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Johanna De Castro Arce, MSc.
Bogotá, Colombia

Oral examination

RARβ *trans*-repression of AP-1 transcription factor in HeLa cervical cancer cells: Consequences on transcription of viral and cellular AP-1 controlled genes

Referees: Prof. Dr. Christine Clayton
Prof. Dr. Claus-Hobe Schröder

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Abbreviations

AF-1	Activation function 1
AF-2	Activation function 2
AP-1	Activator protein 1
atRA	all-*trans*-retinoic acid
CDK	Cyclin dependent kinase
CoA	Co-activator
DBD	DNA-binding domain
ERK	Extracellular signal-regulated protein kinases
FCS	Fetal calf serum
Ha-ras	Harvey rat sarcoma virus oncogene
HAT	Histone acetyltransferase
HDAC	Histone de-acetylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK	Glycogen synthase kinase
HPV	Human papilloma virus
JNK	Jun N-terminal kinase
LBD	Ligand-binding domain
MAPK	Mitogen-activated protein kinases
MEKK1	MAPK kinase kinase
MKP	MAPK phosphatase
MKK	MAPK kinase
MMP	Metalloproteinase
N-CoR	Nuclaer receptor co-repressor
NR	Nuclear receptor
NLS	Nuclear localization signal
P	Phospho
POH1	Human Pad1 homolog
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
Rb	Retinoblastoma protein
Skp-2	S-phase kinase associated protein 2 (p45)
SMRT	Silencing mediator for RXR and TR
TNFα	Tumor necrosis factor α
TR	Thyroid receptor
TRE	TPA responsive element
URR	Up stream regulatory region

I. INTRODUCTION

The present work analyzes the interaction between the retinoic acid receptor beta (RARβ) with the transcription factor AP-1 in the context of HPV-induced carcinogenesis. Before providing evidence how constitutive RARβ expression abrogates AP-1 activity in cervical carcinoma cells, I shall give a brief overview about the individual key regulatory proteins and their function in normal eukaryotic cells.

1.1 Activator protein 1 (AP-1) transcription factor

1.1.1 General aspects

The transcription factor AP-1 (activator protein 1) is compose of heterogeneous dimeric proteins consisting of members of Jun, Fos and ATF-2 families. Once bound to DNA, the *trans*-activation function of a given dimeric complex can vary, which can be explained by differences in phosphorylation by upstream protein kinases as well as protein/protein interaction with other cellular factors. As a consequence, distinct sets of genes are targeted, suggesting that individual members of the AP-1 family have specific functions in AP-1 regulated cellular processes (Angel *et al*., 2001).

In vitro, dimers formed by Fos and Jun bind with the highest affinity to an asymmetric heptanucleotide recognition sequence TGA(C/G)TCA (TRE) and with slightly slower affinity to a symmetric octanucleotide TGACGTCA (CRE) (Figure 1). The TRE element is found in a wide range of promoter and enhancer regions embedded in different regulatory contexts. Variation in recognition sequences may contribute to the differential functions of different Fos-Jun family dimers at various regulatory elements (Chinenov and Kerppola, 2001).

The repertoire of Fos-Jun proteins in a given cell is subject to changes in response to various extracellular stimuli. Through dimerization mediated by the leucin zipper, the seven family members can form 18 different homo and heterodimers. The number of detectable Fos-Jun dimers varies among different cell types (Chinenov and Kerppola, 2001). Table 1

	c-Jun	JunB	JunD	c-Fos	FosB	Fra-1	Fra-2
c-Jun	+	+	+	+	+	+	+
JunB		+	+	+	+	+	+
JunD			+	+	+	+	+
c-Fos				-	-	-	-
FosB					-	-	-
Fra-1						-	-
Fra-2							-

Table 1: Dimer combinations of Fos and Jun family proteins capable of forming stable AP-1 transcription factor complexes.

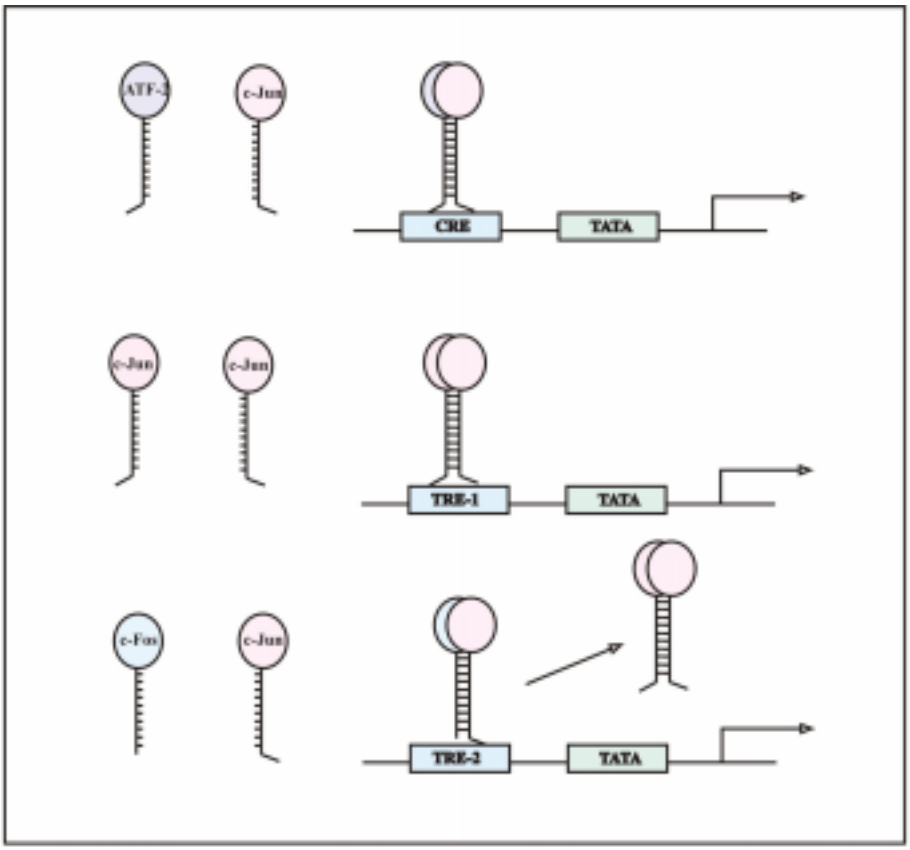


Figure 1 The expression of AP-1 responsive genes is affected by the response element itself (TRE-1 and -2 represent slight variations in the recognition sequence), the relative DNA binding affinities of the different AP-1 dimers (Fos-Jun higher affinity than Jun-Jun for TRE-2 sequence) and the AP-1 composition in any given time (ATF-2-Jun, Jun-Jun or Jun-Fos).

The functions of Fos-Jun family proteins depend on the specific cell type in which they are expressed and are also affected by the specific signals that elicit their expression. Thus, the functions of AP-1 must be mediated by mechanisms that depend on the cellular context in which they are expressed. The mechanisms that give cell specificity include selective dimerization, interaction with other regulatory proteins and post-translational modifications. Moreover extracellular stimuli can modulate the abundance of the AP-1 proteins by controlling the transcription of their genes, the stability of their mRNAs and the activity and stability of the specific Fos-Jun proteins (Karin *et al*., 1997; Chinenov and Kerppola, 2001).

1.1.2 AP-1 family members function and regulation

The main components of AP-1 transcription factors can be divided into three major families: Fos, Jun and ATF.

The Fos family contains the following members, all of them characterized as 'immediately early' genes because this response occurs in the absence of new protein synthesis:

i. c-Fos: *c-fos* protein and mRNA are undetectable in most quiescent cells and require stimulation by hormones, serum mitogens or other ligands to reach easily detectable levels (Distel and Spiegelman, 1990). Stimulation of the cells induces *c-fos* transcription very rapidly and transiently. Several elements mediate c-*fos* induction: CRE (cAMP response ele-

ment), SIE (Sis-inducible enhancer) and SRE (Serum response element). SRE is important for *c-fos* induction by a large variety of extracellular stimuli, which lead to extracellular signal-regulated kinase (ERK) activation (Karin *et al*., 1997). In addition to transcriptional control, the abundance of c-Fos is regulated at the level of mRNA stability. Adenylate and uridylate-rich elements (AREs), major protein-coding region determinant of instability (mCDR) and ARE-/mCDR-binding proteins can alter the stability of *c-fos* mRNAs. ARE and mCDR elements determine the mRNA turn-over by deadenylation of the poly(A) tail and nuclease degradation of the mRNA body. In the case of mCDR elements the process is coupled to translation (Chen *et al*., 1995; Grosset *et al*., 2000; Chen *et al*., 2002). Finally, the c-Fos content is regulated by protein stability through the ubiquitin-proteasome pathway, which contributes to determine the duration of the AP-1 response (Tulchinsky, 2000).

ii. FosB: As *c-fos*, *fosB* is rapidly and transiently induced after stimulation with serum, growth factors and phorbol esters but becomes undetectable in 3h (Tulchinsky, 2000). The *fosB* gene encodes two funtionally distinct proteins by the production of alternately spliced transcripts. The long protein FosB, differs from FosB2 by the presence of a C-terminal extension. FosB is more potent activator of transcription and neoplasic transformation than FosB2 (Skinner *et al*., 1997; Herdegen and Waetzig, 2001; Jochum *et al*., 2001).

iii. Fra-1/Fra-2: Functional TRE sequences have been described in the promoter of *fra-2* and within the intronic enhancer of *fra-1*, suggesting that the transcription of both genes can be regulated via a positive auto-regulatory loop. Fra-1 and Fra-2 lack the C-terminus *trans*-activating domain. This combine with the delayed synthesis in response to stimuli, suggest they might be inhibitory factors which in certain circumstances may limit the duration of the AP-1 response (Tulchinsky, 2000).

The following members compose the Jun family all of them ‘immediately early’ genes:

i. c-Jun: The *c-jun* gene is expressed in many different cell types at low levels and its expression is enhanced in response to many stimuli via protein kinase C pathway, growth factors, UV irradiation or cytokines. *c-jun* is regulated at transcriptional, post-transcriptional and post-translational levels. The *c-jun* promoter region is highly conserved and contains potential binding sites for several transcription factors including Sp-1, nuclear factor-*jun*, CCAAT transcription factor and AP-1 itself. Induction of *c-jun* expression is mediated through a TRE-like site located in the proximal region of the promoter; a c-Jun/ATF-2 heterodimer rather than a conventional c-Jun/c-Fos AP-1 dimer preferentially recognizes this site. Despite its inducible expression, most cell types contain a certain basal level of c-Jun protein prior to stimulation (Mechta-Grigoriou *et al*., 2001). It has been demonstrated that constitutive expression from the *c-jun* promoter is driven

by ATF-2 or an ATF-2 –dimerization partner distinct from c-Jun under resting conditions (Steinmüller *et al*., 2001). The phosphorylated form of c-Jun subsequently induces its own transcription through positive auto-regulation (Berry *et al*., 2001; Mechta-Grigoriou *et al*., 2001). *c-jun* mRNA instability maybe mediated by an ATTTA signal sequence in the 3' untranslated region of the mRNA (Vogt and Bos, 1990; Chen *et al*., 2002). Additional layer of complexity to the c-Jun regulation is at level of translation. The *c-jun* mRNA has a extremely long 5' untranslate region which is GC rich. Tipically mRNAs with long GC rich 5' untranslate regions are translated extremely inefficiently, but in the case of *c-jun* mRNA have been shown that the translation proceed through internal ribosome entry. The complex folding of the 5' untranslate region creates a structure functionally defined as an internal ribosome entry segment (IRES). IRES has been implicated as a mechanism to allow translation of messages under stress conditions in which overall cellular protein synthesis is compromised (Sehgal et al., 2000). Finally, at protein level c-Jun itself is unstable having a 90 minutes half-life. It carries a PEST motif which is a sequence involved in the rapid destruction of short-lived proteins, and is *in vivo* a substrate for multi-ubiquitination and proteasome degradation (Musti *et al*., 1997).

ii. JunB: The sequence analysis of *junB* promoter reveals several potential regulatory elements, which include Stat3, CRE, SRE and CAAT enhancer-binding domains. JunB differs in biological properties from c-Jun and was defined as a negative regulator of cell growth. This functional difference is due to changes in aminoacid sequences, which decrease its dimerization potential and eliminate the JNK phosphorylation sites, and therefore decrease its *trans*-activation capacity (Mechta-Grigoriou *et al*., 2001).

iii. JunD: In contrast to *c-jun* and *junB*, *junD* is refractory to serum stimulation and its basal expression remains high in many cell types. The promoter contains several identified binding sites: CRE, Sp-1, Octamer motif, CAAT-box, a GC-rich region and AP-1 consensus site. The octamer motif is the major regulator of *junD* expression and is specifically recognized by the ubiquitous Oct-1 protein (Mechta-Grigoriou *et al*., 2001). There are two predominant JunD isoforms generated by alternative initiation of translation: Full-length (JunD-FL) and a shorter protein (ΔJunD) (Short and Pfarr, 2002)

ATF (activating transcription factor) family members: ATF-2, ATF-3 and B-ATF. Unlike Jun and Fos, expression of ATF-2 is constitutive. The activity of ATF-2 is regulated post-translationally by phosphorylation after JNK or p38 mitogen-activated protein kinase induction, and it preferentially interacts with CRE rather than TRE binding sites (Karin *et al*., 1997; Chinenov and Kerppola, 2001).

1.1.3 Modulation of AP-1 function- MAP kinase pathway

AP-1 activation is caused by the increased expression of Fos and Jun proteins after extracellular stimuli but also by phosphorylation. Fos and Jun are signal converters; they are activated by external signals, altering transcription of specific genes triggering the cellular response. The signal is internalized in the cell by a transduction cascade involving the Mitogen-acivated protein kinases (MAPKs). MAPKs connect cell-surface receptors to critical regulatory targets within the cell, such as AP-1 family members. They also respond to chemical and physical stresses, controlling cell survival and adaptation. Mammals express at least three different regulated groups of MAPKs (see figure 2), extracellular signal-related kinases ERK1/2, Jun amino-terminal kinases JNK1/2/3, p38 proteins p38α/β/γ and ERK5. Each MAPK is activated by specific MAPK kinase (MAPKK): MEK1/2 for ERK, MKK3/6 for p38, MKK4/7 for JNK and MEK5 for ERK5. Up-stream each MAPKK can be activated by more than one MAPKK kinase, increasing the complexity and diversity of MAPK signaling (Chang and Karin, 2001). Additionally, in order to generate the appropriate output signal, MAPK modules have evolved strategies to communicate with other pathways. This cross-talk can affect signaling properties and information flow and can modify the specificity of the pathways involved (Schaeffer and Weber, 1999). The duration of the response after MAPK activation is regulated by MAPK phosphatases (MKPs) which are rapidly induced by the same stimuli that activate the MAPK pathways (Camps *et al*., 1999).

MAPK cascades contain at least three protein kinases that work in series and these three enzymes comprise a module (see Figure 2).

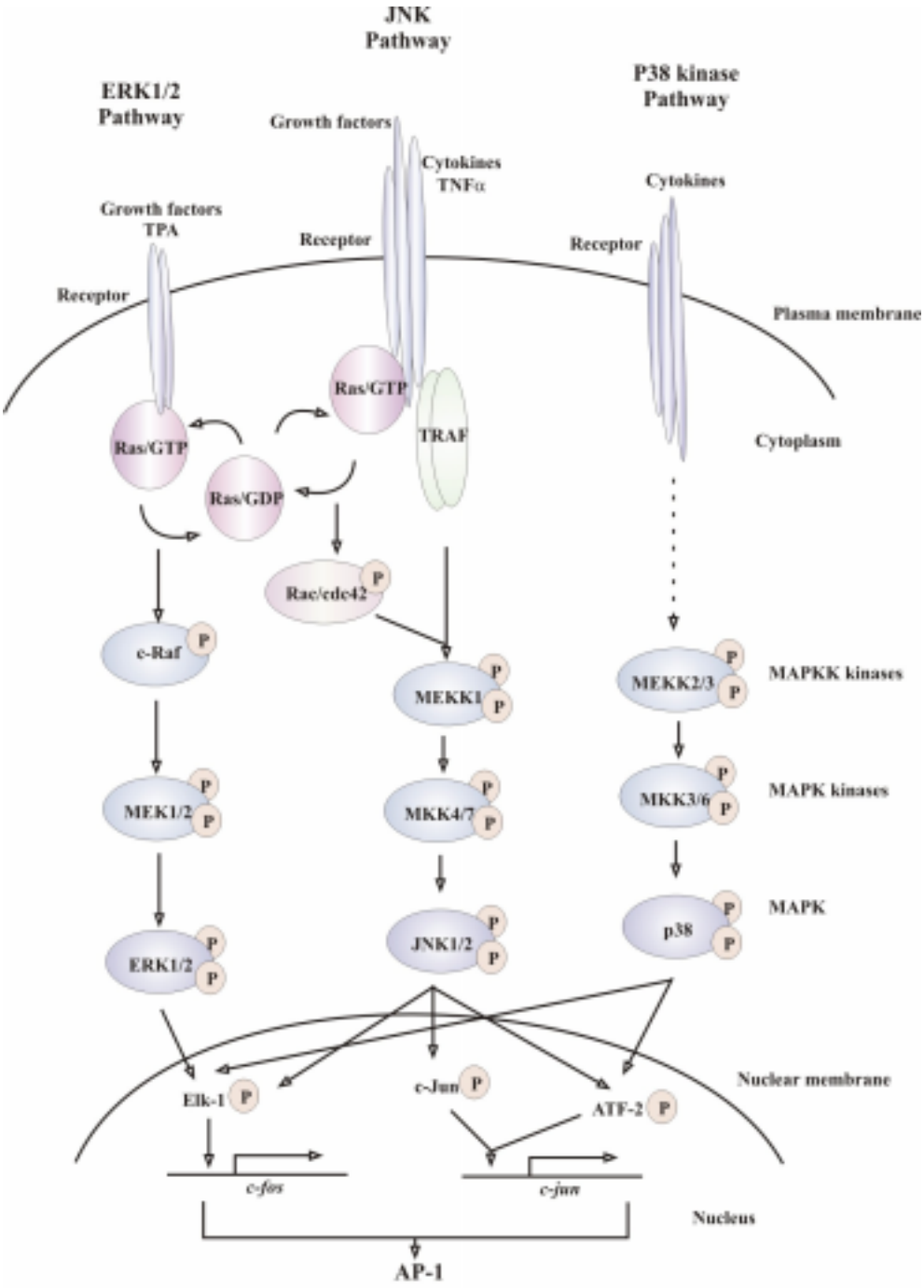


Figure 2 MAPK signaling pathways (for details, see text section 1.1.3).

1.1.3.1 Signaling through extracellular signal-regulated protein kinase (ERK) pathway

ERK1/2 are among the protein kinases most commonly activated in signal transduction pathways. They have particularly been linked to cell proliferation, but have important roles in many other events (English *et al*., 1999). Activation of the ERK signaling pathway, either by growth factors or by activating mutations in cytoplasmic effectors such as Ras and Raf, results in AP-1 activation.

The Ras cascade is classical ERK activator. There are three *ras* genes that control cellular proliferation: Ha-ras, K-ras and N-ras. They encode nearly identical 21 KDa proteins collectively known as Ras (Marshall, 1995). Ras proteins are positioned at the inner surface of the plasma membrane where they serve as binary molecular switches to transduce extracellular ligand-mediated stimuli into the cytoplasm to control signal transduction pathways influencing cell growth, differentiation and apoptosis. Ras biological activity is controlled by a regulated GDP/GTP cycle. Single aminoacid substitutions, known as oncogenic activation, at position 12, 13 or 61 unmask Ras transforming potential and create mutant proteins that are insensitive to stimulation. Consequently, these oncogenic Ras mutant proteins are locked in the active state. Active Ras forms a high affinity complex with Raf, activating it by a not-fully-understood mechanism. Raf then phosphorylates and activates MAPKK-MEK1/2 via direct association with the catalytic domain. Activated MEKs function as a dual specificity kinases and phosphorylate MAPK-ERK1/2 to activate them. Once activated, ERK1/2 proteins translocate to the nucleus where they phosphorylate and activate a variety of substrates that include Elk-1 transcription factor. This finally leads to the stimulation of the *c-fos* and other promoters (Campbell *et al*., 1998).

1.1.3.2 Signaling through the p38 pathway

The first p38 subgroup member was discovered as a lipopolysaccharide (LPS)–induced tyrosine phosphoprotein and as the target of a drug developed to inhibit LPS-induced tumor necrosis factor α biosynthesis. Four p38-like MAPKs are known, often activated by cellular stresses and, as a result, referred to as stress-activated protein kinases. In addition to their roles in cytokine biosynthesis, they are implicated in many other events (English *et al*., 1999).

1.1.3.3 Signaling through the Jun N-terminal kinase (JNK) pathway

JNK regulates AP-1 by at least two mechanisms: first, JNK increases expression of Fos proteins by Elk-1 phosphorylation (Wisdom, 1999). Second, c-Jun activity is post-translationally up-regulated by JNK-mediated phosphorylation at serines residues within c-Jun activation domain (Wisdom, 1999). This event increases the *trans*-activating potential and DNA binding activity of c-Jun protein. Additionally, phosphorylation increases the stability of c-Jun, resulting in an increment of its half-life in 2-3-fold in comparison with a dephosphorylated c-Jun (Musti *et al*., 1997). Although c-Jun is an exclusive JNK substrate, JNK can also phosphorylate and activate other transcription factors. ATF-2 is one of them. It heterodimerizes with c-Jun, stimulating *c-jun* mRNA expression. Therefore, through activation of both c-Jun and ATF-2, JNK can regulate the abundance and activity of c-Jun (Barr and Bogoyevitch, 2001).

Following the exposure of cells to external factors, JNK activation has been routinely measured by protein kinase activity towards the transcription factor c-Jun. The JNK pathway can be activated by different stimuli (Figure 3):

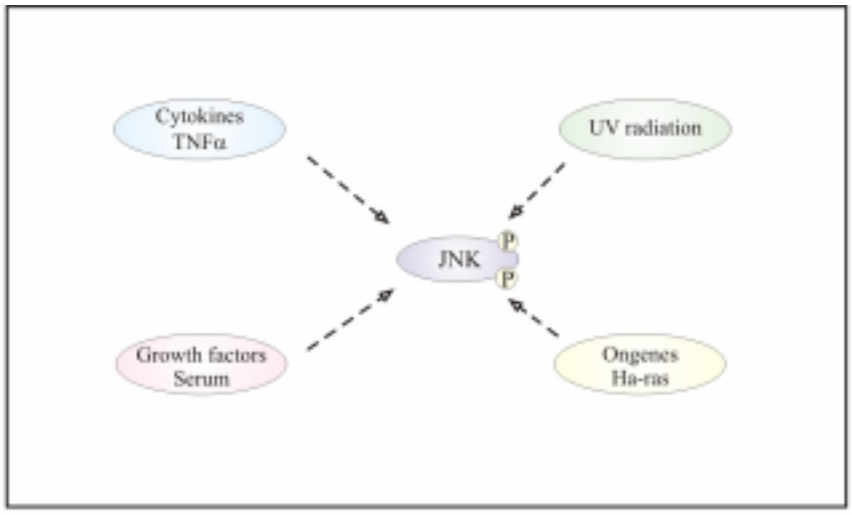


Figure 3 JNK pathway can be activated by different stimuli.

i. Tumor necrosis factor α (TNFα) is a potent activator of JNK signaling and AP-1 (Wisdom, 1999). The binding of TNFα to its receptors TNFR1 and TNFR2 generate a cascade of signals through association with TNF-receptor-associated factors TRAF. The N-terminal effector domain of TRAFs can interact with the MAPKK kinase, MEKK1, and activate it leading to phosphorylation and activation of the MAPK kinase MKK4/7, which finally phosphorylates and activates the JNK, resulting in c-Jun phosphorylation at serines 63 and 73. This phosphorylation event consequently activates AP-1, which in turn induce genes involved in chronic and acute inflammatory responses. The p38 MAPK pathway can be also activated by TNFα (Figure 2, Baud and Karin, 2001).

ii. In addition to proinflamatory stimuli, JNK activity is stimulated by certain growth factors. It has been reported that activation of JNK by serum is dependent on MEKK1 phosphorylation and activation, because the JNK activation is considerably reduced in Mekk1$^{-/-}$ cells (Xia *et al*., 2000).

iii. Ras activation induces JNK phosphorylation and activation through Rac/cdc42 via MEKK1 leading to c-Jun and ATF-2 phosphorylation. Althoug Ha-ras function is essential for JNK activation by growth factors (but not by cytokines as TNFα), oncogenically activated Ha-ras leads only to a partial JNK activation (Figure 2, Minden *et al*., 1995; Dhanasekaran and Reddy, 1998).

iv. MEKK1 is the key kinase in the pathway of JNK activation. Many stimuli converge on this kinase resulting in JNK activation. MEKK1 is a serine-threonine kinase which, upon activation, binds and phosphorylate MKK4/7, which phosphorylates and activates JNK (Karin, 1995). It has been demonstrated that two threonine residues at the positions 560 and 572 in the sequence of MEKK1 are essential for catalytic activity. When Thr 560 and Thr 572 were individually mutated to alanine residues, MEKK1 lost a significant portion

of its catalytic activity both in terms of autophosphorylation and phosphotransferase activity toward MKK4/7 (Siow *et al*., 1997). Additionally, it has been suggested that the N-terminal domain of the protein participates in its regulation which explains why a mutant lacking the amino acids 1-352 exhibit constitutive activation (Minden *et al*., 1994).

