lowag C, Neuberg M, Muller R. (1989). Trans-repression of the mouse c-fos promoter: a novel mechanism of Fos-mediated trans-regulation.*Cell*, **59(6),** 999-1007

Lucibello FC, Slater EP, Jooss KU, Beato M, Muller R. (1990). Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. *EMBO J.*, **9(9),** 2827-34

Mangelsdorf DJ and Evans RM (1995). The RXR heterodimers and orphan receptors. *Cell*, **83(6),** 841-50. Review

McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ, O'Malley BW. (1999). Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol.* **69(1-6),** 3-12. Review

Miner JN, Diamond MI, Yamamoto KR. (1991). Joints in the regulatory lattice: composite regulation by steroid receptor-AP1 complexes. *Cell Growth Differ.*, **2(10),** 525-30. Review.

Meyer T, Carlstedt-Duke J, Starr DB. (1997). A weak TATA box is a prerequisite for glucocorticoid-dependent repression of the osteocalcin gene. *J Biol Chem.* **272(49),** 30709-14

Moras D, Gronemeyer H. (1998). The nuclear receptor ligand-binding domain: structure and function. *Curr Opin Cell Biol.*, **10(3)**:384-91. Review

Muchardt C. and Yaniv M. (1993). A human homologue of Saccharomyces cerevisiae SNF2/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor. *EMBO J*., **12(11),** 4279-90

Mumberg D, Lucibello FC, Schuermann M, Muller R. (1991). Alternative splicing of fosB transcripts results in differentially expressed mRNAs encoding functionally antagonistic proteins. *Genes Dev.,* **5(7),** 1212-23

Nakabeppu Y, Ryder K, Nathans D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell*, **55(5),** 907-15

Nakajima T, Uchida C, Anderson SF, Lee CG, Hurwitz J, Parvin JD, Montminy M. (1997). RNA helicase A mediates association of CBP with RNA polymerase II. *Cell,* 90(6), 1107-12

Neigeborn L and Carlson M. (1984). Genes affecting the regulation of SUC2 gene expression by glucose repression in Saccharomyces cerevisiae. *Genetics,* **108(4),** 845-58

Nissen RM and Yamamoto KR. (2000). The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev*., **14(18)**, 2314-29

Offringa R, Gebel S, van Dam H, Timmers M, Smits A, Zwart R, Stein B, Bos JL, van der Eb A, Herrlich P. (1990). A novel function of the transforming domain of E1a: repression of AP-1 activity. *Cell,* **62(3),** 527-38

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y (1998) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, **87(5),** 953-9

Okada S, Wang ZQ, Grigoriadis AE, Wagner EF, von Ruden T. (1994). Mice lacking c-fos have normal hematopoietic stem cells but exhibit altered B-cell differentiation due to an impaired bone marrow environment. *Mol Cell Biol.,* **14(1),** 382-90

Onate YA, Finnegan MG, Hales BJ, Johnson MK. (1993).Variable temperature magnetic circular dichroism studies of reduced nitrogenase iron proteins and [4Fe-4S]+ synthetic analog clusters. *Biochim Biophys Acta*., **1164(2),** 113-23

Papavassiliou AG, Treier M, Bohmann D. (1995). Intramolecular signal transduction in c-Jun. *EMBO J.*, **14(9),** 2014-9

Papoulas O, Beek SJ, Moseley SL, McCallum CM, Sarte M, Shearn A, Tamkun JW. (1998). The Drosophila trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. *Development*, **125(20),** 3955-66

Passegue E, Jochum W, Schorpp-Kistner M, Mohle-Steinlein U, Wagner EF. (2001). Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking junB expression in the myeloid lineage. *Cell*, **104(1),** 21-32

Pearce D and Yamamoto KR. (1993). Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. *Science.* **259(5098)**, 1161-5

Periyasamy S and Sanchez ER. (2002). Antagonism of glucocorticoid receptor transactivity and cell growth inhibition by transforming growth factor-beta through AP-1-mediated transcriptional repression. *Int J Biochem Cell Biol.,* **34(12),** 1571-85

Petrij F, Giles RH, Dauwerse HG, Saris JJ, Hennekam RC, Masuno M, Tommerup N, van Ommen GJ, Goodman RH, Peters DJ, et al. (1995). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature*. **376(6538)**, 348-51

Picard D and Yamamoto KR. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO J*., **6(11),** 3333-40

Ponta H, Cato AC, Herrlich P. (1992). Interference of pathway specific transcription factors. *Biochim Biophys Acta*., **1129(3),** 255-61. Review.