1.1.3.4 Other phosphorylation events that regulate AP-1 activity independent of MAPK

c-Jun is phosphorylated on 5 to 7 serine and threonine residues. One enzyme that can regulate c-Jun post-translationally independent of the MAPK pathway is the glycogen synthase kinase-3 (GSK-3). Two serines and one threonine residues were identified as targets of the GSK-3 *in vivo*. The residues are located directly upstream of the basic region of the c-Jun protein, the part of the protein responsible for DNA binding (Figure 4). This close proximity between the phosphorylation sites and the DNA-binding domain suggest that phosphorylation might alter DNA-binding. Incubation of bacterially expressed, unphosphorylated, c-Jun protein with GSK-3 led to a dramatic decrease in its DNA-binding activity (Angel and Karin, 1991).

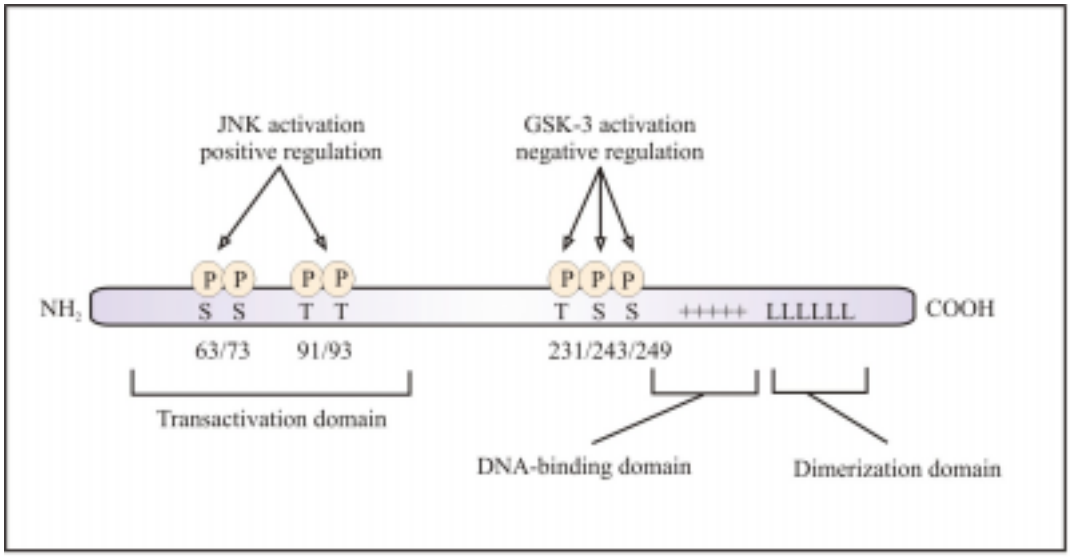


Figure 4 Schematic representation of the structure of human c-Jun. Phosphorylated serine (S) or threonine (T) residues, as well as the leucine zipper (++++ LLLL) are indicated. The numbers represent the amino acid position of the phosphorylation sites. The approximate position of the transactivation domain, DNA-binding domain and dimerization domain are indicated.

1.1.4 AP-1 proteins as modulators of neoplastic transformation

Many cytoplasmic transforming proteins, such as Ras, activate AP-1 due to their ability to activate ERK and JNK signaling pathways. This together with the ability of overexpressed Fos and Jun proteins to elicit cellular transformation in some systems has provided circumstantial evidence that AP-1 functions as an important nuclear modulator of transformation. For example, c-Jun mutant cells are resistant to transformation by activated Ras and the induction of invasive skin cancers by the combination of an activated Ras transgene and topical application of phorbol esters is markedly impaired in c-Fos null mice (Wisdom, 1999).

Additional evidence for the c-Fos role in malignancy progression was obtained from the determination of the AP-1 composition in tumorigenic and non-tumorigenic human cells. It was shown that the characteristic AP-1 pattern in HPV-18-positive tumorigenic cells is mainly c-Jun/c-Fos, while non-tumorigenic HPV-18-positive cells have Fra-1/c-Jun dimers. Stable transfection with a vector expressing c-Fos in non-tumorigenic HPV-18-positive cells converts them to tumorigenicity as tested by hetero-transplantation in nude mice (Soto *et al*., 1999).

1.2 Retinoic acid receptor β

1.2.1 General aspects

Clinical and experimental approaches have shown that vitamin A or retinol, and its biologically active derivatives or retinoids, exert a variety of profound effects on vertebrate development, cellular differentiation and homeostasis. These structurally simple molecules (figure 5) can exert pleiotropic effects through a set of retinoic acid nuclear receptors. The retinoic acid receptors belonging to the super-family of nuclear ligand-activated transcriptional regulators that include steroid hormones, thyroid hormone and vitamin D3 receptors. Diversity in the control of gene expression by retinoid signals is generated through complexity at different levels of the signaling pathway. The existence of several forms of retinoids, whose synthesis may be cell-specifically modulated, represents a first level of complexity in the signaling pathways (for review, see Chambon, 1996).

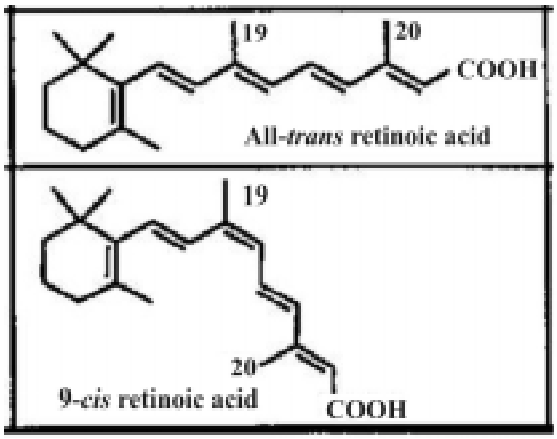


Figure 5 Chemical structure of all-*trans* and 9-*cis* retinoic acid

Two families of nuclear receptors, the retinoic acid receptors RARs and the rexinoid receptors RXRs, each consisting of three isotypes (α/β/γ) encoded in separate genes, have the potential to transduce the retinoic acid signal *in vivo*. Isoforms of each RAR also arise in a tissue-related pattern as a result of differential promoter use and alternative splicing (Napoli, 1996). The RAR family (RARα, RARβ and RARγ) is activated both by all-*trans* retinoic acid (atRA) and by 9-*cis* retinoic acid (9cRA), whereas the RXR family (RXRα, RXRβ and RXRγ) is activated exclusively by 9cRA (for review, see Chambon, 1996).

Purified RARs do not bind efficiently to the retinoic acid response elements (RARE) located on the promoter regions of genes controlled by retinoids. The purification of proteins that

stimulate RAR binding led to the demonstration that RXRs form heterodimers with RARs in solution, and this increases the binding activity. Further analysis suggested that RAR/RXR heterodimers are the functional units that transduce the retinoid signal *in vivo* (for review, see Chambon, 1996).

1.2.2 Structural organization

The structure of retinoic acid nuclear receptors is highly conserved, consisting of six regions denoted A/B, C, D, E and F (Figure 6) (Mangelsdorf and Evans, 1995). The N-terminus (region A/B) varies in its size and amino acid sequence and is sometimes lacking. The DNA-binding domain (DBD) (region C) is highly conserved in its amino acid sequence, the hinge region D again is variable and the ligand-binding domain (LBD) (region E) is well conserved. Some nuclear receptors contain a C–terminal domain of unknown function (region F). Regions C and D participate in DNA binding; region E contains the main nuclear receptor dimerization interface and the ligand-binding function. A ligand-independent activation function AF-1 has been assigned to the A/B region and the ligand-dependent activation function AF-2 is located in the C-terminal end of the region E.

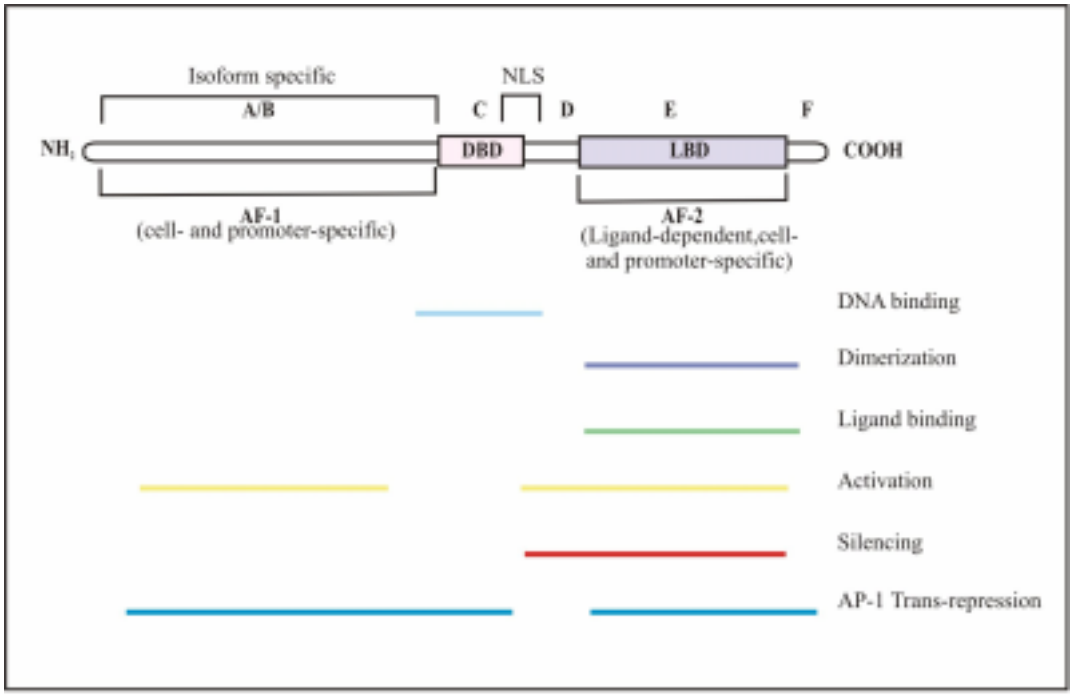


Figure 6 Schematic illustration of the structural and functional organization of retinoic acid receptors. The C and E are conserved regions while A/B, D and F are divergent. AF, transcription activation functions AF-1 is constituvely-active and AF-2 is ligand-inducible. NLS nuclear localization signal, DBD DNA-binding domain and LBD ligand-binding domain.

1.2.3 Activation and repression by RAR/RXR

Nuclear receptors can both activate and repress transcription. This results from the ability of most nuclear receptors to change their conformation by binding to their cognate ligands, which leads to the formation of different complexes. Retinoic acid receptors can bind to DNA in their unliganded state and actively repress transcription, whereas when a ligand is bound, they strongly activate transcription (Figure 7).

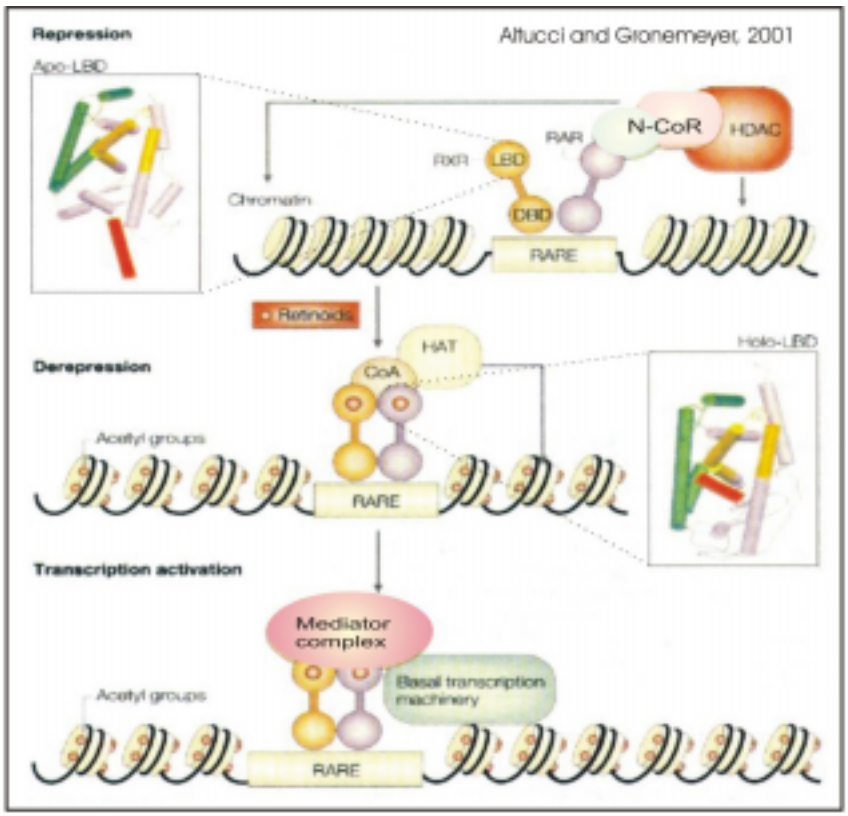


Figure 7 Mechanisms of transcriptional repression and activation by RAR-RXR. In the absence of ligand, co-repressor complexes bind to the RAR-RXR heterodimers. The co-repressors recrute histone deacetylases which remove acetyl groups from nucleosomal histones resulting in chromatin condensation and gene silencing. Binding of the ligand or phosphorylation ligand-independent events induces conformational change destabilizing the co-repressor binding and allowing interaction with co-activators and recruting histone acetyl transferases. The histones acetylation induce chromatin decondesation by nucleosomal repulsion. Through a mediator protein-containing complexes the basal transcriptional machinery is recruted leading to an increased frequency of transcriptional initiation.

i. Ligand-dependent transcriptional activation: Many complexes and cofactors interact with RARs in a ligand-dependent way (Rosenfeld and Glass, 2001; Lee *et al*., 2001; Xu *et al*., 1999; Torchia *et al*., 1998). Functional analysis in cells suggest that liganded RARs activate transcription by the following mechanisms (a) Chromatin remodeling

by the histone acetyl-transferase activity of cofactors or ATP-dependent chromatin remodeling machines and (b) pre-initiation complex recruitment or stabilization (Dilworth and Chambon, 2001).

ii. Ligand-independent transcriptional activation: The ligand-independent activation function (AF-1) can be induced by phosphorylation, leading to interaction with several cofactors in the absence of ligand (Rochette-Egly *et al*., 1997; Bastien *et al*., 2000). Additionally, conformational changes induced by protein-protein contact with another transcription factor can cause activation of unliganded nuclear receptors (Tolon *et al*., 2000).

iii. Nuclear receptor-mediated repression: Unliganded RARs can interact with nuclear co-repressor complexes such as N-CoR and SMRT (Hörlein *et al*., 1995; Chen and Evans, 1995). These proteins contain a highly conserved domain, which is able to actively repress transcription. The co-repressors interfere with transcriptional activation by (a) Inhibiting pre-initiation complex formation or similarly locking the pre-initiation complex into an inactive conformation and (b) deacetylating histone tails in order to create a hypo-acetylated closed form of chromatin (Laherty *et al*., 1998; Heinzel *et al*., 1997; Alland *et al*., 1997).

1.2.4 Retinoic acid receptor β isoforms

Three different RARβ isoforms have been described in humans: RARβ1, RARβ2 and RARβ4 (Figure 8). Each isoform has identical B-F domains (see figure 6); however, the A domain varies drastically both in size and aminoacid sequence. Two promoters, along with alternative splicing, produce the different isoforms. RARβ2 and RARβ4 arise from the same promoter called P2, which contains a strong retinoic acid responsive element (RARE). The 5' untranslated region of the RARβ4 transcript is spliced out including the ATG start codon. Therefore RARβ4 translation is initiated from an internal CUG codon resulting in an isoform that lacks all but 4 of the A domain amino acids. In humans, the P1 promoter gives rise to only one transcript, RARβ1, which appears to be expressed only in fetal tissues (Soprano *et al*., 2000). Retinoid treatment induces the expression of RARβ2 and RARβ4 at the transcriptional level via the RARE element. In contrast, the transcription of RARβ1 is unaffected by retinoic acid treatment (Mendelsohn *et al*., 1994). The function or importance of the different isoforms of RARβ is unknown. Nevertheless a growing body of evidence supports the hypothesis that retinoic acid receptor β2 is a tumor suppressor gene. RARβ2 expression has been reported to be silenced in many malignant tumors, but molecular mechanisms are still poorly understood (Yang *et al*., 2001).

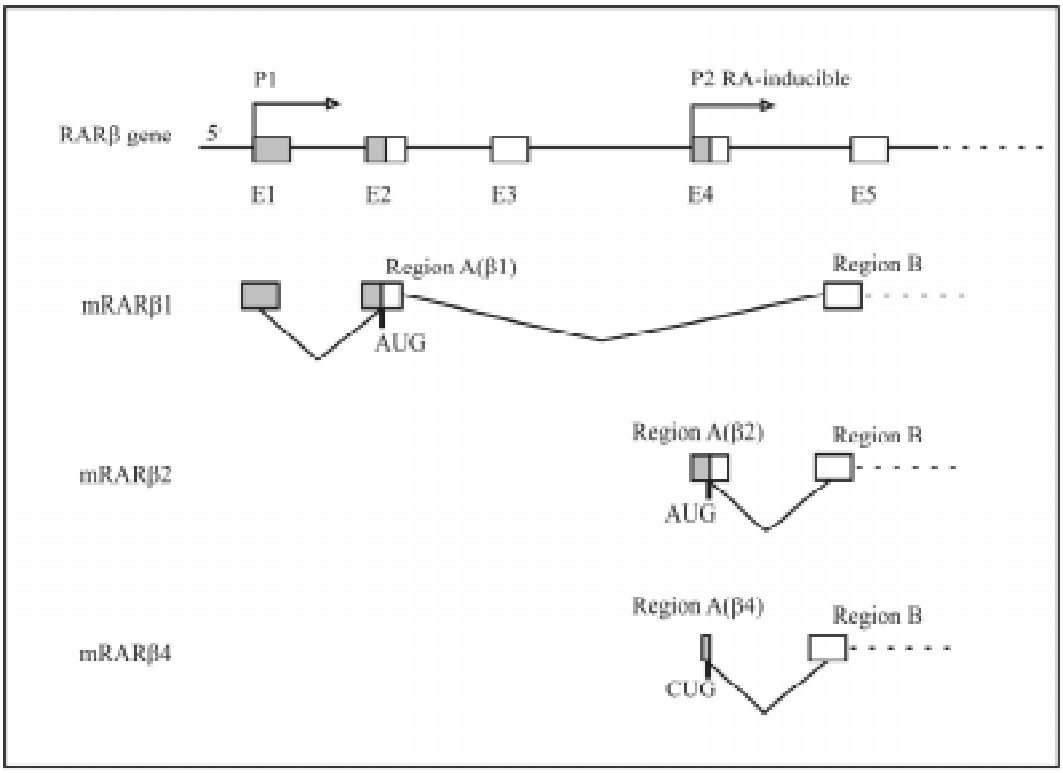


Figure 8 Mechanism for generate RARβ isoforms. Schematic organization of the 5' region of the RARβ gene and major isoforms. Exons are indicated by boxes (E1 to E5). White and gray boxes represent translated A region sequences and the 5'-UTR, respectively. Region A and region B represent the isoform-specific A region and common B region (A/B domain).

1.2.5 Retinoic acid receptor β2 and cancer

As was mention RARβ2 can act as tumor suppressor, since loss of its expression is associated with human tumor progression (Altucci and Gronemeyer, 2001; Hayashi *et al*., 2001). RARβ is silenced in various forms of cancer such as breast, oral, lung, esophageal, gastric, prostata, etc (Qiu *et al*., 1999; Virmani *et al*., 2000; Altucci and Gronemeyer, 2001; Hayashi *et al*., 2001; Nakayama *et al*., 2001b; Vourlekis and Szabo, 2003).

In 40% of cervical squamous cell carcinomas, expression of RARβ2 mRNA was significantly decreased. The deficiency in the expression of RARβ2 was associated with *de novo* methylation of the promoter and the region of the first exon, close to the retinoic acid response element RARE (Figure 8). It has been proposed that this methylation is an early event during cervical carcinogenesis (Ivanova *et al*., 2002).

RARβ2 is the most frequently retinoid receptor lost in different epithelial cancers evaluated to date, the expression is often lost through epigenetic changes of both maternal and paternal alleles (promoter hyper- and aberrant methylation) and/or loss of heterozigosity on chromosome 3 combined with epigenetic modification of the remaining allele (Yang *et al*., 2001). This event correlates with tumor progression and retinoic acid unresponsiveness.

1.2.6 Retinoic acid receptor β2 re-expression, retinoic acid treatment and tumor regression

RARβ2 expression can be re-induced in responsive cancer cells by pharmacological treatment with retinoids. Antisense studies blocking the RARβ2 induction after retinoic treatment reveal that

the induction of RARβ2 by retinoids may be an early step in the cascade of events leading to growth inhibition (Sun *et al*., 2000). Based on this and other findings it has been proposed that the inhibitory effect of retinoids on cell growth is correlated with the induction of RARβ2 in response to retinoid treatment.

Likewise, when RARβ2 is overexpressed in retinoid-insensitive cell lines, the cells acquire retinoid sensitivity leading to growth arrest (Lee *et al*., 2000). Moreover, the ectopic overexpression of RARβ2 in different kinds of cancer induces profound growth inhibition in the absence of additional retinoid, and increases the retinoid sensitivity. Cells stably expressing RARβ2 demonstrate irreversible growth arrest following one week of retinoid treatment (Cheung *et al*., 1998).

The gene programme(s) regulated by RARβ2 is/are entirely unknown, but is tempting to speculate that its function might be related to two functionalities that distinguish RARβ2 from the other receptors: first, RARβ2 interacts only inefficiently with co-repressors, so RARβ2-RXR heterodimers are more responsive to retinoids than for example RARα-RXR heterodimers. Second, RARβ2 constitutively represses AP-1, in contrast to the ligand-dependent cross talk of the other retinoid and rexinoid receptors. It is possible that these activities are unfavorable for rapid tumor growth (Altucci and Gronemeyer, 2001).

1.2.7 AP-1 *trans*-repression, the second mode of RARβ2 action

In addition of its function as transcriptional regulator RARβ2 exhibit a second mode of action through the cross talk repression based on protein-protein interaction (Figure 9).

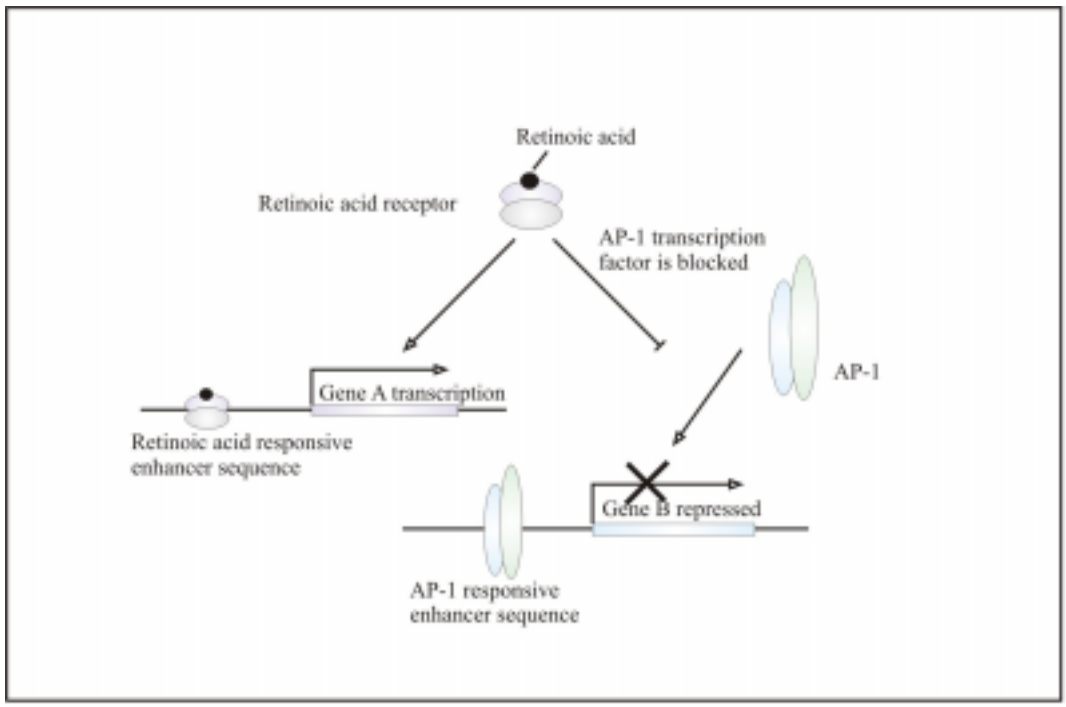


Figure 9 Model of dual action of retinoic acid receptors. (1) They activate the transcription of genes whose enhancers enable RAR to bind. (2) They repress the activity of the AP-1 transcription factor.

RAR-AP-1 repression is mutual since overexpression of AP-1 components repress the transcriptional and *trans*-repressional activity of the RARs (Yang *et al*., 1997). Conversely, active RARs can down regulate AP-1 stimulated transcription, independent of DNA-binding, and is thus distinct from conventional modes of transcription factor action. It has been demonstrated that the AP-1 *trans*-repression accounts for the anti-tumor activity of retinoids.
All three RAR subtypes (α, β, γ), could effectively inhibit the induced AP-1 activity, and the activity of the oncogenes *c-jun* and *c-fos* on AP-1-containing reporter genes, in the presence of retinoic acid. However, only RARβ shows a strong retinoic acid-independent inhibition of AP-1 activity, whereas inhibition of AP-1 by RARα and RARγ is retinoic acid dependent. The RARα and RARγ activation induce RARβ expression, consequently RARβ might function as the main anti-AP-1 retinoic receptor.
Analysis of RARβ protein domains shows that both the N-terminal portion (A/B and C domains) and the C-terminal portion (E/F domain) are involved in the inhibition of AP-1 transcriptional activity. Additionally, the A/B domain of RARβ is responsible for retinoic acid independent repression of AP-1 transcriptional activity (Lin *et al*., 2000). Consequently analysis of RARβ2 and RARβ4 AP-1 *trans*-repression activity reveal that RARβ4 has not anti-AP-1 activity. Since RARβ4 lacks all but 4 amino acids in its A domain (Figures 6 and 8), these results suggest that the A domain plays an important role in mediating AP-1 *trans*-repression activity in the RARβ2 molecule (Soprano *et al*., 2000).

1.3 Human Papillomavirus 18 and cervical cancer

1.3.1 Human Papillomavirus

Papillomaviruses are small, non-enveloped icosahedral particles that contain a circular 8Kbp double-stranded DNA, associated with cellular histones. They are a heterogeneous group of small tumor viruses that infect exclusively epithelial cells. There are more than 85 different fully sequenced genomes (zur Hausen, 1999). Based on their association to benign or malignant lesions, papillomaviruses are subdivided in low and high-risk groups respectively. High-risk HPVs can induce immortalization in human keratinocytes *in vitro* (Dürst *et al*., 1987) and have been found associated with cervical cancer (de Villiers, 1994). The most prevalent high-risk HPV type found in cervical cancer is HPV-16 (50-60% of all positive cases) followed by HPV-18 (10-20%).
The viral genome organization is highly conserved and the can be functionally divided in 3 different regions (Figure 10):

i. "Upstream regulatory region" (URR): this region contains the origin of DNA replication, promoter elements driving gene expression and the epithelial-specific transcriptional enhancer.

ii. Early region: contains the genes encoding non-structural regulatory proteins responsible for viral DNA replication (E1/E2), transcriptional self-regulation (E2) and cellular transformation and immortalization (E5, E6 and E7, the viral oncogenes).

iii. Late region: encodes the structural proteins, required for viral assembly (major capsid protein L1 and minor capsid protein L2).

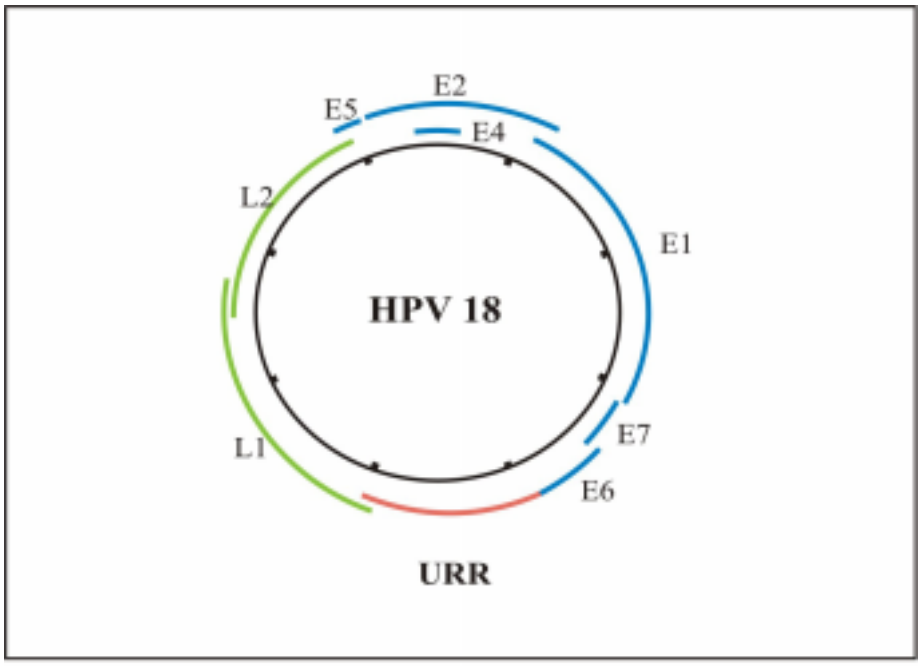


Figure 10 Genomic organization of the Human papillomavirus 18. Red the URR, up-stream regulatory region, Blue early region and Green late region.

HPV enters basal cells at sites of epithelial disruption (Iftner *et al*., 1992). Once penetrating basal cells, the virus can persist as a non-integrated episome in a latent state or can induce benign tumors, papillomas, in a permissive cycle. Alternatively, the HPV genome can be integrated into the host genome, disrupting the negative feedback of the E2 protein on the URR and preserving only the E6/E7 expression. Integration seems to be an early event in the multi-step progression to cervical cancer (Park *et al*., 1995), resulting in the up-regulation of HPV expression of oncogenes E6/E7 and in the increase of the E6/E7 mRNA half-life due to generation of viral-cellular fusion transcripts (Schwarz *et al*., 1985; Jeon *et al*., 1995).

Although E6/E7 expression is sufficient for immortalization of primary human keratinocytes *in vitro* (Woodworth *et al*., 1989; Münger *et al*., 1989), progression to malignancy requires further cellular dysregulation events such as Ras mutations or *c-fos* overexpression (Medina-Martinez *et al*., 1997; Soto *et al*., 1999). Continuous E6/E7 expression is then required to maintain the malignant and proliferative phenotype both *in vivo* and *in vitro* (Knebel Doeberitz *et al*., 1988).

A number of functions have been reported for E6 and E7. E6 binding to the cellular protein p53 through the E6-associated protein, a protein ligase, targets p53 for ubiquitination and proteasome degradation. This leads to chromosomal instability with resulting mutational consequences for HPV-positive cells. E7 binds to retinoblastoma (Rb) and Rb-related proteins inducing their phosphorylation and enhancing their degradation by ubiquitination. This releases transcription factors of the E2F family, activating transcription of genes regulating cell proliferation (for review, see zur Hausen, 2000).

1.3.2 Cellular control of Human Papillomavirus oncogene transcription

The upstream regulatory region URR is 800-1000bp long and contains the *cis*-regulatory elements involved in viral transcription and replication.

Functionally, the HPV-18 URR can be divided in 3 parts:

i. 5'-terminal portion of still unknown function, which only marginally contributes to the activity of the promoter.

ii. Central 230-bp constitutive enhancer essential for the promoter activity.

iii. Promoter proximal region containing basically the promoter region at the 3'-terminus of the URR. From this promoter, transcription of both E6/E7 genes is initiated to give a polycistronic mRNA.

Transcription from the URR is most active in epithelial cells (Hoppe-Seyler and Butz, 1994).

1.3.2.1 Transcription factors interacting with the HPV-18 URR: Role of AP-1

Only one of the factors that interact with the URR is virally-encoded, the others are cellular transcriptional regulators (Figure 11).

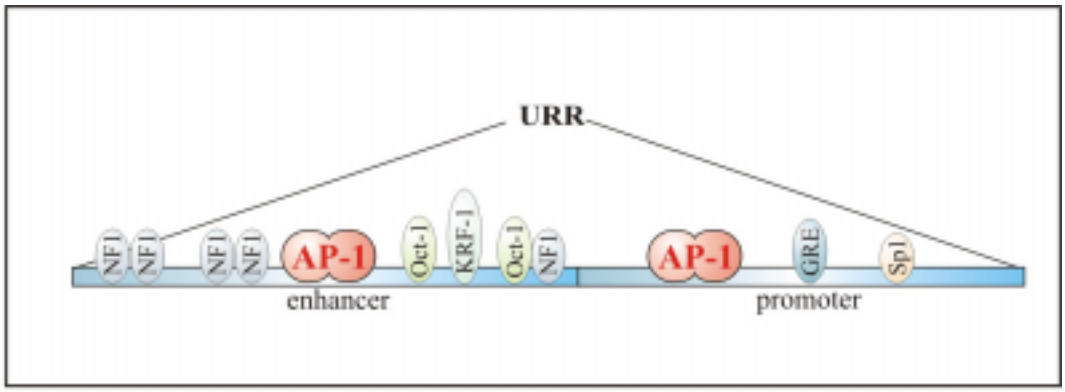


Figure 11 Schematic representation of the HPV-18 URR.

i. Viral E2 protein: Exists in 2 forms, full length behaving as a transcriptional *trans*-activator and truncated form responsible for the transcriptional repression. The repression occurs through displacement of transcription factors at the promoter region. Thus E2 may negatively regulate its own synthesis. After integration this regulation is lost (Bernard and Apt, 1994).

ii. Nuclear Factor 1 (NF-1): There are three NF-1 binding sites. Mutational analysis of these in the natural context of the complete URR or in HPV-18 enhancer subfragments upstream of a heterologous promoter indicated that they only marginally contribute to transcriptional activation (Hoppe-Seyler and Butz, 1994; Bernard and Apt, 1994).

iii. Octamer-binding transcription factor 1 (Oct-1): The HPV-18 URR contains at least 2 low affinity binding sites for cellular Oct-1. While the 5'-terminal Oct-1 recognition sequence overlaps with the binding site for the epithelial cell-specific transcription factor KRF-1 (see below), the 3'-terminal octamer motif is immediately adjacent to a half palindromic NF-1 binding motif at the 3'-border of the constitutive enhancer (Hoppe-Seyler and Butz, 1994; Bernard and Apt, 1994).

iv. Epithelial cell-specific transcription factor 1 (KRF-1): Because this factor is absent in non-epithelial cells has been hypothesized to contribute to the tissue specificity of the HPV-18 enhancer. Mutational inactivation analyses indicate that the activity of this element can vary significantly between different epithelial cell types (Hoppe-Seyler and Butz, 1994).

v. Sp-1: This element has been shown to be strictly required for efficient stimulation of the promoter. Transcriptional activation by Sp-1 is dependent on the functional interplay with the HPV enhancer elements, as the promoter proximal portion of the URR by itself is not able to transcriptionally stimulate the homologous or heterologous promoters (Hoppe-Seyler and Butz, 1994).

vi. Hormone receptors: Glucocorticoid-responsive elements bound by the glucorticoid receptor or progesterone receptor, mediate transcriptional stimulation. Mutation of this element results in the complete loss of transcriptional activation by dexamethasone. In the absence of hormone stimulation the mutation of GRE motif led to a consistent 2-3 fold up-regulation of the basal activity of the URR (Hoppe-Seyler and Butz, 1994).

vii. Activator Protein 1 (AP-1): There are 2 AP-1 binding sites, one within the constitutive enhancer and one within the promoter proximal region. Mutational inactivation of these sites in the context of the complete HPV-18 URR leads to an almost complete loss of the transcriptional activity. Indicating that AP-1 plays a key role in the activation of HPV transcription. Furthermore, mutational analyses indicate that the integrity of both AP-1 elements within the URR is required for efficient activation of the promoter (Hoppe-Seyler and Butz, 1994; Bernard and Apt, 1994).

So far it appears that the AP-1 transcription factor is the main regulator determining the efficiency of HPV expression and in turn in the net amount of the viral onco-proteins E6/E7. The AP-1 composition differs considerably between immortalize and malignant HPV-positive cells (Soto *et al*., 1999). Somatic cell hybridization between different malignant cervical carcinoma HPV-positive cells established complementation groups on the basis of AP-1 composition. Intriguingly, non-malignant hybrids revealed an AP-1 composition characteristically for uninfected normal cells, while malignant somatic cell hybrids still retained an AP-1 pattern typically for the malignant parental counterparts (Soto *et al*., 2000).

1.3.3 Retinoic acid treatment, HPV-18 E6/E7 oncogenes down regulation and growth inhibition of cervical cancer cells

Defects in the RARβ gene expression may be one pathway leading to the escape from growth regulation (Si *et al*., 1996). The stable transfection of RARβ2 in HeLa cells effectively inhibits cell proliferation and anchorage-independent growth (Si *et al*., 1996; Geisen *et al*., 2000). Additionally, nude mice hetero-tranplantation of this HeLa RARβ clones showed a reduced tumor growth (Geisen *et al*., 2000). These experiments demonstrate that, at least in the HeLa system, RARβ is a growth inhibitor both *in vitro* and *in vivo* being the RARβ expression inversely correlated with the tumor growth potential.

Several retinoic acid-induced effects has been postulated to explain the growth inhibition, proteolysis of cyclin D1 (Sueoka *et al*., 1999; Dragnev *et al*., 2001), reduced expression of c-*myc* and increased expression of the cdk inhibitor p27 (Weber *et al*., 1999; Dimberg *et al*., 2001). However, specifically in HPV-positive cervical cancer, the retinoic acid can transcriptionally repress the HPV-18 E6/E7 promoter and this event correlates with the negative effect on cell proliferation. Analysis of HPV-18 URR deletions mutants revealed that the retinoic acid-mediated repression probably occurs via regulatory elements located in the central enhancer and furthermore a fragment of the HPV-18 central enhancer confers retinoic acid-dependent repression on the heterologous timidine-kinase promoter (Bartsch *et al*., 1992). However, the presence of retinoic acid response elements or retinoic acid receptor binding sites have not been identified in the HPV-18 URR (MatInspector search and Blast analysis). Therefore is likely that the inhibition of the HPV-18 E6/E7 expression is due to interference with other transcription factors bound to this region. It has been shown that AP-1 is an essential activator of HPV-18 URR bound to the central enhancer and retinoic acid receptors cause a AP-1 *trans*-repression. The aim of this work was to reveal whether exists any relation between the retinoic acid receptor β expression, the AP-1 *trans*-repression and the HPV-18 E6/E7 down-regulation.

Aims of this study

In order to clarify the interplay between retinoic acid receptor β expression, AP-1 activity and HPV-18 E6/E7 modulation in cervical cancer cells, the following goals were pursued:

i. Identify if retinoic acid treatment induces AP-1 *trans*-repression.

ii. If AP-1 is *trans*-repressed by retinoic acid receptor β, clarify by which mechanisms *trans*-repression is mediated.

iii. Identify the mechanism by which retinoic acid negatively regulates HPV-18 E6/E7 expression.

iv. Evaluate if the sole expression of retinoic acid receptor β can leads to HPV-18 E6/E7 down-regulation

v. Find a relation between AP-1 induction and HPV-18 E6/E7 expression in cervical carcinoma cells.

II. MATERIALS AND METHODS

2.1 Abbreviations

AP-1	Activating protein 1
ATP	Adenosin 5'-triphosphate
atRA	All-*trans*-retinoic acid
bp	Basepair
BSA	Bovine serum albumin
CMV	Cytomegalovirus
cDNA	Complementary DNA from mRNA
DEPC	Diethylpyrocarbonat
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleosine-5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E-64	N-N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl-agmatine
ECL-Reagent	Enhanced Chemiluminescence Reagent
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis[β-aminoethylether]-N, N, N', N'-tetraacetic acid
EMSA	Electrophoresis mobility shift assay
FCS	Fetal Calf Serum
Forw	Forward
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPV	Human papillomavirus
HRP	Horse radish peroxidase
IE4/5	Herpes virus enhancer
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kbp	Kilobasepair
KDa	Kilodalton
LB-Medium	Luria-Bertoni Medium
M	Molar
MEKK1Δ	Mitogen-activated protein kinase kinase kinase 1 constituvely active
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
min	Minute
MMP	Metalloproteinase
MOPS	3-[N-Morpholino] propanesulfonic acid
mRNA	Messenger RNA

OD	Optical density
PAGE	Polyacrilamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pefabloc SC	4-(2-Aminoethyl)-benzolsulfonylflourid
POH1	Human Pad1 homolog protein
PVDF	Polyvinylidene fluoride
RAR α	Retinoic acid receptor α
RAR β	Retinoic acid receptor β
Rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolution per minute
RSV	Rous sarcoma virus
RT	Reverse transcription
sec	Seconds
SDS	Sodium dodecyl sulfate
SDS-PAGE	Denaturant polyacrilamide gel electrophoresis
TEMED	N, N, N', N'-Tetramethylethylendiamine
t-RNA	Transference RNA
TNF-α	Tumor necrosis factor α
Tris	Tris(hydroxymethyl)-aminomethane
v/v	Volume percentage
w/v	Weight percentage

2.2 Materials

2.2.1 Plasmids

Plasmid	Insert	References
pHFβA-1	β–Actin	Gunning et al., 1983
pbact ΔRARβ	(actin)-RARβ	Geisen et al., 2000
pSV-β–Galactosidase	(SV40)-β-galactosidase	Promega
pAP-1-Luc (pTRE-Luc)	(TRE)-Fire fly Luciferase	Stratagene
Plasmid D1	(RARE)-Fire fly Luciferase	de Thé et al., 1990
pRJA1	(RSV)-c-Jun	
pHSV Ha-ras	(IE4/5)-Ha-ras mutant val 12	Geller, 1988
pCMV5-MEKK1Δ	(CMV)-MEKK1 Δ	Lin et al., 1995
pSG-Fra-1	Fra-1 cDNA	
pCI-neo-Fra-1AS	(CMV)-Fra-1 antisense	
pCDNA3-HA.POH1	(CMV)-POH-1	Spataro et al., 1997
pBR322-HPV-18	HPV-18	

The promoter for each expression vector is mention in backets

2.2.2 Antibodies

Antibody	Company	Epitope	Use
Actin (Clone 4) 69100 Lot 7979E Mouse IgG	ICN Biotech.	Chicken gizzard actin as immunogen	Western 1:10000
ATF-2 sc-6233X Lot A198 Rabbit	Santa Cruz Biotech.	N-terminus of human ATF-2 (amino acids 1-96)	Western 1:10000
P-ATF-2 sc-8398X Lot F229 Mouse IgG	Santa Cruz Biotech.	Amino acid sequence containing phosphorylated Thr-71 of ATF-2 of human origin	Western 1:10000
c-Jun sc-1694X Lots C319 and H101 Rabbit	Santa Cruz Biotech.	N-terminus of c-Jun p39 of human origin (amino acids 1-79)	Western 1:10000
P-c-Jun sc-822X Lot H199 Mouse IgG	Santa Cruz Biotech.	Amino acids 56-69 of c-Jun of human origin	Western 1:10000 EMSA 0.1µg/µl
Jun B sc-73X Lot E278 Rabbit	Santa Cruz Biotech.	N-terminal domain of mouse Jun B p39	Western 1:5000
Jun D sc-74X Lot L037 Rabbit	Santa Cruz Biotech.	C-terminus of mouse Jun D p39	Western 1:5000
c-Fos sc-52X Lot E286 Rabbit	Santa Cruz Biotech.	N-terminus of human c-Fos (amino acids 3-16)	Western 1:10000 EMSA 0.1µg/µl

Antibody	Company	Epitope	Use
Fra-1 sc-605X Lot F229 Rabbit	Santa Cruz Biotech.	N-terminus of Fra-1 of rat origin	Western 1:10000 EMSA 0.1 µg/µl
JNK cat#9252 Lot 1 Rabbit	Cell Signaling Tech., Inc.	Full length p54 SAPK/JNK2 fusion protein	Western 1:1000
P-JNK cat#9251 Lot 4 Rabbit	Cell Signaling Tech., Inc.	Synthetic phospho-Thr183/Tyr185 peptide corresponding to human SAPK	Western 1:1000
p53 sc-126 Lot B261 Mouse IgG_{2a}	Santa Cruz Biotech.	N-terminal epitope between amino acid residues 11-25 of p53 of human origin	Western 1:5000
p21 sc-397 Lot I080 Rabbit	Santa Cruz Biotech.	C-terminus of p21 of human origin	Western 1:500
p27 cat#k25020-050 Lot 610241 Mouse IgG_1	Transd. Lab	Epitope between amino acid residues 1-197 of mouse p27	Western 1:1000
Retinoblastoma cat# 554136 Lot M065303 Mouse IgG_1	PharMingen	Epitope between amino acid residues 332-344 of the human Rb	Western 1:500
Cyclin D1 sc-246 Lot D129 Mouse IgG_1	Santa Cruz Biotech.	Recombinant human Cyclin D1	Western 1:1000
Cycline E sc-247 Lot J294 Mouse IgG_{2b}	Santa Cruz Biotech.	Recombinant human Cyclin E	Western 1:1000
Skp-2 sc-1567 Lot G287 Goat	Santa Cruz Biotech.	N-terminus of Skp-2 of human origin	Western 1:1000
Cdk2 sc-6248 Lot L239 Mouse IgG_1	Santa Cruz Biotech.	Epitope corresponding to amino acids 1-298, full length Cdk2 of human origin	Western 1:1000
MKP-1 sc-1102 Lot 051 Rabbit	Santa Cruz Biotech.	C-terminus of MAP kinase phosphatase 1 of mouse origin	Western 1:500
GSK-3α/β sc-7291 Lot A222 Mouse IgG_{2a}	Santa Cruz Biotech.	Epitope corresponding to amino acids 1-420, full length GSK-3β of *Xenopus* origin Reactive with GSK-3α and β	Western 1:200
RARα sc-551X Lot H011 Rabbit	Santa Cruz Biotech.	C-terminus of RARα1 of human origin	Western 1:3000
RARβ sc-552 Lot F081 Rabbit	Santa Cruz Biotech.	C-terminus of RARβ2 of human origin	Western 1:300