Riethmacher D, Brinkmann V, Birchmeier C. (1995). A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proc Natl Acad Sci.*, **92(3)**, 855-9

Rottem S, Barile MF. (1993). Beware of mycoplasmas. *Trends Biotechnol.* **11(4),** 143-51. Review

Ryder K, Lanahan A, Perez-Albuerne E, Nathans D. (1989). jun-D: a third member of the jun gene family. *Proc Natl Acad Sci.,* **86(5),** 1500-3

Sakai DD, Helms S, Carlstedt-Duke J, Gustafsson JA, Rottman FM, Yamamoto KR. (1988). Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. *Genes Dev.*, **2(9),** 1144-54

Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS Jr. (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science,* **270(5234),** 283-6

Schorpp M, Jager R, Schellander K, Schenkel J, Wagner EF, Weiher H, Angel P. (1996). The human ubiquitin C promoter directs high ubiquitous expression of transgenes in mice. *Nucleic Acids Res.*, **24(9),** 1787-8

Schorpp-Kistner M, Wang ZQ, Angel P, Wagner EF. (1999). JunB is essential for mammalian placentation. *EMBO J.*, **18(4),** 934-48

Schreiber M, Baumann B, Cotten M, Angel P, Wagner EF. (1995). Fos is an essential component of the mammalian UV response. *EMBO J*, **14(21)**, 5338-49

Schreiber M, Wang ZQ, Jochum W, Fetka I, Elliott C, Wagner EF. (2000). Placental vascularisation requires the AP-1 component fra1. *Development,* **127(22),** 4937-48

Schule R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. (1990). Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell.*, **62(6)**, 1217-26

Shyu AB, Greenberg ME, Belasco JG. (1989). The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes Dev.*, **3(1),** 60-72

Smeal T, Angel P, Meek J, Karin M. (1989). Different requirements for formation of Jun: Jun and Jun: Fos complexes. *Genes Dev.*, **3(12B)**, 2091-100

Smeal T, Binetruy B, Mercola DA, Birrer M, Karin M. (1991). Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature.*, **354(6353),** 494-6

Smith CL, Onate SA, Tsai MJ, O'Malley BW. (1996). CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proc Natl Acad Sci.*, **93(17),** 8884-8

Soriano P, Montgomery C, Geske R, Bradley A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell*, **64(4),** 693-702

Stern M, Jensen R, Herskowitz I. Five SWI genes are required for expression of the HO gene in yeast. *J Mol Biol*., **178(4),** 853-68

Sternberg N and Hamilton D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol.*, **150(4),** 467-86

Stein B, Angel P, van Dam H, Ponta H, Herrlich P, van der Eb A, Rahmsdorf HJ. (1992) Ultraviolet-radiation induced c-jun gene transcription: two AP-1 like binding sites mediate the response. *Photochem Photobiol*., **55(3),** 409-15

Stromstedt PE, Poellinger L, Gustafsson JA, Carlstedt-Duke J., (1991) The glucocorticoid receptor binds to a sequence overlapping the TATA box of the human osteocalcin promoter: a potential mechanism for negative regulation. *Mol Cell Biol.*, **11(6),** 3379-83

Subramaniam N, Cairns W, Okret S. (1997). Studies on the mechanism of glucocorticoid-mediated repression from a negative glucocorticoid response element from the bovine prolactin gene. *DNA Cell Biol*., **16(2)**, 153-63

Tanaka T, Akira S, Yoshida K, Umemoto M, Yoneda Y, Shirafuji N, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T. (1995). Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell*., **80(2),** 353-61

Teurich S. and Angel P. (1995). The glucocorticoid receptor synergizes with Jun homodimers to activate AP-1-regulated promoters lacking GR binding sites. *Chem Senses*, **20(2),** 251-5. Review.