Antibody	Company	Epitope	Use
POH-1 Rabbit		Polyclonal antibody kindly provided by Dr. Chris Norbury, Oxford University	Western 1:300
HPV-18 E7 sc-1590 Lot D228 Goat	Santa Cruz Biotech.	N-terminus of Human papilloma virus type 18 early protein E7	Western 1:200
Rabbit cat#W401B Lot11259001 IgG-HRP	Promega	Second antibody, conjugated to horseradish peroxidase	Western 1:10000
Mouse cat#W4021 Lot 80096 IgG-HRP	Promega	Second antibody, conjugated to horseradish peroxidase	Western 1:10000
Goat sc-2020 Lot K169 IgG-HRP	Santa Cruz Biotech.	Second antibody, conjugated to horseradish peroxidase	Western 1:10000
Mouse isotypic specific IgG_1-HRP sc-2060 Lot J151	Santa Cruz Biotech.	Second antibody, conjugated to horseradish peroxidase	Western 1:10000
Mouse isotypic specific IgG_{2a}-HRP sc-2061 Lot C292	Santa Cruz Biotech.	Second antibody, conjugated to horseradish peroxidase	Western 1:10000
Mouse isotypic specific IgG_{2b}-HRP sc-2062 Lot A279	Santa Cruz Biotech.	Second antibody, conjugated to horseradish peroxidase	Western 1:10000

2.2.3 PCR Primers

Gene	Sequence	Annealing Temperature	Number of cycles
RARβ	Forw 5'-GGAATCGATGCCAATACTGTCGACTCC-3 Rev 5'-GGCAAAGGTGAACACAAGGTC-3'	59°C	35
RARα	Forw 5'-ACCCCCTCTACCCCGCATCTACAAG-3' Rev 5'-CATGCCCACTTCAAAGCACTTCTGC-3'	65°C	30 Gianini *et al.*, 1997
fra-1	Forw 5'-GCGCCTAGGCCTTGTATCTCCCTTTCCCC-3' Rev 5'-CCGCTCGAGGCGAGGAGGGTTGGAGAGCC-3'	65°C	35
c-fos	Forw 5'-AACTTCATTCCCACGGTCAC-3' Rev 5'-CCTTCTCCTTCAGCAGGTTG-3'	55°C	35
jun-B	Forw 5'-GCCCTTCTACCACGACGACTC-3' Rev 5'-CTGCACCTCCACCGCTGCCA-3'	63°C	35
jun-D	Forw 5'-GGTGCCCGACGTGCCGAGCTT-3' Rev 5'-GTACGCCGGGACCTGGTGC-3'	61°C	35
c-jun	Forw 5'-GCATGAGGAACCGCATCGCTGCCTCCAAGT-3' Rev 5'-GCGACCAAGTCCTTCCCACTCGTGCACACT-3'	55°C	35 Garret *et al.*, 2002
poh-1	Forw 5'-GGTTGTTGGCTTTCTGGTGT-3' Rev 5'-ATGCCTGGATAGATGGCTTG-3'	55°C	35

Gene	Sequence	Annealing Temperature	Number of cycles
mmp-1	Forw 5'-ATGCTGAAACCCTGAAGGTG-3' Rev 5'-CTGCTTGACCCTCAGAGACC-3'	55°C	35
mmp-13	Forw 5'-TTGAGCTGGACTCATTGTCG-3' Rev 5'-GGAGCCTCTCAGTCATGGAG-3'	55°C	35
mmp-3	Forw 5'-GCAGTTTGCTCAGCCTATCC-3' Rev 5'-GAGTGTCGGAGTCCAGCTTC-3'	57°C	35
GAPDH	Forw 5'-TGGATATTGTTGCCATCAATGACC-3' Rev 5'-GATGGCATGGACTGTGGTCATG-3'	65°C	35 Griffiths *et al.*, 1997

2.2.4 Oligonucleotides for electromobility shift assays, EMSA

Oligo	Sequence	Origin
AP-1 Consensus	5'-cgcttgatgactcagccggaa-3'	Collagenase gene Lee *et al.*, 1987
Oct-1 Consensus	5'-tgtcgaatgcaaatcactagaa-3'	Scheidereit *et al.*, 1988
AP-1 Enhancer	5'-cgcacctggtattagtcatttcc-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)
AP-1 Promoter	5'-gaactataatatgactaagctgtgc-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)
NF-1	5'-ccattggcgcgcctctttggcgcatac-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)
KRF-1	5'-tgcttgcataactatatccactcccatgt-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)
Oct-1/NF-1	5'-ttaagctaattgcatacttggcttgtacaa-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)
GRE	5'-aggttgggcagcacatactatactttc-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)
Sp-1	5'-gtagtatataaaaaagggagtgaccga-3'	HPV-18 URR (Butz and Hoppe-Seyler, 1993)

2.2.5 Solutions and Buffers

Ampicillin

50 mg/ml in water
Store at –20°C

Biogel-Suspension

1 X TE buffer
5 mM sodium chloride
0.2 %(w/v) SDS
50 %(w/v) Biogel

Blocking buffer (Western blot)

1 X TBS pH 7.6
5 %(w/v) Milk powder
0.1 %(v/v) Tween 20
Store at 4°C

BSA

1 µg/µl in water for protein quantification curve Store at –20°C

Buffer A (Cytoplasmic protein extraction)

10 mM HEPES pH 7.9
10 mM Potassium chloride
0.1 mM EDTA pH 8.0
0.1 mM EGTA pH 7.9
Store at –20°C
Before use add proteases and phosphatases inhibitiors

Buffer C (Nuclear protein extraction)

20 mM HEPES pH 7.5
400 mM Sodium chloride
1 mM EDTA pH 8.0
1 mM EGTA pH 7.9
Store at –20°C
Before use add proteases and phosphatases inhibitiors

Charcoal stripped fetal calf serum

500 ml fetal calf serum
25 g active charcoal
Mix 30 min at room temperature
Spin down and sterile filtration
Store at –20°C

***Chloroform/Isoamylalcohol* (24:1)**

24 parts chloroform
1 part isoamyl alcohol
Store light protected at 4°C

Denaturing solution (Southern blot)

0.5 N sodium hydroxide
1.5 M sodium chloride

***Denhardt´s Solution* (250X)**

5 %(w/v) BSA
5 %(w/v) Ficoll type 400
5 %(w/v) Polyvinylpyrrolidon 25

DEPC Water

0.1 %(v/v) DEPC
1L water

DMEM complete

500 ml DMEM
10 % Fetal Calf Serum
100 U/ml Penicillin
100 µg/ml Streptomycin

***DNA lysis buffer* (3X)**

1.5 %(w/v) SDS
150 mM Tris/HCl pH 7.8
150 mM EDTA

DTT

0.1 M stock solution
Prepare fresh

EDTA

0.5 M EDTA pH 8.0

EGTA

0.25 M EGTA pH 7.9

***EMSA Binding buffer* (5X)**

50 % Glycerin
60 mM HEPES pH 7.9
20 mM Tris pH 8.0
300 mM Potassium chloraide
5 mM EDTA
100 µg/ml BSA
Before use add proteases and phosphatases inhibitors

E-64

2.5 mg/ml Stock solution
in 50 % ethanol/50 % bidest water
Store at –20°C

Freezing medium (Cell culture)

10 %(v/v) DMSO
30 %(v/v) FCS
60 %(v/v) Medium (DMEM)
Store at –20°C

G-418 (Geneticin sulfate)

375 mg/ml stock solution
in DMEM complete
Store at –20°C

Hybridization solution (Northern and Southern blot)

50 % Formamide
10 U/ml t-RNA
5X SSC
0.1 % Denhardt´s solution
50 mM sodium phosphate buffer pH 6.5
1%(w/v) SDS

Hybridization washing buffer (Northern and Southern blot)

2 X SSC
0.1 % SDS

***Laemmli buffer SDS-PAGE* (10X)**

0.25 M Tris Base
1.9 M Glycine
1 %(w/v) SDS

LB-Medium

10 g sodium chloride
10 g Bacto-Trypton
5 g yeast extract
add Water to 1 liter, pH 7.2

LB-ampicillin plates

LB-Medium
2 %(w/v) Bacto-Agar
Add 50 mg ampicillin per liter

Luciferase reaction buffer **(10X)**

250 mM Glycin-glycin
150 mM Magnesium sulfate
pH 7.8 Store at 4°C
Before use dilute to 1X and
add 5 mM ATP

Luciferin solution **(100X)**

10 mg in 1.42 ml of luciferase
reaction buffer
Store light protected at –70°C
Before use prepare a 1X suspension
in reaction buffer

MOPS **(20X)**

400 mM MOPS
100 mM sodium acetate
20 mM EDTA
pH 7.0 (DEPC water)
Store light protected

Neutralization solution (Southern blot)

1 M Tris pH 7.4
1.5 M sodium chloride

PBS

123 mM sodium chloride
17,6 mM di-sodium hydrogenphosphate-
dihydrate
10 mM potassium dihydrogenphosphate
pH 7.2-7.8

Pefabloc SC

23.8 mg/ml stock solution
Store at –20°C

Poly (dI/dC) Poly (dI/dC)

1 mg/ml (10U=500µg)
in TNE buffer 10 min at 45°C annealing
and cooling at room temperature
Store at –20°C

***RNA loading buffer* (2X)**

50 % Formamide
2.2 M Formaldehyde
1 %(w/v) Ficoll (type 400)
0.02 %(w/v) Bromophenol blue
1 X MOPS buffer pH 7.0
(DEPC water)
Store at –20°C

RNase A

10 mg/ml RNase A
10 mM Tris pH 7.5
15 mM Sodium chloride
80°C/20 min, store at –20°C

SDS

10 %(w/v) in water

SDS total protein extraction buffer

50 mM Tris pH 6.8
10 % Glycerin
2 %(w/v) SDS
0.74 M 2-Mercaptoethanol

SOC medium

2 % (w/v) Bacto-Trypton
0.5 % (w/v) yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM Glucose

Sodium fluoride

500 mM Sodium fluoride
Store at –20°C

Sodium ortho-vanadate

10 mM Sodium ortho-vanadate
pH 10 Store at –20°C

Sodium Phosphate buffer

1 M sodium dihydrogenphosphate (3 parts)
1 M di-sodiumhydrogenphosphate (2 parts)
pH 7.0

***SSC* (20X)**

3 M sodium chloride
0.3 M tri-sodium citrate dihydrate
pH 7.0

***TAE* (50X)**

2 M Tris/HCl
0.25 M sodium acetate
0.05 M EDTA pH 8.0
pH 7.8

***TBE* (10X)**

0.9 M Tris base
0.9 M Boric acid
0.02 M EDTA pH 8.0

***TBS* (1X)**

10 mM Tris/HCl pH 7.5
100 mM sodium chloride

TBST

1 X TBS pH 7.6
0.1 %(v/v) Tween 20

***TE* (1X)**

10 mM Tris-HCl
1 mM EDTA pH 8.0

***TNE* (1X)**

10 mM Tris/HCl pH 8.0
1 mM EDTA
100 mM sodium chloride

TNFα

100 U/µl Stock solution
10 µg in 200 µl bidest water
add DMEM complete to 10 ml
Store at –70°C

***Towbin* (1X)**

25 mM Tris Base
192 mM Glycine
0.1 %(w/v) SDS
15 %(v/v) Methanol

Trypan blue

0.25 % in PBS

***Western blot loading buffer* (5X)**

10 % SDS
5 mg Bromophenol blue
12.5%(v/v) 2-Mercaptoethanol
5 mM EDTA pH 8.0
50 % Glycerin
300mM Tris pH 6.8
Store at –20°C

2.2.6 Cell lines

HeLa:

Tissue:	Adenocarcinoma; cervix.
Morphology:	Epithelial
Age stage:	31 years
Ethnicity:	Black
Growth properties:	Adherent
HPV presence:	Type 18 (about 50 copies per cell)
Reference:	Boshart *et al.*, 1984; Schwarz *et al.*, 1985

HeLa RARβ clones (β1 and β2):
HeLa cells stably transfected with retinoic acid receptor β2, under the control of the β-actin promoter, were single cell cloned and expanded. Two of those clones were analyzed in this study (Geisen *et al.*, 2000).

2.2.7 Chemicals and reagents

[α^{32}P] dCTP 10 mCi/ml	Amersham, Life Science
Acetic acid glacial	Merck, Darmstadt
Acrylamide/bis-Acrylamide (29:1), 30%	Sigma, Deisenhofen
Active charcoal	Serva, Heidelberg
Agarose	Invitrogen, Karlsruhe
All-trans-retinoic acid	Sigma, Steinheim
Ammonium acetate	Fluka BioChemika, Deisenhofen
Ampicillin	Roche Diagnostics, Mannheim

Ammonium persulfate	Sigma, Deisenhofen
Bacto-Agar	Gibco Life Technologies
Bacto-Tryptone	Difco, Detroit
BenchMark (protein marker)	Gibco Life Technologies
Biogel P30	BioRad, München
Boric acid	Sigma, Deisenhofen
Bovine Serum Albumin	Sigma, Deisenhofen
Bradford-Reagent (Bio-Rad Protein Assay)	BioRad, München
Bromophenol Blue	Sigma, Deisenhofen
Calcium chloride (dihydrate)	Sigma, Deisenhofen
Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132)	Calbiochem, Bad Soden
Chloride acid	Merck, Darmstadt
Chloroform	Merck, Darmstadt
CLONfectin	Clontech
Cycloheximide	Sigma, Deisenhofen
Diethylpyrocarbonat (DEPC)	Sigma, Deisenhofen
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Dithiothreitol (DTT)	Sigma, Deksenhofen
DNA Markers Gene Ruler 100bp DNA-Ladder	MBI Fermentans, Vilnius
DNA 6X loading buffer	MBI Fermentans, Vilnius
Dulbecco's modified Eagle's Medium (DMEM)	Gibco, Eggenstein
E-64	Roche Diagnostics, Mannheim
ECL-Reagent	NEN Lifescience Products Inc
EDTA	Roche Diagnostics, Mannheim
EGTA	Sigma, Deisenhofen
Ethanol absolute	Merck, Darmstadt
Ethidium bromide	Fluka, BioChemika
Fetal calf serum	Gibco Life Technologies
Formaldehyde	Merck, Darmstadt
Formamide	Fluka BioChemika, Deisenhofen
[γ^{32} P] dATP 10 mCi/ml	Amersham, Life Science
Geneticin (G-418 Sulfate)	Calbiochem, Bad Soden
Glycerin	Merck, Darmstadt
Glycine	Sigma, Deisenhofen
HEPES	Gerbu, Gaiberg
Hexanucleotide	Amersham-Pharmacia, Freiburg
Hydrochloric acid	Merck, Darmstadt
Isoamyl alcohol	Merck, Darmstadt
Klenow-Fragment (DNA polymerase I, 5000U/ml)	New England Biolabs, Frankfurt
LB-Medium	Gibco Life Technologies
Magnesium chloride	Merck, Darmstadt

2-Mercaptoethanol	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
Milk powder	Roth, Karlsruhe
MOPS	Gerbu, Gaiberg
Nonidet-P40	Roche Diagnostics, Mannheim
Nucleotides (dNTPs for PCR)	Roche Diagnostics, Mannheim
PBS	Gibco, Eggenstein
Pefabloc SC	Roche Diagnostics, Mannheim
Penicillin 10000U/ml/Streptomycin 10000µg/ml	Gibco Life Technologies
Poly (dI-dC) Poly (dI-dC)	Amersham-Pharmacia, Freiburg
Potassium acetate	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt
Potassium hydrogen phosphate	Roth, Karlsruhe
Potassium hydroxide	Merck, Darmstadt
2-Propanol, Isopropanol	Merck, Darmstadt
Random primers	Roche Diagnostics, Mannheim
Restriction enzymes	Amersham-Pharmacia, Freiburg
RNAse A (90 U/mg)	Serva, Heidelberg
Roti-Phenol	Roth, Karlsruhe
Sodium acetate	Merck, Darmstadt
Sodium azide	Sigma, Deisenhofen
Sodium citrate	Sigma, Deisenhofen
Sodium chloride	Merck, Darmstadt
Sodium dihydrogen phosphate (monohydrate)	Merck, Darmstadt
Sodium dodecyl sulfate	Sigma, Deisenhofen
Sodium fluoride	Merck, Darmstadt
Sodium hydroxide	Roth, Karlsruhe
Sodium ortho-vanadate	Sigma, Deisenhofen
SuperScript II (Rnase H (-) 200U/µl)	Gibco Life Technologies
Taq platinum DNA Polymerase (5U/µl)	Invitrogen, Karlsruhe
TEMED	Gibco Life Technologies
T4 Polynucleotide kinase (10000U/ml)	New England Biolabs, Frankfurt
T4 DNA ligase	New England Biolabs, Frankfurt
t-RNA	Sigma, Deisenhofen
Tripsin/EDTA	Gibco Life Technologies
Triton X-100	Sigma, Deisenhofen
Triton X-100 cell lysis buffer (3X)	DB PharMingen, Heidelberg
Trizma-Base	Sigma, Deisenhofen
TNF-α	Strathmann Biotech, Hannover
Tween 20	Sigma, Deisenhofen
Trypan blue	Sigma, Deisenhofen
Urea	Merck, Darmstadt

2.2.8 Laboratory equipment

Analytic Scale AE 160	Mettler, Gießen
Analytic Scale basic	Sartorius, Göttingen
Centrifuge 2K15	Sigma, Deisenhofen
Centrifuge Biofuge, Varifuge RF	Heraus, Hanau
Developer machine Curix 60	AFGA
Electrophoresis camera	Renner GmbH, Dannstadt
Geiger counter LB 1210B	Berthold, Wildbad
Gel drier 483	Bio-Rad, München
Hybridization Oven, Hybrid Mini	Bachofer, Reutlingen
Incubator (for cells) B5061 EC/CO_2, Forma Scientific	Heraeus, Hanau
Luminometer, Lumat LB 9501	Berthold
Microscope CK2	Olympus
Minifuge	Heraeus, Hanau
Mini-PROTEAN II (minigels western)	BioRad, München
Neubauer count chamber	Bender and Hobein, Bruchsal
PCR Thermo Cycler MultiCycler PTC-2000	M.J. Research
pH-meter Calimatic 765	Knick
Phosphorimager®	Molecular Dynamics, Sunnyvale, CA
Phosphorimager Cassette	Molecular Dynamics, Sunnyvale, CA
Photometer Ultraspec 3000	Amersham-Pharmacia, Freiburg
Pipette boy acu	Integra Biosciences
Pipettes p2.5, p10, p20, p200, p1000	Eppendorf, Hamburg
Power supply EPS 600	Amersham-Pharmacia, Freiburg
Scale 1216 MP	Sartorius, Göttingen
Sonifier 250	Branson/Heinemann, Schwäbisch Gmünd
Sterile Hood (BioGard Hood)	Baker Company, Sandford
Thermomixer 5436	Eppendorf, Hamburg
Trans-illuminator 254-366 nm	Vetter, Wiesloch
Vortex	Heidolf, Rust
Water Bath	Julabo, Seelbach
Western blot transfer camera Hoefer, Semiphor	Amersham-Pharmacia, Freiburg

2.2.9 Others

Autoradiography cassettes (Kodak)	Sigma, Deisenhofen
Cell culture flasks	Costar
Cell culture plates	Greiner, Nürtingen
Cryo-tubes	Greiner, Nürtingen
Eppendorf Tubes	Eppendorf, Hamburg

Films Hyperfilm™ ECL	Amersham-Pharmacia, Freiburg
Glass pipettes	Renner, Darmstadt
Glass wool	Serva, Heidelberg
Nylon membranes (GeneScreen Plus™)	NEN Lifescience Products Inc
Paper filters	Schleicher and Schüll, Dassel
Pipet Tips (Sterile)	Becton and Dickinson, Heidelberg
Pipet Tips	Greiner, Nürtingen
Photometer plastic Cuvettes	Greiner, Nürtingen
Polypropilene conical tubes	Falcon, Becton-Dickinson
PVDF-membranes (Immobilon P)	Millipore, Eschborn
Sterile filters	Millipore, Eschborn
X-ray films	Fuji
Whatman 3MM paper filter	Schleicher and Schüll, Dassel

2.2.10 Kits

Absolutely RNA™ RT-PCR Miniprep kit	Stratagene
Effectene™ transfection Reagent	Qiagen, Hilden
Hexalabel Plus™ DNA Labeling kit	MBI Fermentans, Vilnius
High Sensitivity β-galactosidase Assay kit	Stratagene
One shot® TOP 10	Invitrogen
Plasmid Purification kit	Qiagen, Hilden
Qiaquick Gel Extraction kit	Qiagen, Hilden
SAPK/JNK Assay kit	Cell Signaling Tech.

2.3 Methods

2.3.1 DNA probes preparation

2.3.1.1 Competent bacteria transformation (Dagert and Ehrlich, 1979)

Commercially available One-shot® TOP 10 competent cells were thawed on ice, 2ng of DNA were added, mixed, and incubated for 30 minutes on ice. Later, cells were incubated at 42°C for 30 seconds and chilled on ice for 2 minutes. The cells were diluted l:5 in pre-warmed SOC-Medium and incubated for 1 hr at 37°C under constant shaking. Twenty and two hundred microliters of the transformed cells were plated on LB-ampicillin plates and incubated at 37°C overnight. Next day, an individual colony was picked, transferred to 10ml ampicillin-containing LB-medium and incubated for about 8h at 37°C.

After the incubation 800µl of this bacterial suspension were mixed with 200µl of 87% glycerin-sterile solution, transfer into cryo-tubes and frozen at -70°C for its posterior use. In order to obtain quantitative amounts of plasmids, the "QIAGEN maxiprep kit" was used following the attached instruction manual. The concentration of DNA was determined photometrically by measuring the optical density (OD) at 260/280 nm. 1 OD unit corresponds to 50µg DNA/ml.

2.3.1.2 Plasmid-DNA restriction analysis

See sections 2.3.4.3 DNA restriction analysis and 2.3.4.4 DNA electrophoresis and Southern blot

2.3.1.3 DNA extraction from agarose gel "QIAquick gel extraction kit"

DNA fragments were extracted from agarose gels using the "QIAquick gel extraction kit" (Qiagen). The protocol allows the extraction of DNA fragments in size ranges between 100 bp to 10 Kbp. After agarose gel electrophoresis the DNA fragment was cut out from the gel, transferred into an eppendorf tube and subsequently treated exactly as described in the attached instruction manual.

2.3.2 Techniques of cell culture

2.3.2.1 Cell culture

Cervical carcinoma cell line HeLa and HeLa clones stably transfected with pbact-RARβ (Geisen *et al*., 2000) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin.
In order to keep the selection pressure the clones were grown in a media containing 750µg/ml of G-418. Incubation was carried out at 37°C with 5% CO_2 and 95% humidity.
The cell cultures that reached the sub-confluent growth (80-90% confluency) were washed once with PBS and treated with Trypsin/EDTA at 37°C until the cells were removed from the plate. The trypsinization was stopped with the addition of complete DMEM and the cells were dilute between 1/5 to 1/30 in new medium depending on the future necessities.

2.3.2.2 Thaw and freeze down eukaryotic cells

To defrost cells the cryo-vials were warmed up to 37°C, once de-frozen the cell-containing medium was transfer to a conical tube and diluted in DMEM complete medium 1:10. The cells were spinning down during 5min at 2000rpm (Varifuge RF, Heraus), 4°C. Then the pellet was re-suspended in a fresh medium and transferred to a culture flask or plate for incubation at 37°C with 5% CO_2 and 95% humidity.
For freeze down sub-confluent culture plates (80-90% confluency) were trypsinized as in the section 2.3.2.1. The cells were centrifugated 5min at 2000rpm (Varifuge RF, Heraus), 4°C and the pellet was re-suspended in freezing medium. The cell suspension was placed in cryo-vials (app. 10^6 cells/ml/vial) and cooled down to –70°C. Later the cryo-vials containing cells were transferred to the liquid nitrogen to long term conservation.

2.3.2.3 Cell counting

In order to determine the cell concentration (cells/ml) was used the trypan blue dye, which additionally allow to determine the living cells. After trypsinization the cells suspension was transferred to a conical tube. A certain volume of cells suspension was mixed with the trypan blue dye solution 1:1 and counted in a Neubauer chamber.
The number of cells was calculated as:

$$\text{Cells/ml} = \frac{\#\text{ cells counted}}{\#\text{ squares counted}} \times 10^4 \times \text{Dilution factor}$$

2.3.2.4 Cell treatment with retinoic acid, MG-132 and TNFα

i. For retinoic treatment, cells were incubated with 10μM *all-trans*-retinoic acid (atRA) disolved in dimethylsulfoxide (DMSO) as a 10^{-2}M stock solution and kept at –20°C light protected. Four hours before treatment with atRA, the normal culture media was replaced by media containing 10% charcoal-stripped serum in order to reduce the retinoids level present in cell culture medium. The cells were exposed to atRA for 72 h without change of culture media, adding fresh atRA after 48h incubation.

ii. Confluent cells were treated with 20μM of MG-132 dissolved in DMSO during 8h. The stock solution was prepared at 20mM and stored at –20°C.

The final concentration of the solvent DMSO in the culture media was 0.1% (v/v), the control cultures received media containing 0.1% (v/v) DMSO alone and were incubated at the corresponding experimental conditions.

iii. TNFα was resuspended in DMEM complete medium as 100U/μl stock solution and store at –70°C. Confluent cells were treated with 500U/ml during 15 or 30 minutes. Between 15-30 minutes is detected the maximal JNK activation and after 30 minutes is possible to observed the c-Jun phosphorylation.

2.3.3 Protein analysis

2.3.3.1 Nuclear and cytoplasm protein preparation (Schreiber *et al*., 1989)

Nuclear extracts were prepared using the method of Schreiber *et al*. 1989 with the only modification that phosphatases inhibitors and proteases inhibitors were included in the buffers A and C at concentrations suggested by the manufacturer.

Stock	Activity	Final concentration
0.1M DTT		1mM
23.8mg/ml(in H_2O) Pefabloc SC	Serine-proteases inhibitor	0.5mg/ml
2.5μg/ml(in H_2O) E-64	Cysteine-protease inhibitor	5μg/ml
0.5M NaF	Phosphatase inhibitor	1mM
10mM Na_3VO_4	Phosphatase inhibitor	0.2mM

For the nuclear-cytoplasm protein extraction a 60cm^2-culture plate with 90-100% confluence (app. $3x10^6$ cells) was washed twice with isotonic PBS. Then 1.6ml of the hypothonic buffer A was added and the cells were scrapped. The cells suspension was placed into an eppendorf tube and ice-cooled 15min to allow cell swelling. Subsequently, 100μl of the 10% solution of non-ionic detergent Nonident P-40 were added to the cells suspension and the eppendorf were vortex 10sec to get the plasma membrane lysis. After centrifugation at 14000rpm, 1min, 4°C the supernatant contain the cytoplasmic protein and the pellet the intact nuclei, which kept the nuclear protein inside.

The cytoplasm protein-containing extract was alicuoted and stored at –70°C. In this extracts the protein concentration was determined by the Bio-Rad DC (Detergent Compatible) protein assay based on a modified Lowry method (Lowry, 1951). For each sample 2-4µl of cytoplasmic protein extract was diluted in 200µl of bi-destilated water, next 100µl of the reagent A* (1ml Reagent A plus 20µl Reagent C) and 800µl of Reagent B were added, mixed and incubated 15min at room temperature. Finally, the absorbancy of the samples was photometrically measured at 750nm and the concentration was determined using a standard BSA curve as reference.
The nuclei-containing pellet was re-suspended in 200µl of ice-cooled buffer C and placed 15min on ice mixing it every 2min. Finally, the extract was centrifugated at 14000rpm, 5min, 4°C and the nuclear protein containing-supernatant was alicuoted and stored at –70°C. The protein concentration of the nuclear extracts was determined by Biorad Bradford-Method (Bradford, 1976). Two to five microliters of the nuclear protein preparation was diluted in 800µl of bi-destilated water and 200µl of the Biorad reagent was added. After gently mix the absorbancy was measured at 595nm and the protein concentration was determined using defined amounts of BSA as standard.

2.3.3.2 SDS-total protein extract (Klotz *et al*., 1999)

To prepare SDS-total protein extracts, cells were washed twice with cold PBS, 500µl of PBS was added and the cells were scrapped, transferred to an eppendorf tube and centrifugated for 30sec at 13000rpm. The pellet was lysed in SDS-total protein extraction buffer without 2-mercaptoethanol. The lysate was sonified twice 15sec (50% power capacity, position 5) at 4°C. Subsequently, the extract was boiled 5min and later ice-cooled. The protein total extract was divided in 2 aliquots, one without 2-mercaptoethanol for protein quantification and the other was supplemented with 0.74M 2-mercaptoethanol, both were stored at -70°C.
Protein concentration was determined by the Bio-Rad DC (Detergent Compatible) protein assay section 2.3.3.1.

2.3.3.3 SDS-Polyacrylamide gel electrophoresis (Laemmli, 1970; Hames and Rickwood, 1990)

This system is based on a discontinuous gel in which the stacking and separating gel layers differ in salt concentration, pH and acrylamide concentration.
Depending on the size of the protein 10% to 12% acrylamide gels were used, the separating gel contains 0.4M Tris pH 8.8, 0.1% SDS, 10-12% Acrylamide/Bis-acrylamide (29:1), 0.5% ammonium persulfate and 0.07% TEMED. After 1h of polymerization at room temperature, a 3% stacking gel solution was added, the stacking gel contains 0.125M Tris pH 6.8, 0.1% SDS, 3% Acrylamide/Bis-acrylamide (29:1), 0.5% ammonium persulfate and 0.16 % TEMED.
Between 25-50µg of total protein were mixed with "Western loading buffer", heated at 95°C for 5min and loaded in the gel. The run was performed in "Laemmlis running buffer" and the running time was approximately 2h (15mA until dye front reached separating gel, then 30mA).

2.3.3.4 Western blot "Semidry" (Gallagher *et al*., 1997)

The proteins were transferred from the gel to the polyvinylidene fluoride PVDF-membranes using a semidry blotting system. After electrophoresis the transfer stack was assembly: over the semi-dry blotter were placed 9 sheets of whatman 3MM filter paper cut to the gel dimensions and soaked in "Towbin buffer", on them the pre-activated membrane and on top the gel followed by other 9 sheets of whatman 3MM paper soaked in "Towbin buffer". The transfer proceeded at 0.8-1.2mA/cm^2 at 4°C for 70min, depending on the protein size.

Then PVDF-membrane was incubated 1 hour at room temperature in "Blocking solution", and subsequently incubated overnight at 4°C with the first antibody (diluted in blocking solution or BSA following the manufacturer recommendations). Next day, the membrane was washed five times with TBST 5min each, followed by the incubation with the secondary antibody in blocking solution for 1h at room temperature. Finally, the membrane was washed again as before, incubated 2min with the ECL reagents and exposed on Hyperfilm^{TM} ECL films.

In order to use the same filter for re-incubation with additional antibodies, membranes were "stripped" in 200mM NaOH for 5min and washed with water and TBS. Then were ready to block again and repeat the process with the selected antibodies.

2.3.3.5 Kinase assay: "SAPK/JNK assay kit" (Figure 12)

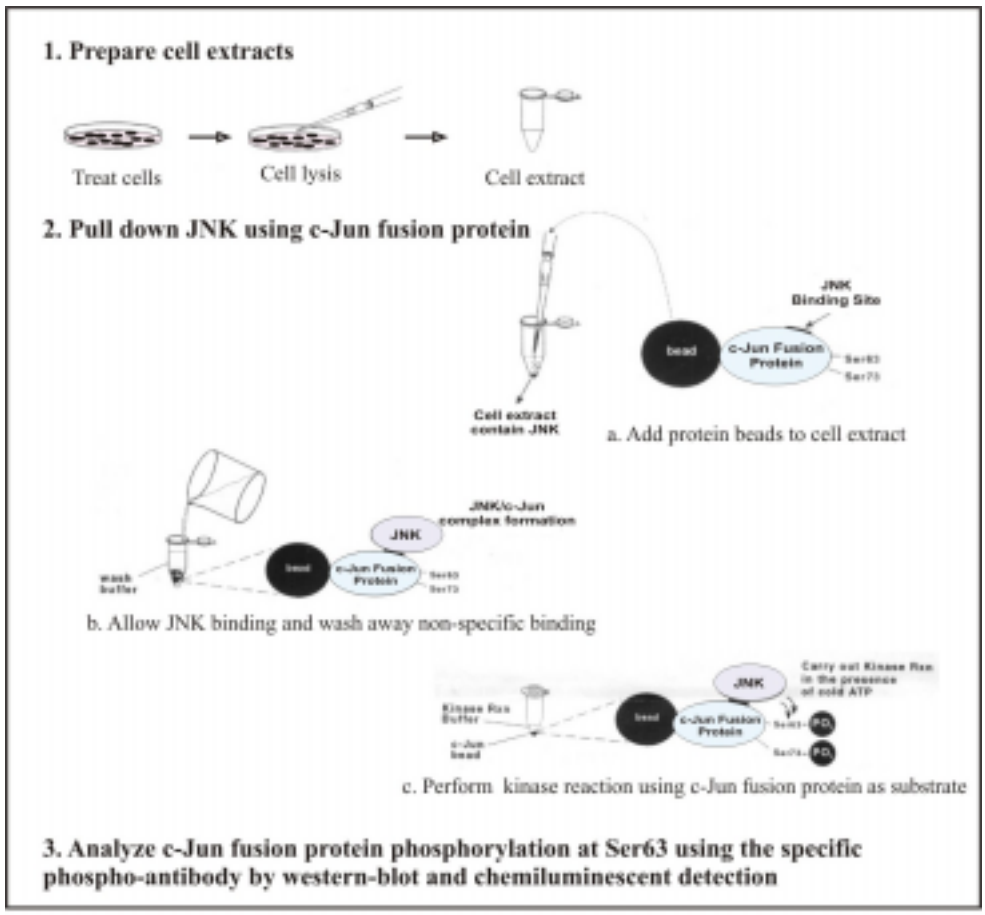


Figure 12 Jun N-terminal kinase assay kit overview

JNK activity was assayed using the SAPK/JNK assay kit (Cell Signalling Technology) following the manufacturer instructions. Cells treated for 15min with TNFα, 8h with proteasome inhibitor MG-132 or 24h post-transfected with MEKK1Δ were harvested in 1X cell lysis buffer and JNK and phospho-JNK were pull down from the extracts (250µg total protein kinase extracts) using 2µg of c-Jun fusion protein beads. Then pellets were incubated in 1X kinase buffer supplemented with 100µM ATP at 30°C for 30 min. SDS-PAGE and immuno-blot allowed the detection of c-Jun phosphorylation. The controls were un-treated or un-transfected cell extracts. Is important to note that the c-Jun fusion protein is a recombinant protein fusion of c-Jun codons 1-89 and glutathione S-transferase and has a reduced molecular weight (33-35KDa) in comparison to c-Jun cellular protein (40KDa).

2.3.4 Nucleic acids analysis

2.3.4.1 RNA-extraction "Absolutely RNA™ RT-PCR Miniprep kit"

RNA was isolated with the "Absolutely RNA™ RT-PCR miniprep kit" (Stratagene) according to manufacturer instructions. For the RNA extraction a 60cm^2-culture plate with 90-100% confluence (app. 3x10^6 cells) was washed twice with isotonic PBS, 500µl of PBS was added and the cells were scrapped and transferred to an eppendorf tube. Cells suspension was spinning down at 3000rpm (Varifuge RF, Heraus), room temperature, 5min. The cells pellet was re-suspended in 600µl of Lysis buffer supplemented with 4.2µl of 2-mercaptoethanol and vortex repeatedly to ensure that the viscosity of the lysate was low. Afterwards, the lysate was filter and mixed with 70% DEPC-ethanol. The filtrate was transferred to a Fiber-Matrix Spin Cup in order to allow binding of the nucleic acids to the matrix. The matrix was washed with low salt washing buffer once and then the DNase treatment proceeded. Next, the matrix was washed once with high salt buffer and twice with low salt buffer and the pure RNA was eluted from the matrix with TE pH 7.5 at 60°C. The concentration of RNA was determined photometrically by measuring the optical density (OD) at 260/280 nm. 1 OD unit corresponds to 40mg RNA/ml.
To check RNA quality, approximately 5µg of RNA was separated on 1% agarose/1X MOPS gel in presence of ethidium bromide under non-denaturing conditions section 2.3.4.5.

2.3.4.2 DNA extraction from eukaryotic cells

Cells were washed twice with PBS, incubated with 3ml "DNA lysis buffer" for 10min at room temperature, scrapped and transferred into 15ml falcon tubes. Proteinase K was added at final concentration of 200ng/µl and incubated at 56°C for 1 hr. Nucleic acid were purified using phenol/chloroform extraction. After centrifugation, the upper DNA-containing aqueous phase was transferred into a new tube where DNA was precipitated after addition of ammonium acetate (2M final concentration) and 0.8 volumes of isopropanol. The mixture was centrifuged and the pellet was washed twice with 70% ethanol, air-dried and resuspended in TE buffer. The concentration of DNA was determined photometrically by measuring the optical density (OD) at 260/280 nm. 1 OD unit corresponds to 50mg DNA/ml. DNA was treated with RNAseA (Ribonuclease A, 10µg/ml) during 10 minutes at 37°C.

2.3.4.3 DNA restriction analysis

For restriction analysis, 1µg of DNA was supplemented with 2U of restriction enzyme in the corresponding buffer as recommended by the manufacturer. After 2 hours at 37°C, DNA was loaded in agarose gels.

2.3.4.4 DNA electrophoresis and Southern blot

Agarose gel electrophoresis is a standard method to separate, identify and purify nucleic acid fragments. The concentration of agarose used for the gel depends on the size of the DNA fragments to be analyzed. By using the fluorescent intercalating dye ethidium bromide at a final concentration of 0.5µg/ml the visualization of the nucleic acids within the gel is possible.

The required amount of agarose was added to the appropriated amount of TAE1X buffer and the mixture was heated to allow all the agarose to dissolve. The solution was cooled down and then was added 0.5µg/ml of ethidium bromide. The gel was poured and allowed to solidify. The gel was transferred to an electrophoresis chamber filled with TAE1X buffer and the samples were loaded after being mixed with agarose loading buffer (6:1). The electrophoresis proceeded between 2 to 8 V/cm. Later, the gel was place on an UV trans-illuminator and photographed.

After DNA restriction and electrophoresis, the agarose/1X TAE gel was incubated for 45min in "denaturing solution".

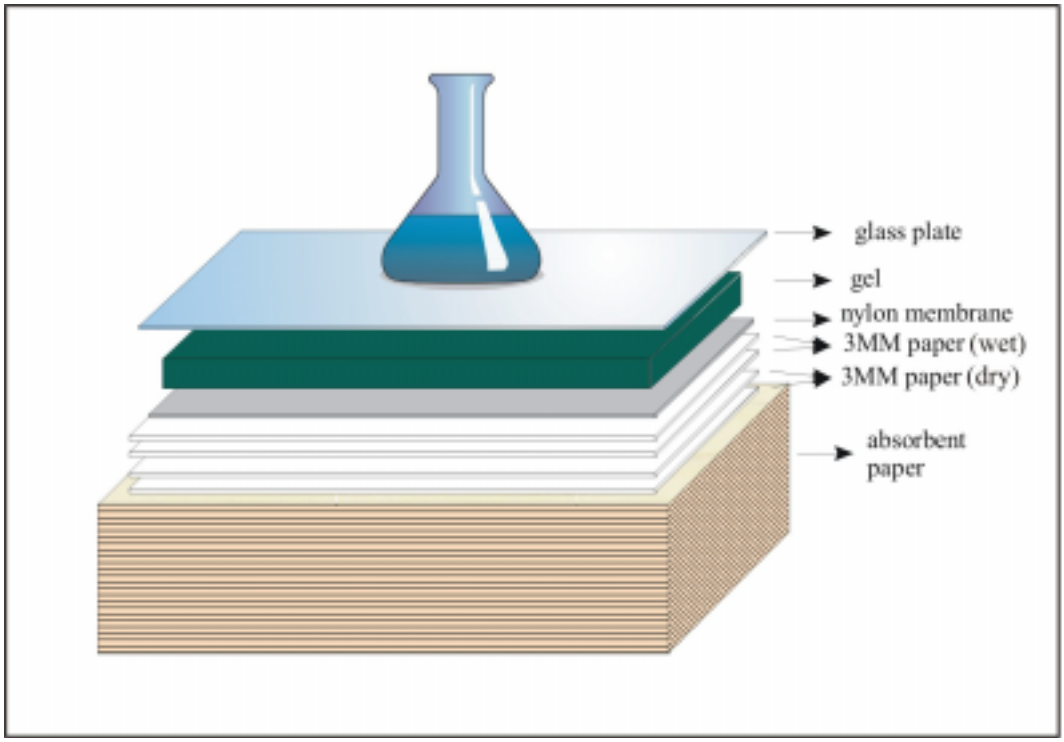


Figure 13: Assembly for nucleic acids transference.

For blotting, a pack of high absorbent paper was used and 4 pieces of 3MM paper were placed on it (first 2 papers dry and then 2 papers wet after soaking in "denaturing-solution"). Gene Screen nylon membrane, previously incubated 1min in bi-destilate water and 15min in denaturing-solution, was placed on the 3MM wet paper. Finally, the agarose gel was laid

on the nylon membrane (see Figure 13). The whole assembly was then covered with plastic paper and left overnight at room temperature. Next day, the nylon membrane was washed 15 minutes in "neutralization solution" and air-dried over 3MM paper. The membrane was then ready for hybridization.

2.3.4.5 RNA electrophoresis and Northern blot (Alwine *et al* 1977)

RNA was separated in 1% agarose/1X MOPS/ethidium bromide gels under non-denaturing conditions. One volume of RNA-loading buffer was added to each probe, heated 10min at 65°C and ice cooled 3min before loading. The gel was run at 60V for 2h and then photographed.
After electrophoresis the gel was incubated for 15 min in 20X SSC buffer. For blotting, a pack of high absorbent paper was used and 4 pieces 3MM paper were placed on it (first 2 papers dry and then 2 papers wet by soaking in 20X SSC buffer). Gene Screen nylon membrane previously incubated 1min in bi-destilated water and 15min in 20X SCC was laid on the 3MM paper. Finally, the agarose gel was deposited over the nylon membrane and the whole assembly was wrapped with plastic paper (see also Figure 13). RNA transfer proceeded over night at room temperature. Next day, the nylon membrane was washed for 5 minutes in 2X SSC and air-dried over 3MM paper. After this step, the membrane was ready for hybridization.

2.3.4.6 Probe labeling "Random-Priming" (Feinberg and Vogelstein, 1983) and Hybridization (Southern, 1975)

The radioactive probes for hybridization analysis were obtained by random priming, in which a mixture of random hexamers is used to prime DNA synthesis *in vitro* from a linear denaturated DNA template. For this purpose the "Hexalabel™ DNA labeling kit" was used. Briefly, 100ng DNA were diluted in 1X reaction buffer, boiled 10min and cooled down on ice. Later, the appropriated nucleotides mix (-dCTP), 25 µCi/µl α^{32}P-dCTP and 5U of Klenow-polymerase were added. The reaction was incubated for 30min at 37°C, then a complete dNTP mix was added and the incubation was prolonged 5min. Subsequently, the unincorporated label was removed by filtration of the TNE-diluted labeling reaction through a Biogel-suspension column.
Blot pre-hybridization was performed in "hybridization buffer" for at least 1h at 42°C, after that denaturated radioactive probe was added to the pre-hybridized membrane and hybridization proceeded over night. Next day, filters were washed twice with "hybridization washing buffer" at 68°C and exposed on Hyperfilm™ ECL films at –70°C.

2.3.4.7 Reverse transcription and polimerase chain reaction: Semi-quantitative RT-PCR

cDNA was obtained from one to five micrograms of RNA by using random primers and SuperScript II reverse transcriptase following the manufacturer instructions. The random primers and the RNA were heated at 70°C for 10min and chilled on ice. Then the mixture was supplemented with 1X reverse transcription (RT) buffer (50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$), 10mM DTT, 500µM dNTPs and incubated at 25°C for 10min for the annealing step. After annealing, 100U of reverse transcriptase SuperScript II was added and the reaction was incubated 50min at 42°C for the first cDNA strand synthesis. Finally, the cDNA was heated to 70°C for 15min and chilled on ice.

PCRs were performed in 50μl final volume containing 10mM Tris-HCl pH 8.3, 200μM dNTPs mix, 500nM of each primer, 5U of Taq platinum polymerase and 1-5μl of cDNA. All PCR reactions were performed for 35 cycles consisting of 30sec at 94°C, 45sec at the corresponding annealing temperature and 30sec at 72°C, with a final extension of 10min. The amount of cDNA and the number of cycles were carefully standarized in order to reach a semi-quantitative indication of the RNA content. The PCR products were analysed in 1-2% agarose gels.

2.3.5 Protein-DNA interaction

2.3.5.1 Electrophoresis mobility shift assay (EMSA)

The electrophoresis mobility shift assay, EMSA determines the binding interaction between DNA and DNA-binding proteins, such as the interaction of transcription factors with the corresponding regulatory regions. The complexes of protein and DNA migrate more slowly through a non-denaturating polyacrilamide gel than unbound double-stranded oligonucleotides. The addition of antibodies to the reaction is commonly used to determine the identity and specificity of the binding protein. If the antibody recognise the DNA-protein complex, it can bind and retard even more the migration of the complex in the gel (Figure 14).