te Riele H, Maandag ER, Berns A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc Natl Acad Sci*., **89(11),** 5128-32

Thepot D, Weitzman JB, Barra J, Segretain D, Stinnakre MG, Babinet C, Yaniv M. (2000). Targeted disruption of the murine junD gene results in multiple defects in male reproductive function. *Development*, **127(1)**, 143-53

Torchia J, Glass C, Rosenfeld MG. (1998). Co-activators and co-repressors in the integration of transcriptional responses. *Curr Opin Cell Biol***., 10(3),** 373-83. Review

Touray M, Ryan F, Jaggi R, Martin F. (1991). Characterisation of functional inhibition of the glucocorticoid receptor by Fos/Jun. *Oncogene.*, **6(7),** 1227-34

van Dam H, Castellazzi M.(2001). Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. *Oncogene*, **20(19),** 2453-64. Review.

Torres RM and Kuhn R., (1997), Laboratory protocols for gene targeting

Vayssiere BM, Dupont S, Choquart A, Petit F, Garcia T, Marchandeau C, Gronemeyer H, Resche-Rigon M. (1997). Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. *Mol Endocrinol.,* **11(9)**, 1245-55

van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, Herrlich P, Zantema A, Angel P, van der Eb AJ. (1993). Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. *EMBO J.*, **12(2),** 479-87

van Dam H, Huguier S, Kooistra K, Baguet J, Vial E, van der Eb AJ, Herrlich P, Angel P, Castellazzi M. (1998). Autocrine growth and anchorage independence: two complementing Jun-controlled genetic programs of cellular transformation. *Genes Dev.* **12(8),** 1227-39

van Tilborg MA, Lefstin JA, Kruiskamp M, Teuben J, Boelens R, Yamamoto KR, Kaptein R. (2000). Mutations in the glucocorticoid receptor DNA-binding domain mimic an allosteric effect of DNA. *J Mol Biol*., **301(4),** 947-58

Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J*., **15(14),** 3667-75

Vogt PK. (1994). In: The Fos and Jun Families of Transcription Factors. *CRC Press:* Boca Raton, FL USA, 203-219

Vogt PK. Jun, the oncoprotein. (2001). *Oncogene*, **20(19),** 2365-77. Review

Wade PA, Wolffe AP. (1997). Histone acetyltransferases in control**.** *Curr Biol***., 7(2),** R82-4. Review

Walfish PG, Yoganathan T, Yang YF, Hong H, Butt TR, Stallcup MR. (1997). Yeast hormone response element assays detect and characterize GRIP1 coactivator-dependent activation of transcription by thyroid and retinoid nuclear receptors. *Proc Natl Acad Sci.,* **94(8),** 3697-702

Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U, Wagner EF. (1992). Bone and haematopoietic defects in mice lacking c-fos. *Nature*, **360(6406)**, 741-5

White MA, Nicolette C, Minden A, Polverino A, Van Aelst L, Karin M, Wigler MH. (1995), Multiple Ras functions can contribute to mammalian cell transformation., *Cell.* **80(4),** 533-41

Whyte P, Williamson NM, Harlow E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. *Cell,* **56(1)**, 67-75

Wilkinson DG, Bhatt S, Ryseck RP, Bravo R. (1989). Tissue-specific expression of c-jun and junB during organogenesis in the mouse. *Development*, **106(3),** 465-71

Wilson CJ, Chao DM, Imbalzano AN, Schnitzler GR, Kingston RE, Young RA. (1996). RNA polymerase II holoenzyme contains SWI/SNF regulators involved in chromatin remodeling. *Cell*, **84(2),** 235-44

Wolthuis RM, Bos JL. (1999) Ras caught in another affair: the exchange factors for Ral. *Curr Opin Genet Dev.*, **9(1),** 112-7. Review

Xu L, Glass CK, Rosenfeld MG. (1999). Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev.*, **9(2),** 140-7. Review.

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M. (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell,* **62(6),** 1205-15

Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, Newsome D, Bronson RT, Li E, Livingston DM, Eckner R. (1998). Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell*, **93(3),** 361-72

Yen J, Wisdom RM, Tratner I, Verma IM (1991). An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. Proc Natl Acad Sci., **88(12),** 5077-81