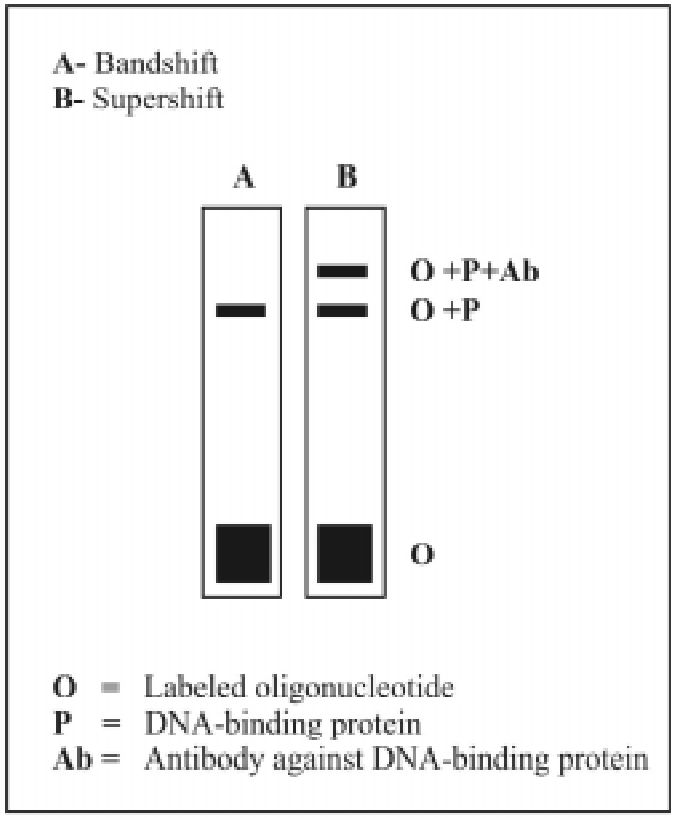


Figure 14 Electrophoresis mobility shift assay

The oligonucleotides used in gel retardation assays were made in an Applied Biosystems (Foster City, CA) synthesizer using phosphoramitide chemistry and they were further purified by HPLC. For electrophoretic mobility shift assays 2μg of each oligonucleotide strand (sense and anti-sense) was annealed in TNE by a PCR program that include 10min at 85°C followed by a

temperature decreased of 0.5°C every 30sec. Two hundred nanograms of annealed oligonucleotides were labelled with ^{32}P-γ-ATP (Amersham, 3000Ci/mmol) and T4 polynucleotide kinase at 37°C, 30min. Non-incorporated label was removed from the labeled oligonucleotides by gel-purification in a 15% polyacrylamid/1X TBE electrophoresis at room temperature. The labelled oligonucleotides were extracted from the polyacrilamide in TNE by shaking over night at 4°C.
Binding assay was performed in a 20µl reaction volume containing 1X EMSA binding buffer, 2µg poly(dI-dC) poly (dI-dC) and 2µg nuclear extract. After 5min at room temperature, 10000 cpm of ^{32}P-γ-ATP 5'-end labelled double-stranded oligonucleotide probe was added. Reaction was incubated for additional 30 min at room temperature.
For super-shift analysis 2µg of the specific antibody was added after the 30min at room temperature and the reaction was further incubated for 1h at 4°C.
DNA-protein complexes were resolved in a 5.5% non-denaturing polyacrylamide gel (29:1), dried and exposed overnight in Fuji medical X-ray films.
Bands were quantified with a Molecular Dynamics (Sunnyvale, CA) Phosphorimager using the "Image Quant" program as software.

2.3.6 Transient transfection analyisis

2.3.6.1 Transient transfection protocol "Effectene"

Transient transfection experiments were used to analyse the effect of some proteins expression over the RARβ-AP-1 *trans*-repression phenomenon or to monitor the AP-1 or RARβ activity under certain conditions.
The transfection experiments were simple, double or triple depending on how many plasmids were introduced into the cells and in all cases the transfection was carried out with "Effectene™" reagent following the manufacturer instructions. For simple transfections $2.5x10^6$ cells were plated in 60-cm^2 dishes the day before transfection. Next day, 2µg of the plasmid DNA was mixed with 300µl of DNA-condensation buffer, 16µl of enhancer and 60µl of effectene, following the incubation time recommended to allow the complexes formation, 3ml of complete DMEM was added and the transfection complexes were poured into the cells. Cells were incubated over night in the presence of the pDNA-transfection-complexes and then were splited in two or three 21-cm^2 plates in order to prepare RNA, total-protein extracts and nuclear-cytoplasm extracts.
In the case of double transfections $2.5x10^6$ cells were plated in 60-cm^2 dishes. Next day, cells were co-transfected with 1.5µg of plasmid DNA (TRE-Luciferase, Promega or RARE-Luciferase D1 (Bartsch *et al*., 1992)) and 0.5µg of SV-40-β-galactosidase as control for transfection, using the same protocol as described for simple transfections.
One day after transfection, cells were splited in two plates (for luciferase and β-galactosidase extract preparation) and between 12-24h later cells were treated with MG-132 (8h) or atRA (4h) using DMSO as control. Cells were harvested and Luciferase activity was measured using a luciferase assay (section 2.3.6.2). Data were normalized to the co-expressed β-galactosidase activity (section 2.3.6.3).
In the case of the antisense Fra-1 expression plasmid transfection 1.5µg of the antisense Fra-1 plasmid were co-transfected with 0.5µg of MEKK1Δ expression plasmid, using the same number of cells and the same transfection conditions as before. One day later, cells were splited in two plates in order to prepare RNA and nuclear-cytoplasm extracts.

Finally for the triple transfection, 2µg of the MEKK1Δ or Ha-ras-mutant expression plasmid were co-transfected with 1.5µg of TRE-Luciferase (Promega) and 0.5µg of SV-40-β-galactosidase. The DNA was mixed with 600µl of DNA-condensation buffer, 32µl of enhancer and 120µl of effectene. Following the incubation time recommended to allow the complexes formation, 3ml of complete DMEM was added and the transfection complexes were applied to $2.5x10^6$ cells plated in 60-cm^2 dishes. Cells were incubated over night in the presence of the pDNA-transfection-complexes and then were splited in two plates for luciferase and β-galactosidase extract preparation. Luciferase activity was measured using a luciferase assay (section 2.3.6.2). Data were normalized to the co-expressed β-galactosidase activity (section 2.3.6.3).
For the "Effectene" protocol the transfection efficiency in HeLa cells was higher than 30% as determined by transfection with expression vector carring the green fluorescence protein.

2.3.6.2 Firefly luciferase reporter gene analysis

After the specific transfection conditions and/or treatments, cells were washed twice with cold PBS and 500µl of "Triton X-100 cell lysis buffer" 1X was added. Cells were incubated 15min on ice and then 5min at room temperature, afterwards cells were scrapped and transferred to an eppendorf tube. The cell lysates were centrifugated 10sec and the clarified extracts were transferred to a fresh eppendorf, frozen down on dry ice and then stored at –70°C.
For the luciferase assay 350µl of the 1X "luciferase reaction buffer" was mixed with 50-100µl of the sample and immediately placed into the luminometer where automatically was injected 100µl of 1X "luciferin solution".
Luciferase activity was calculated in RLU/mg of protein and normalized according to the β-galactosidase specific activity (section 2.3.6.3). The fold induction was calculated with respect the normalized luciferase activity of the specific controls in each transfection case.

2.3.6.3 β-galactosidase normalization "High-Sensitivity β-Galactosidase assay kit"

After the specific transfection conditions and/or treatments, cells were washed once with PBS and 500µl of lysis buffer was added. Cells were frozen at –20°C for 30min and later thawed at room temperature to allow the lysis. The cell lysate was transferred to an eppendorf tube and centrifugated at 12000rpm, 5min, 4°C. The supernatants were collected and placed in a new tube and stored at –20°C.
For the analysis 100µl of the cell extract was mixed with 900µl of the substrate chlorophenol red-β-D-galactopyranoside (CPRG) 1X, vortex 5min and incubated at 37°C in a water bath until the sample became red. The reaction was stopped by the addition of 500µl of stop solution. The time spent in the process since CPRG addition until stop solution addition was recorded. The optical density of the samples was measured at 570nm, and the specific activity of β-galactosidase was calculated as indicated by the manufacturer:

Concentration of chlorophenol red (nmol/ml)= OD x 55 (A)
Amount of chlorophenol red (nmol)= (A) x 1.5ml (reaction volume) (B)
Activity of β-gal (nmol/min or U)= (B)/incubation time at 37°C (min) (C)
Specific activity β-gal (U/mg)= [(C)/protein concentration of the lysate (mg/ml)]/0.1ml (volume of lysate used in the assay)

2.3.7 Retroviral vector construction

2.3.7.1 Cloning of Fra-1 cDNA on pLXIN vector

The Fra-1 insert was excised from the pSG-Fra-1 plasmid by restriction digestion with HincII-EheI and gel purified. Simultaneously the bicistronic retroviral vector pLXIN (Invitrogen) was digested with HpaI in order to open the plasmid generating blunt ends. After gel purification of the digested plasmid, the vector was de-phosphorylated to avoid the re-circularization and the blunt ligation reaction proceeded in a 10:1 insert:vector ratio at 16°C over night.
One-shot® TOP 10 chemically competent cells were transformed with 1-5μl of the ligation reaction and plated in LB-Ampicilline (section 2.3.1.1). Resistant colonies were picked up and grown, plasmid DNA mini-preparations were carried out using the "QIAGEN miniprep kit" following the attached instruction manual and restriction analysis of the plasmids was performed using the restriction enzymes EcoRI which excise the insert and ApaI which determine the insert orientation (sense or antisense). In order to re-confirm the fra-1 cloning, a Southern blot of the digested plasmids was performed and hybridized with fra-1 probe.

2.3.7.2 Transfection of the virus producing cell line RetroPack™ PT67

(Figure 15)

All security rules were followed to avoid any biological contamination in the working area.

The Clontech RetroPack™ PT67 cell line is a mouse fibroblast (NIH 3T3)-derived cell line designed for stably producing high-titer retrovirus. Virus produced by RetroPack PT67 cells express a dualtropic envelope, 10A1, that can infect mouse, rat, human, hamster, mink, cat, dog and monkey cells. Virus produced by these cells can enter target cells via two surface molecules, the amphotropic retrovirus receptor, RAM1 and the GALV receptor. That means if one receptor is not abundantly expressed by a given species or cell type, the alternative receptor may still allow viral entry. Thus virus packaged by RetroPack 67 has a broad mammalian host range.
The RetroPack PT67 cell line was thawed and grown in high glucose DMEM medium. A kill curve for G-418 was performed in order to determine the antibiotic concentration and the time necessary for isolation of resistant transfected virus-producing clones.
For retroviral vector transfection $5x10^5$ PT67 cells were plated in $25cm^2$ flasks 24h before transfection. Two-hours previous transfection the complete high glucose DMEM medium was replaced by 25μM chloroquine-containing high glucose DMEM, which may increases transfection efficiency 2-3 fold.
The recommended transfection agent for the RetroPack PT67 cell line is the "CLONfectin" reagent by Clontech. The fresh liposome-DNA containing solution was prepared following the manufacturer instructions. The CLONfectin reagent was re-suspended in HEPES-NaCl buffer dropwise at concentration of 1μg/μl, vortex gently and placed on ice. Then the solution A (10μg of pDNA and 300μl serum-free high glucose DMEM) was mixed with the solution B (10μg of CLONfectin and 300μl serum-free high glucose DMEM) gently and this transfection solution was incubated at room temperature 30min. Subsequently, 2.4ml of serum-free high glucose DMEM was added and mixed. After removing the chloroquine-containing medium the CLONfectin/DNA/DMEM solution was gently applied to the cells.

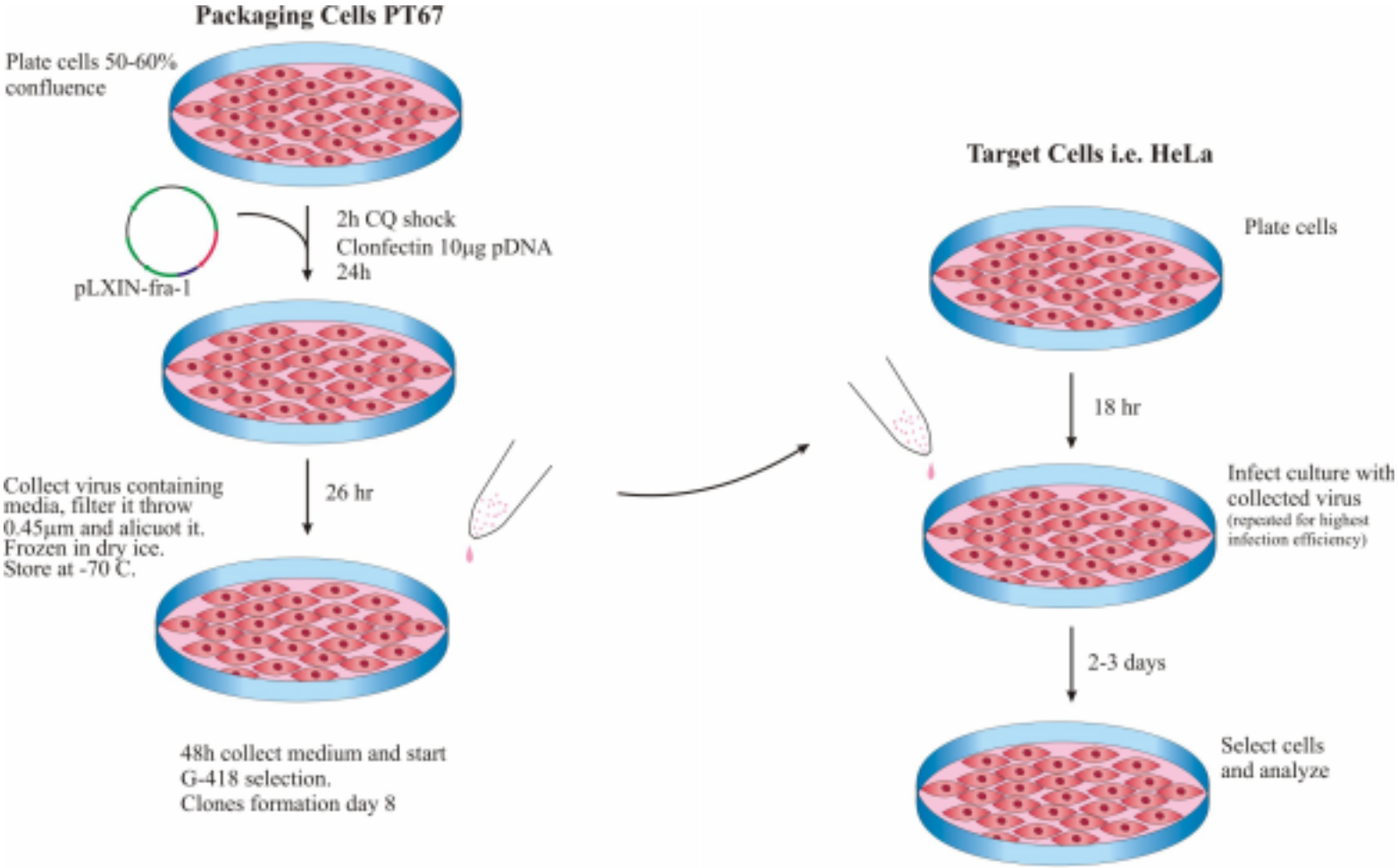


Figure 15 Cells transfection and infection with the retroviral vector pLXIN-fra-1

The flasks were incubated at 37°C for 3h. Later, the CLONfectin/DNA-containing medium was removed and the cells were washed once with pre-warmed PBS. Finally, 3ml of complete high glucose DMEM was added to proceed the incubation 24h.
The positive transfectants selection was started 48h post-transfection by the replacement of the complete DMEM by G-418 (600μg/ml)-containing DMEM in the cell cultures.

2.3.7.3 Virus collection and storage

(Figure 15)

All security rules were followed to avoid any biological contamination in the working area.

Twenty-six hours post-transfection was started the virus-containing-medium collection. The virus-containing-medium was aspirated from the culture flask and filtered through 0.45-μm cellulose acetate filter (low protein binding to preserve the virus). The filtrate was alicuoted in cryo-tubes and frozen in dry ice. Then the cryo-tubes were stored at –70°C until be used.

2.3.7.4 Infection of HeLa cells

(Figure 15)

All security rules were followed to avoid any biological contamination in the working area.

For HeLa infection $2x10^5$ cells were plated in $25cm^2$ flasks. The next day a pool of Fra-1 carrying viruses was prepared and two dilutions, 1:2 and 1:10, of the virus-containing-medium were tested to infect the HeLa cells. The infection mix contained the virus-containing-medium at the corresponding dilution in complete DMEM and 4mg/ml of Polybrene (Sigma), which reduces charge repulsion between the virus and the cellular membrane. As control HeLa cells were infected with a pool of viruses carrying the empty vector pLXIN. Twenty-four hours post-infection the DMEM was replace by DMEM-G-418 (1mg/ml) in order to start the Fra-1 expressing-cells selection, two days later the non-infected cells started to die. The selection proceeded for seven days more, then the resistant clones were pooled and nuclear-cytoplasm extracts and RNA were prepared.

III. RESULTS

3.1 RARβ *trans*-repression of AP-1 transcription factor

3.1.1 RARβ expression in HeLa RARβ transfected cells

In HeLa cells has been shown low expression of RARα and RARγ and almost undetectable levels of RARβ (Si *et al*., 1996). In order to determine the effect of RARβ constitutive expression in HeLa cells, they were stably transfected with a pbact RARβ expression vector (Geisen *et al*., 2000). This plasmid contains the cDNA of the human RARβ2 gene under the control of the human β actin promoter. The transfected cells were selected for G-418 resistance, cloned and expanded. Dr. Elisabeth Schwarz (DKFZ, Heidelberg) kindly provided the clones for further research.

The expression of the RARβ exo-gene in HeLa clones was analyzed at RNA and protein levels. As a control, HeLa cells under un-stimulated conditions and after 3 days of treatment with *all-trans*-retinoic acid (atRA) at pharmacological dose of 10μM were used.

Figure 16 shows that in HeLa cells, cultured in media supplemented with charcoal stripped serum (in order to reduce the levels of retinoids contained in the serum), the RARβ expression was undetectable both at RNA and protein level. However, RARβ expression in HeLa cells was increased after 3 days of retinoic acid treatment. As expected, the clones expressed higher levels of RARβ RNA and protein in the absence of exogenous ligand addition. Consistent with a previous report (Geisen *et al.*, 1997), the induction of RARβ expression, endogenously by atRA or exogenously by the transgene had no effect on the biosynthesis of RARα.

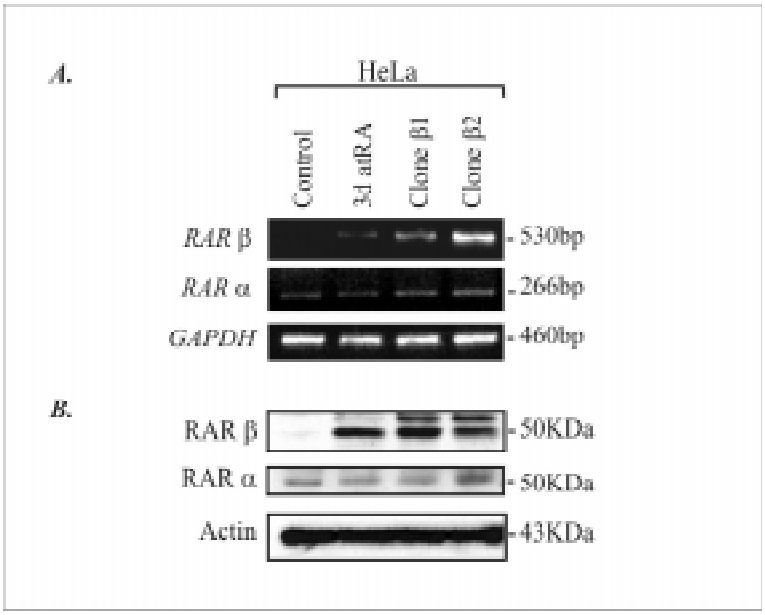


Figure 16 Induction of the endogenous RARβ expression by atRA and stable ectopic expression of RARβ in HeLa cells. ***A***. Semiquantitative RT-PCR products by RARβ, RARα and GAPDH-specific primer sets. Control represents untreated cells; 3d atRA, cells treated with *all-trans*-retinoic acid for about 72h; clone β1 and β2, the stable transfected clones that express the receptor in the absence of exogenous added ligand. ***B***. Immunoblot analysis of nuclear extracts (25μg per lane) separated in two identical SDS-10% polyacrylamide gels followed by electrotransfer and incubation with specific antibodies. Equal loading and protein transfer was confirmed by incubating the filters with an anti actin antibody.

The functionality of endogenous and exogenous RARβ protein was tested by transient transfection of luciferase gene under the control of the promoter region of the RARβ gene. Figure 17 shows the induction of luciferase expression after 4h of retinoic acid treatment. The induction shows that the stably transfected gene generate a functional protein. Additionally, as shown in panel B, it is interesting that the endogenous activity of the RARE promoter is much higher in the clones when are compared to HeLa cells. This implies a different basal activity between RARβ clones and HeLa with respect to the expression of genes driven by RARE containing promoters in the absence of ligand.

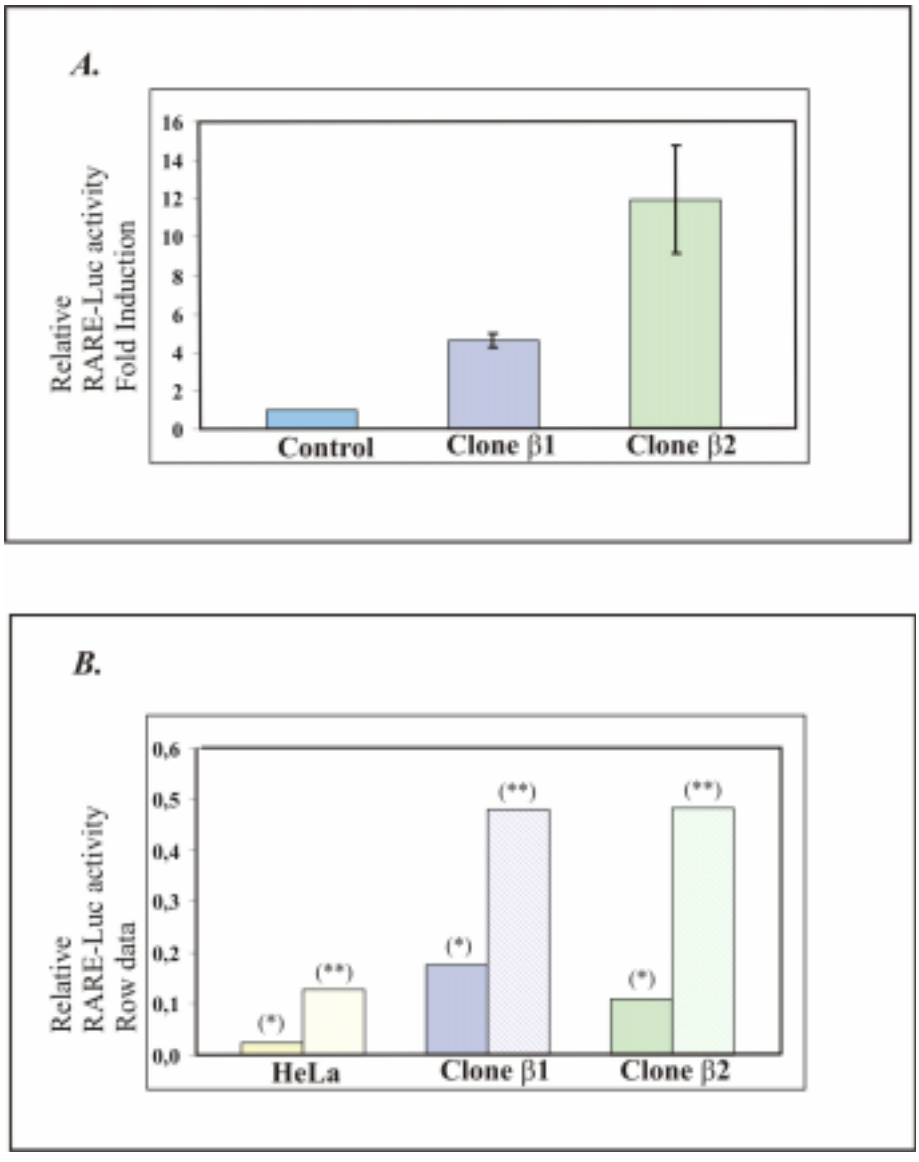


Figure 17 RARE-Luciferase activity in HeLa and the stable transfected clones. RARE-Luciferase reporter construct, was cotransfected with SV-40-β-galactosidase. One-day post transfection, cells were splitted in two identical plates. After 40h, one of the plates was treated with 10μM atRA for 4h. ***A***. The cells were assay for luciferase activity and each value was normalized according to the β-galactosidase activity and compared with the untreated control to define the fold induction. The untreated controls were normalized and arbitrarily set as 1. Data represent the results from three independent experiments. ***B***. A representative experiment showing the row data for the luciferase activity in order to compare the basal (*) and induced (**) promoter activation in HeLa and HeLa RARβ clones.

3.1.2 Ectopic RARβ expression leads to a selective reduction of AP-1

RARβ is known to be a negative regulator of AP-1 transcription factor activity (Van de Klundert *et al*., 1995; Huang *et al*., 1997) but this has not been tested so far in cervical cancer cells. Figure 18 shows a reduction between 70-80% of the AP-1 content in the clones in comparison with HeLa parental cells in the absence of exogenous added ligand. HeLa cells treated for 3 days with retinoic acid show a reduction in the AP-1 content of about 60%. Note that the reduction of AP-1 was selective since the binding of the transcription factor Oct-1 to its cognate recognition sequence shows no variation between the clones and the parental HeLa cells.

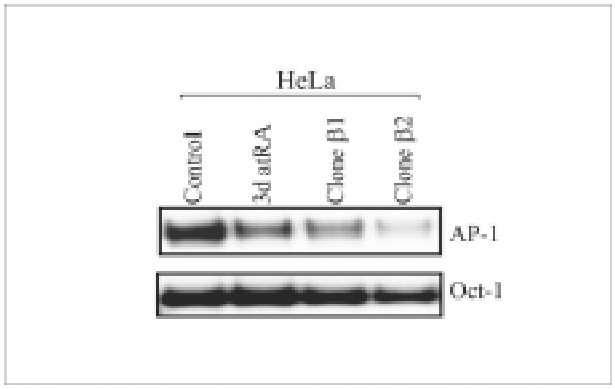


Figure 18 The AP-1 content in the HeLa RARβ clones is reduced in the absence of retinoic acid treatment. EMSA using ^{32}P-labeled oligonucleotides specific for AP-1 or Oct-1 sequences. Nuclear extracts of HeLa untreated cells (control), HeLa treated 3d with atRA or HeLa RARβ clones were analyzed. Quantitation of the AP-1 content was performed by PhosphorImager.

3.1.2.1 Effect of RARβ expression on individual AP-1 family members

To analyze why AP-1 was down-regulated, the mRNA and protein levels of AP-1 family members was determined (Figure 19). AP-1 content of c-Jun, Fra-1 and c-Fos in HeLa and HeLa RARβ clones was quantified by PhosphorImager analysis. RARβ cells have 85-92% less c-Jun in the AP-1 than the HeLa parental cells, the Fra-1 content was approximately 2 times more for RARβ clones in comparison with HeLa and c-Fos was almost unaltered between HeLa and HeLa RARβ clones. The cellular content of JunB, JunD and ATF-2 was comparable between HeLa and HeLa RARβ clones, and no significant differences were observed.

Since c-Jun has been reported as the main dimerization partner in HeLa cells (Soto *et al*., 1999; Soto *et al*., 2000) and Jun family members are necessary for the binding of AP-1 to DNA, one can conclude that the down regulation of AP-1 in the RARβ clones is mainly due to the strong reduction of c-Jun protein. As *c-jun* mRNA is present in the RARβ clones at detectable levels, the c-Jun protein reduction must be a post-transcriptional phenomenon.

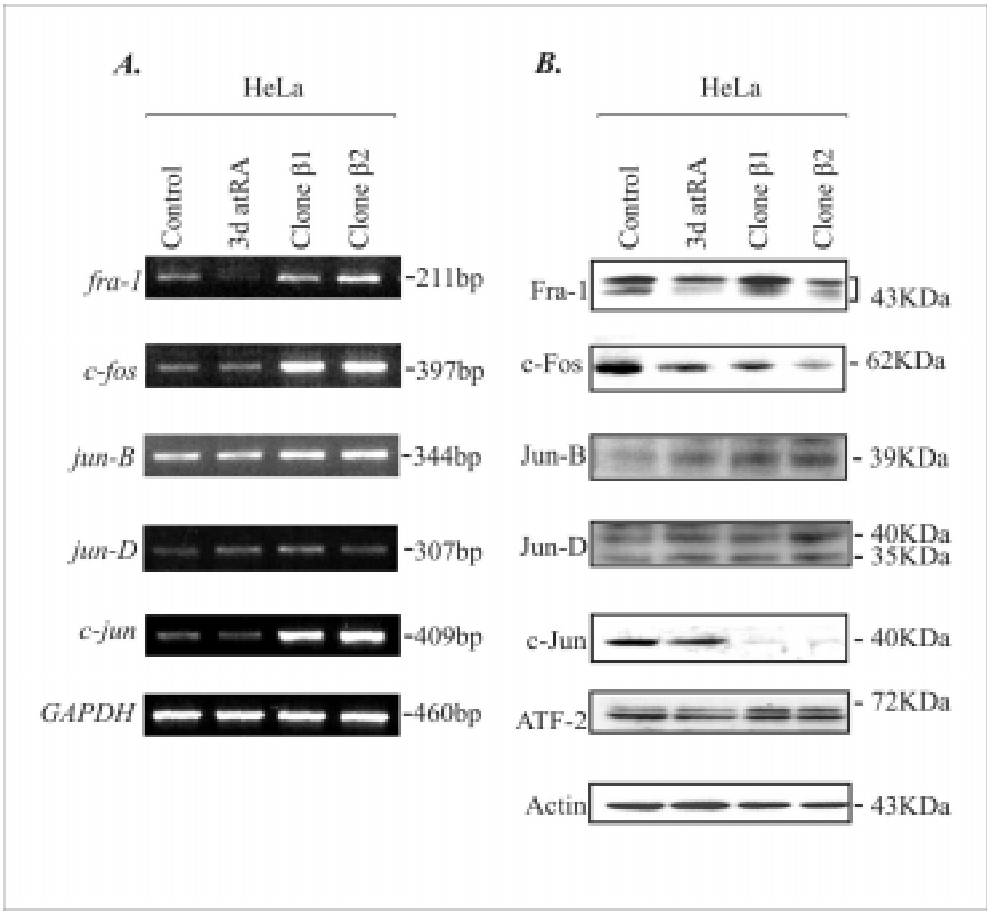


Figure 19 Reduced AP-1 content in RARβ clones due to post-transcriptional down regulation of c-Jun. ***A***. Semi-quantiative RT-PCR. ***B***. Western blot analysis for different members of the AP-1 family, the sizes of PCR products and molecular weight of the proteins are indicated.

3.1.2.2 Mechanism of c-Jun down regulation

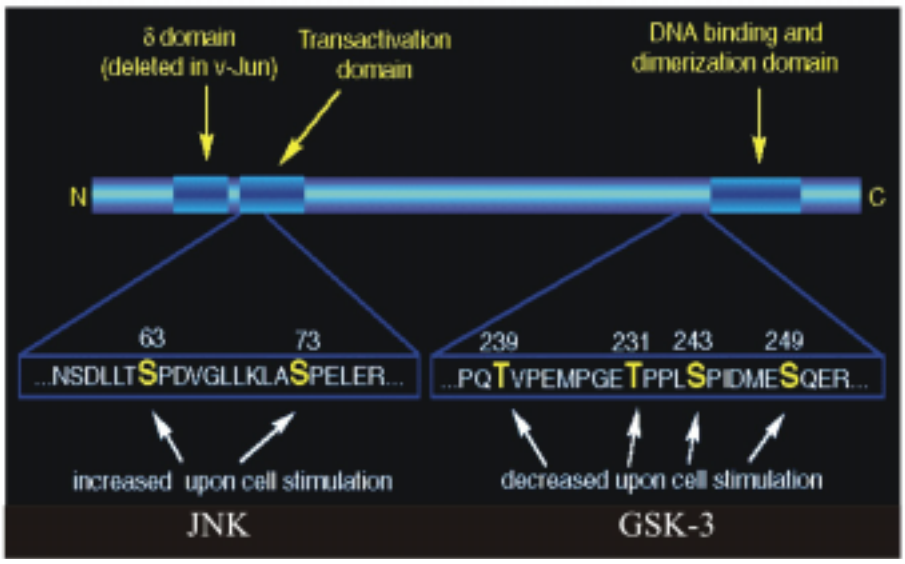


Figure 20 Schematic representation of c-Jun phosphorylation sites at the N and C-terminal domains

As we observe c-Jun down regulation in the RARβ clones, the role of Jun N-terminal kinase (JNK) in the c-Jun degradation was analyzed (Musti *et al*., 1997). Two possibilities can be envisioned: first

the JNK is unable to be activated (phosphorylated) and this is the reason why c-Jun is degraded. Second, the JNK is activated but the presence of RARβ leads to an increase in the content of MAPK phosphatase 1 (MKP-1), which de-phosphorylates the activated JNK and blocks its function resulting in the c-Jun instability. Additionally, it has been suggested that retinoic acid can mediate activation of glycogen synthase kinase 3 (GSK-3), I therefore tested whether c-Jun phosphorylation by GSK-3 (RARβ activated) can result in diminished DNA binding and c-Jun degradation (Figures 20 and 21).

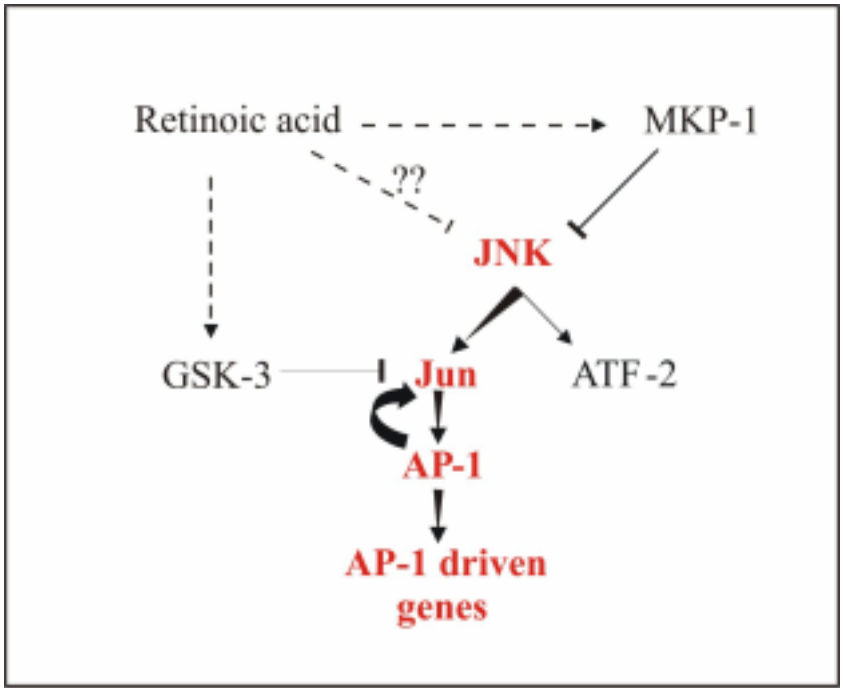


Figure 21 Schematic interplay between retinoic acid and kinases c-Jun related

To induce JNK activation tumor necrosis factor α (TNFα) treatment was used. Differential JNK activation (phosphorylation), MKP-1 expression (at protein level) and GSK-3 inactivation (phosphorylation) between HeLa and HeLa RARβ clones was evaluated. Figure 22 shows that JNK is activated in the clones to higher levels than in HeLa parental cells, MKP-1 expression is comparable and the active form of GSK-3, isotypes α and β, is present in all cell lines at similar levels.

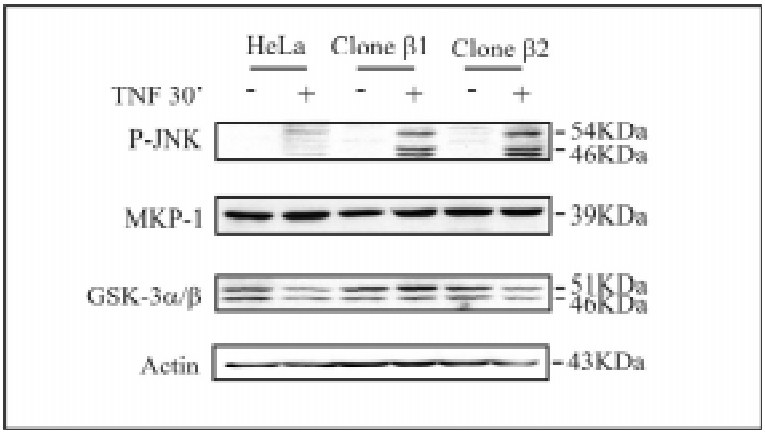


Figure 22 Western blot analysis for kinases and phosphatases related in the c-Jun stabilization. Thirty micrograms of total extract per lane were separated in SDS-10% polyacrylamide gels. Equal loading and protein transfer were confirmed by incubating the filters with anti-actin antibody. The filters reprobed for the different antibodies were stripped in 0.2N NaOH.

These results indicated that JNK activation is not responsible for the c-Jun down regulation which is in contrast to other systems reported in the literature (Lee *et al*., 1998; Moreno-Manzano *et al*., 1999; Gonzalez *et al*., 2000; Li *et al*., 2001). Additionally, the GSK-3 activation is not playing a role in the RARβ effect on c-Jun stability.

To check if the activation of JNK and the phosphorylation of c-Jun was coupled or whether RARβ interfere between JNK activation and c-Jun phosphorylation, an activity test using exogenous c-Jun was performed. In the figure 23 the activity test reveals that phospho-JNK is active and able to phosphorylate exogenous added c-Jun protein. Consequently, the absence of the c-Jun protein in the HeLa RARβ clones is not due to the inactivation, inhibition or dysregulation of the JNK.

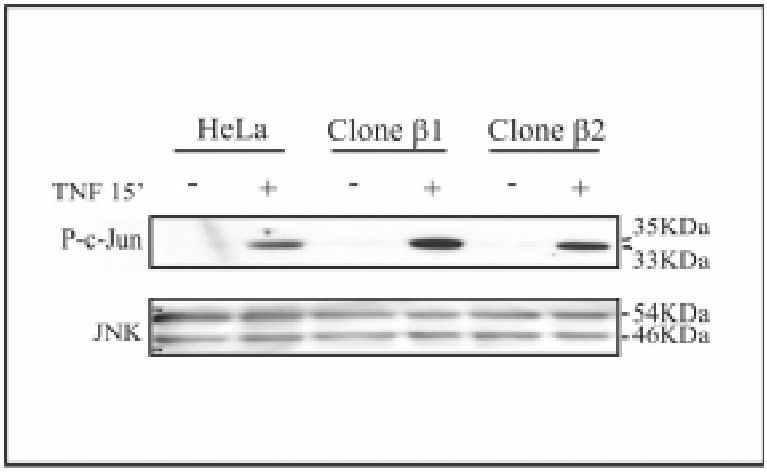


Figure 23 JNK activity of HeLa parental cells and HeLa RARβ clones after 15' TNFα treatment. JNK from cell extracts was pulled down using c-Jun fusion protein beads follow by the kinase reaction in the presence of ATP. The JNK activity was analyzed by Western blot using a specific phospho-c-Jun antibody. Note a slight reduction in the molecular weight of the c-Jun fusion protein in comparison with the cellular c-Jun (see materials and methods section 2.2.3.5). Equivalent loading and protein transfer were confirmed by incubating the filters with an anti total JNK antibody.

3.1.3 Increase of c-Jun protein leads to AP-1/DNA-binding reconstitution in HeLa RARβ clones

As was showed in the previous sections, AP-1 reduction was mainly due to c-Jun down regulation. Next I checked whether an increase in the c-Jun content in the cells could result in AP-1 binding reconstitution in the HeLa RARβ clones. The different strategies used in order to reach this aim will be analyzed in the next sections.

3.1.3.1 Overexpression of exogenous c-Jun in HeLa and HeLa RARβ clones

The first strategy used to increase AP-1 was c-Jun overexpression by transient transfection in HeLa and HeLa RARβ clones. The electrophoretic mobility shift assay EMSA showed a 2-fold increase in AP-1 binding in the RARβ clones as well as in HeLa parental cells. However, the c-Jun and P-c-Jun in the clones remain undetectable in Western blot in contrast to the HeLa parental cells, in which c-Jun content is highly increased and phospho-c-Jun is detectable after protein overexpression without JNK activation (Figure 24).

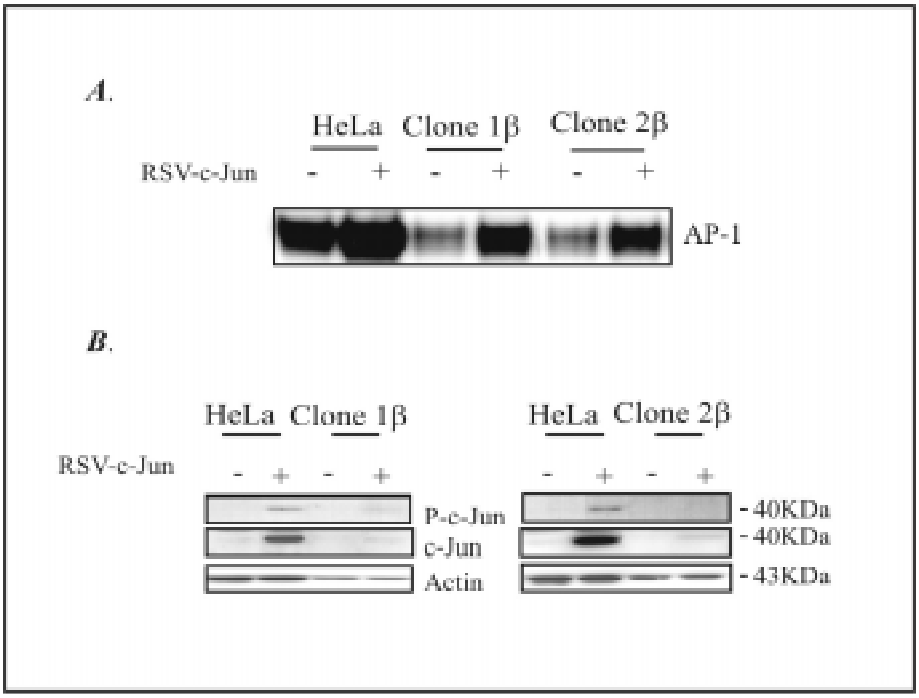


Figure 24 c-Jun overexpression in HeLa and HeLa RARβ clones. ***A***. Nuclear extracts of HeLa and HeLa RARβ clones control and transiently transfeted with RSV-c-Jun were prepared for EMSA with AP-1 oligonucleotide. ***B***. Western blot for c-Jun and phospho-c-Jun was performed using the same extracts, in which the amount of protein was normalized to the β-galactosidase leading to different intensity of the actin signal. Amount of protein and transfer was monitored incubating the filters with anti-actin antibody, the molecular masses of the proteins are indicated.

The results suggest that the transfected protein is unstable in the HeLa RARβ clones, since in contrast to HeLa parental cells, it can not be accumulated, even under overexpressed conditions as is shown in the Western blot. However, although c-Jun was not visible under Western blot conditions, using EMSA a highly sensitive method, it is possible to detect an increase in the AP-1 complex. At this point, it was postulated that c-Jun is regulated via differential half-life in HeLa and HeLa RARβ clones. In the following experiments the aim was stabilize the c-Jun protein and accumulate it by different mechanisms in order to restore AP-1 levels in the HeLa RARβ clones.

3.1.3.2 Tumor necrosis factor α treatment in HeLa and HeLa RARβ clones

As was shown in figures 22 and 23, TNFα treatment induces phosphorylation and activation of JNK. However, it was not tested whether this has an effect on c-Jun protein accumulation.

EMSA super shift for c-Jun revealed a clear increase in the AP-1 content in the cells after TNFα treatment. More than half of the AP-1 signal is shifted after the addition of the c-Jun antibodies (Figure 25 panel A).

In contrast, Western blot results of RARβ clones (panel B) did not show any increase in the content of c-Jun after TNFα treatment. Nevertheless, phosphorylated c-Jun was augmented in comparison with the control untreated cells. This rise in phospho-c-Jun can explain the increase of binding between AP-1 complex and its recognition sequence (Figure 25 panel A).

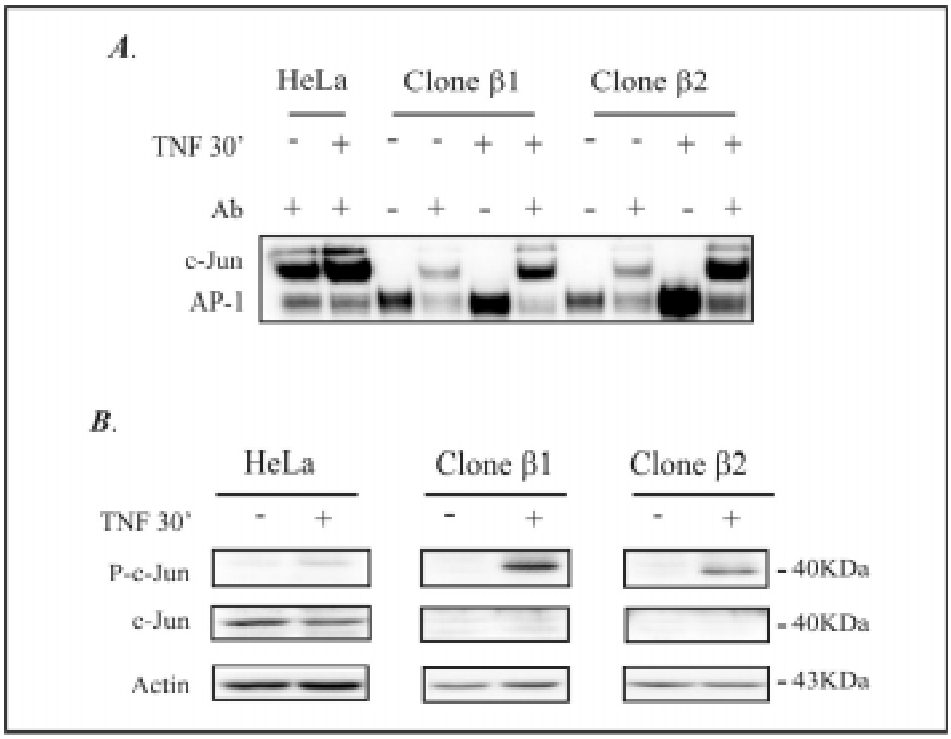


Figure 25 TNFα treatment in HeLa and HeLa RARβ clones. ***A***. AP-1 EMSA using nuclear extracts from HeLa and HeLa RARβ clones control and 30 min treated with TNFα. For super-shift 1μg of antibody anti-phospho-c-Jun was added. ***B***. Western blot for c-Jun and phospho-c-Jun was performed using the same extracts. Equal loading and transfer was monitoring incubating the filters with an anti-actin antibody. The molecular weight of the proteins are indicated.

It has been shown that treatment of the cells with *all-trans*-retinoic acid (atRA) impairs JNK activation by inhibiting its phosphorylation. Section 3.1.2.2 showed that RARβ is not inhibiting the JNK phosphorylation or activity but it was still unclear whether the exogenous addition of the RARs ligand could inhibit JNK in HeLa cervical cancer cells. To test this possibility, HeLa RARβ clones were treated for 3 days with atRA 10μM and then with TNFα (500U/ml) 30 minutes. The results of the figure 26 shows that the phosphorylated form of JNK is comparable with and without atRA treatment, indicating that retinoic acid treatment has no effect on JNK activation by TNFα in HeLa RARβ clones. This finding is in contrast to observations made in other cancer models (Lee *et al*., 1998; Moreno-Manzano *et al*., 1999; Li *et al*., 2001).

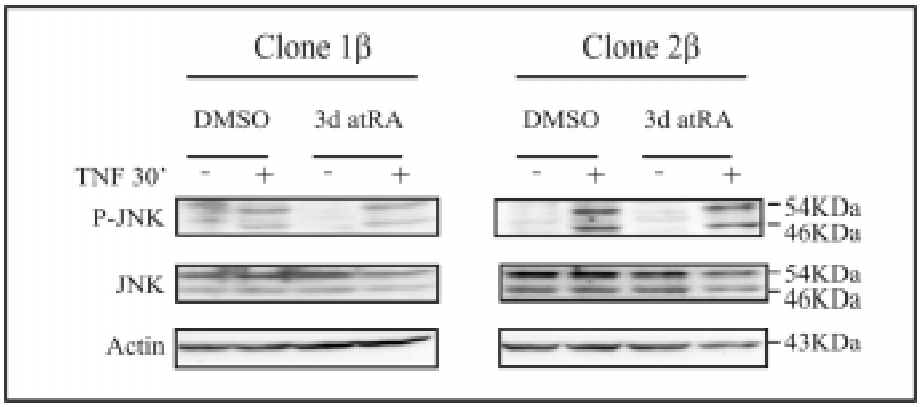


Figure 26 JNK activation in HeLa RARβ clones control and TNFα induced after 3 days treatment with retinoic acid. Total SDS extracts were prepared from cells treated with DMSO or atRA after JNK activation by TNFα. Forty micrograms of total extract per lane were separated in two identical SDS-10% polyacrilamide gels. An anti-actin antibody was used as a control for protein loading and transfer.

JNK activation by TNFα was not able to stabilize the c-Jun protein to the levels detected in HeLa parental cells, and c-Jun remained undetectable after TNFα treatment by Western blot. The increase of AP-1 can be explained by the phosphorylation of the c-Jun present in the cells (Figure 25), which lead to a higher affinity of AP-1 binding, nevertheless an undetectable increase of c-Jun protein can not be ruled out.

3.1.3.3 Proteasome inhibitor treatment of HeLa and HeLa RARβ clones activates the JNK pathway

Since proteasome inhibitors can induce the activation of the Jun-N-terminal kinase (JNK) (Meriin *et al*., 1998; Nakayama *et al*., 2001a), HeLa and HeLa RARβ clones were treated with proteasome inhibitor MG-132 for 8h. As depicted in the figure 27 panel A a high c-Jun accumulation in the RARβ clones was observed. Moreover, c-Jun and ATF-2 were phosphorylated (panel A) and as consequence of the auto regulatory loop the level of *c-jun* mRNA expression was increased (panel B). This can be considered as an indirect evidence of JNK activation by MG-132 and suggests that the increase of c-Jun was not only due to an accumulation of the protein by proteasome blocking but additionally involved *de novo* synthesis. To determine whether JNK was activated by MG-132 treatment, an activity assay was performed. The panel C shows that MG-132 treatment activates JNK and confirms that in HeLa cervical cancer cells RAR receptors do not inhibit JNK activation.

Is important to note that c-Jun induction and accumulation has no effect in the RARβ transgene expression at mRNA or protein levels (Figure 27 panels A and B).

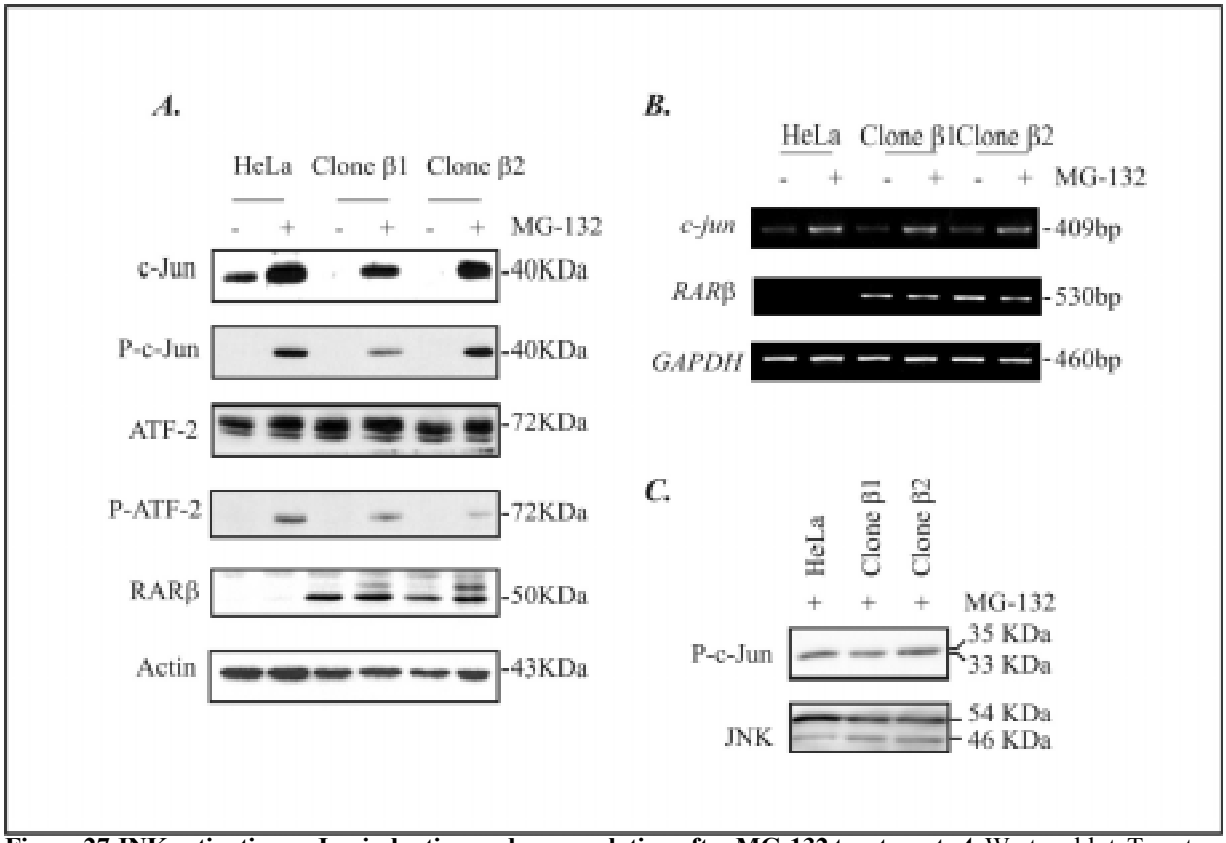


Figure 27 JNK activation, c-Jun induction and accumulation after MG-132 treatment. ***A***. Western blot: Twenty-five micrograms nuclear extracts from cells treated 8h with 20μM MG-132 (+) and untreated controls (-). After electrotransfer, the blots were incubated with the c-Jun, phospho-c-Jun, ATF-2, phospho-ATF-2 and RARβ antibodies. Equal loading was confirmed with an anti-actin specific antibody. ***B***. mRNA expression of c-Jun and RARβ genes

after 8h MG-132 treatment detected by semi-quantitative RT-PCR. The products were separated in 1% agarose gels. ***C***. JNK activity after 8h MG-132 treatment. JNK from cell extracts was pulled down using c-Jun fusion protein beads follow by the kinase reaction in the presence of the ATP. The JNK activity was determined by Western blot using a specific phospho-c-Jun antibody. The cells untreated showed not kinase activity and were unable to phosphorylate exogenous added c-Jun (see figure 23).

The effect of the c-Jun accumulation on AP-1 binding was analyzed by EMSA. As expected according to PhosphorImager analysis the AP-1 content increased in RARβ clones after MG-132 treatment to 70-150% (being HeLa parental cells 100%, figure 28 panel A). To exclude a non-specific general up regulation of other transcription factors, Oct-1 binding is shown. Addition of anti-phospho-c-Jun antibodies shifted about 50% of the AP-1 signal (Figure 28 panel B).

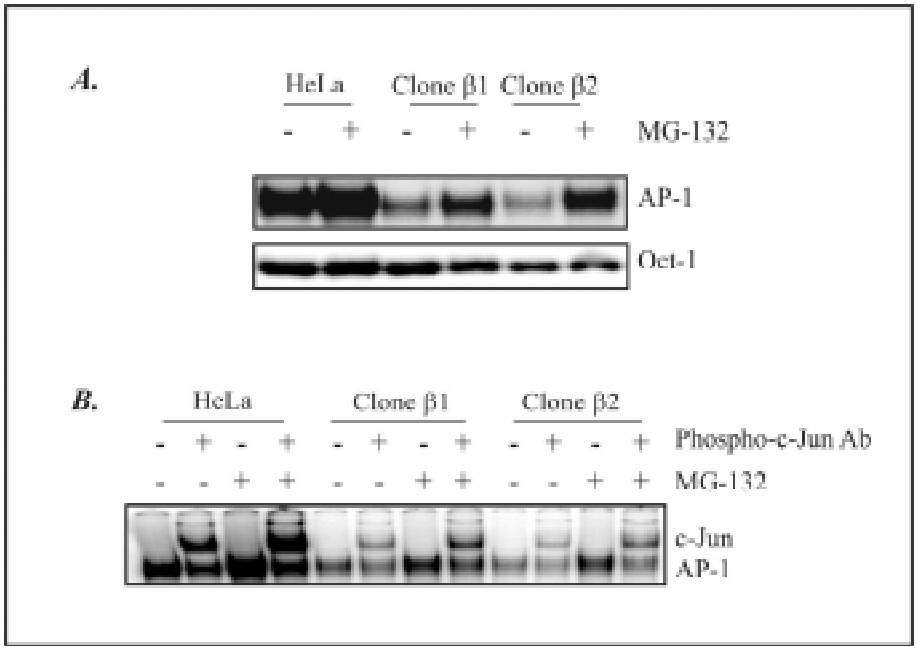


Figure 28 AP-1 reconstitution after c-Jun accumulation by MG-132 treatment. ***A***. EMSA using ^{32}P-labeled oligonucleotides specific for AP-1 or Oct-1, 2μg of nuclear extracts from cells control or treated 8h with MG-132 were used for the binding reaction. Cells treated 8h with 20mM MG-132 (+) and untreated controls (-). ***B***. Super shift analysis of the c-Jun content. After 30min incubation at room temperature of nuclear extracts with oligonucleotides specific for AP-1 1mg of c-Jun monoclonal antibody was added following by an extra incubation of 60min at 4°C.

In order to test whether the accumulated c-Jun protein was functionally reconstituted in the AP-1 complex, cells were transfected with a plasmid containing the luciferase gene under the control of TPA responsive element (TRE-Luc). The results are presented in the figure 29. There was 5-7 fold induction when stimulated cells are compared with control cells, showing that AP-1 restored after c-Jun induction and accumulation by MG-132 was functionally active.

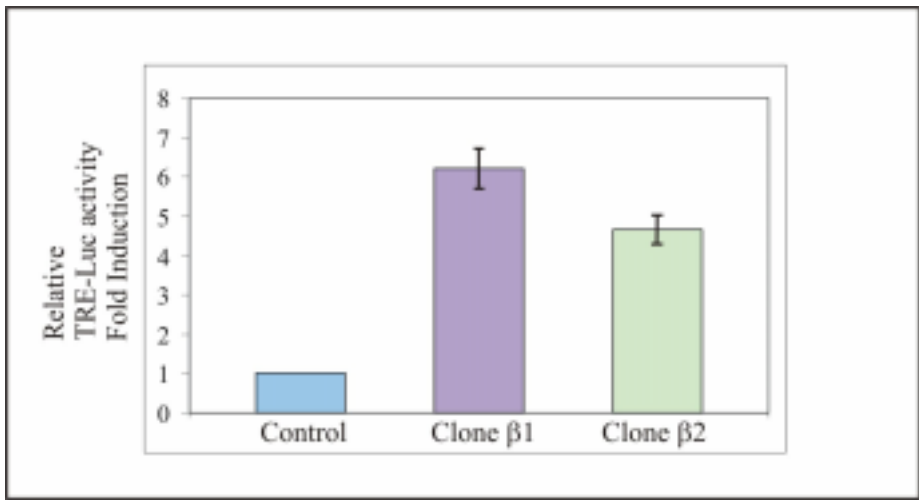


Figure 29 AP-1 activity using TRE-Luciferase reporter assay in the RARβ clones treated with MG-132. Cells were co-transfected with plasmids TRE-Luc and SV-40-β-galactosidase. One day after transfection HeLa RARβ clones were splited in two identical plates. Thirty eight hours post transfection one of the plates was treated with 20μM MG-132 for 8 h. Cell extracts were assayed for luciferase activity, each value was normalized according to the β-galactosidase activity and compared with the untreated control to define the fold induction in each case. The untreated controls were normalized and arbitrarily set as 1. Data represent the results from three independent experiments.

The MG-132 experiments showed that is possible to re-induce c-Jun protein expression in the HeLa RARβ clones, by blocking proteasome degradation, and suggested that the activation of the JNK is involved. Next it was analyzed whether a sustained induction of JNK is enough to revert the RARβ effect.

3.1.3.4 Serum starvation and stimulation results in c-Jun induction and AP-1 binding reconstitution in HeLa RARβ clones

Own results from the laboratory have shown that the TNFα stimulus is strong but transient: the duration of JNK activation is less than 60 minutes. I therefore tested a more sustained stimulus to find out if this could cause c-Jun accumulation (stabilization).

To achieve this aim the cells were starved for 24h and stimulated by 10% fetal calf serum (FCS) addition for 2h. In figure 30 panel A is an EMSA in which AP-1 binding is reconstituted after serum induction with an increment in the signal of 3-4 fold.

In order to determine if this increment in AP-1 correlates with c-Jun accumulation a Western blot for c-Jun was performed. In figure 30 panel B a clear protein accumulation can be visualized after serum induction. This correlates with an increase in the synthesis of mRNA (Figure 30 panel C).

fra-1 mRNA synthesis is transcriptionally activated by AP-1 through a consensus AP-1 site found in the first intron of the *fra-1* gene (Bergers *et al*., 1995; Griffiths *et al*., 1998). To monitor Fra-1 expression a Western blot was performed. The figure 30 panel B shows that Fra-1 protein after serum stimulation was induced, which also suggests functionality of the reconstituted AP-1 complex.

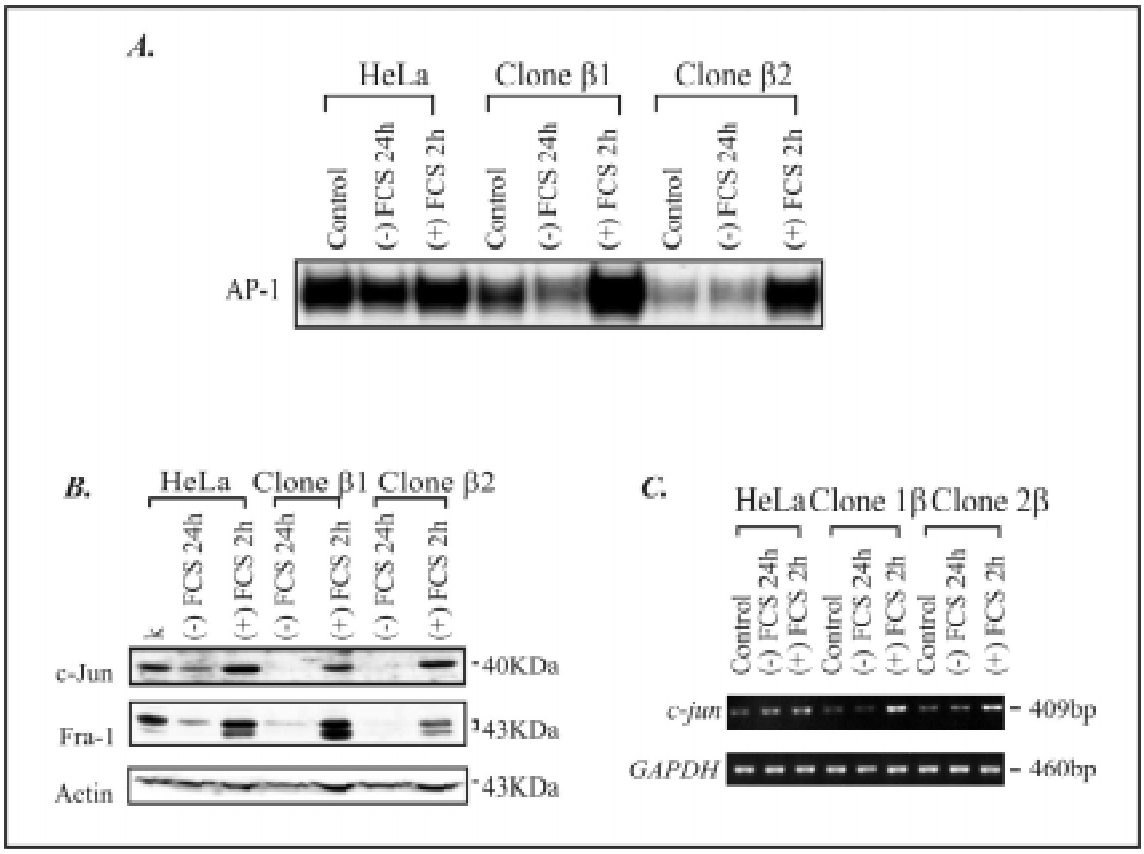


Figure 30 c-Jun induction and AP-1 reconstitution by serum stimulation. ***A***. EMSA for AP-1. Cells were depleted from serum and then stimulated for 2h with 10% FCS. Nuclear extracts were prepared and 2µg of protein was mixed with the probe. Controls are untreated cells; (-) FCS 24h, cells depleted from serum during 24 hours and (+) FCS 2h, cells depleted from serum 24h and then stimulated with 10%FCS for 2 hours. ***B***. Western blot analysis from the same extracts. Twenty-five micrograms of the nuclear protein was loaded per lane. The filters were probe with c-Jun, Fra-1 and anti-actin antibodies. ***C***. Semi-quantitative RT-PCR for *c-jun* using RNA from the same preparations. GAPDH was used as internal control.

In contrast to the transient JNK stimulation of TNFα treatment, the longer FCS stimulus sufficed to induce and accumulate c-Jun protein to detectable levels in the HeLa RARβ clones.

In order to test if this reconstitution of AP-1 can be abrogated by an activated RARβ receptor, HeLa and HeLa RARβ clones were treated 3 days with *all-trans*-retinoic acid (atRA), starved 24 hours and stimulated by serum 10% during 2 hours. The results are presented in the figure 31. The atRA treatment followed by serum induction led to AP-1 binding reconstitution but the pre-treatment with atRA diminished the effect of the serum stimulation nearly 2.5 to 3.0 times. Nevertheless, the AP-1 content after serum stimulation under atRA treatment is higher in comparison with un-stimulated cells. In consequence was observed a reconstitution of the AP-1 binding in the presence of atRA which argue against a blocking of the JNK pathway due to retinoid treatment.

However, there was a slight reduction in the c-Jun protein content after serum stimulation in atRA pre-treated cells, which could be the reason for the reduction in the AP-1 content under such conditions. It is important to remember that retinoic acid treatment stimulate all retinoic acid receptors and the reduction in the AP-1 binding reconstitution may not exclusively be due to RARβ driven mechanism.

It is surprising that although c-Jun content in the clones is slightly reduced, the phosphorylated form of c-Jun is comparable between serum stimulated cells in the presence or absence of retinoic acid treatment. Evidently Jun-N-terminal kinase (JNK) is not the target for the RARβ receptor. Additionally, is suggested that phospho-c-Jun is not degraded in the clones and this correlates with reports that JNK phosphorylation of c-Jun leads to stabilization of the protein (Musti *et al*., 1997). When the protein is not phosphorylated it becomes a substrate for proteasome degradation, in a process which is RARβ driven (Figure 19).

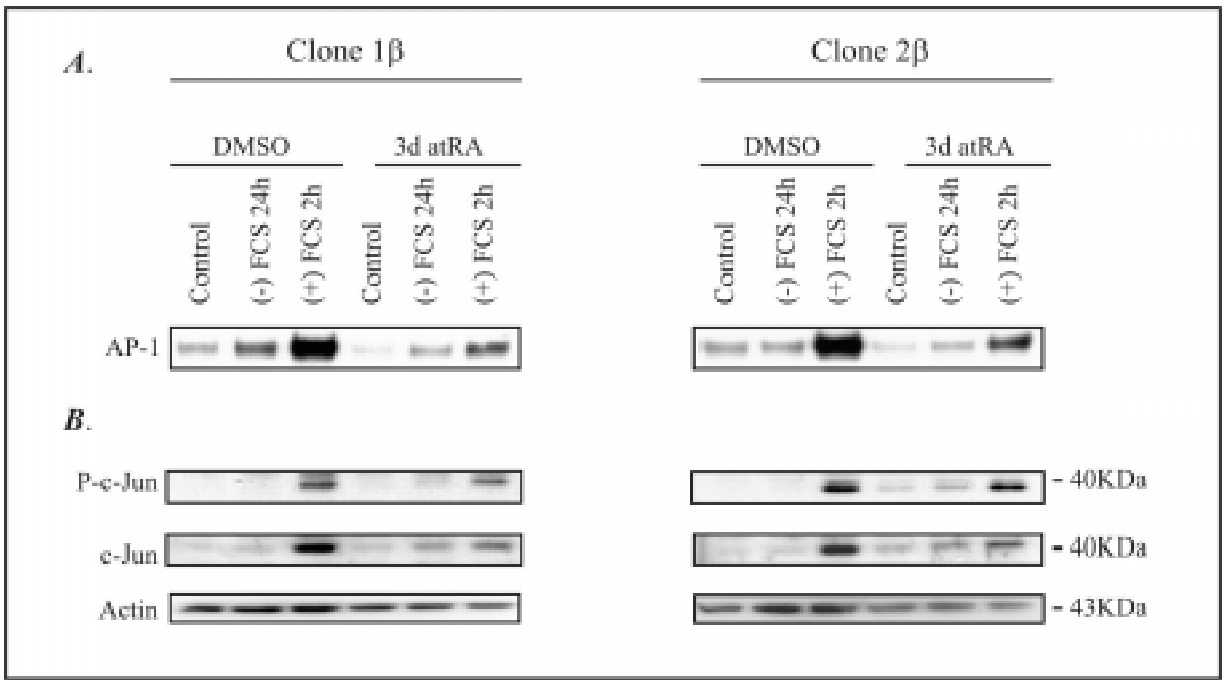


Figure 31 Serum induction after retinoic acid treatment. Cells were treated with pharmacological dose of *all-trans*-retinoic acid (10μM) during 3 days and simultaneously starved from serum 24 hours and induced by 10%FCS for 2 hours. After the treatment cells were harvested and nuclear extracts were prepared. ***A***. EMSA for AP-1 oligo was carried out with 2μg of the nuclear extracts. ***B***. Western blot for c-Jun and phospho-c-Jun was performed using the same extracts. Equal loading and transfer was monitored with an anti-actin antibody. The molecular masses are indicated.

The fetal calf serum stimulation leads to mitogen-activated protein kinase (MAPK) activation, including JNK phophorylation and activation through the protein kinase C pathway (Furuse *et al*., 1997). Based on the previous results it is possible to suggest that c-Jun accumulation depends on its stabilization, which is mainly due to c-Jun phosphorylation by JNK. The stimulus leading to JNK activation must be strong and sustained to allow the stabilization of c-Jun molecules by phosphorylation. This phosphorylated c-Jun will enhance the *c-jun* gene expression by a feedback process and will result in further c-Jun accumulation.

3.1.3.5 Constitutively active proteins that belong to the JNK pathway cause c-Jun induction and AP-1 binding reconstitution in HeLa RARβ clones

Two proteins can activate Jun-N-terminal kinase (JNK) pathway: Ha-ras^{mt} protein (12 Gly->Val) and a constitutive active mutant of the MEKK1 (mitogen-activated protein kinase kinase 1). MEKK1Δ lacks the N-terminal regulatory domain (Minden *et al*., 1994; Lin *et al*., 1995). MEKK1 is described as the most potent activator of JNK (Xia *et al*., 2000) (Figure 32).

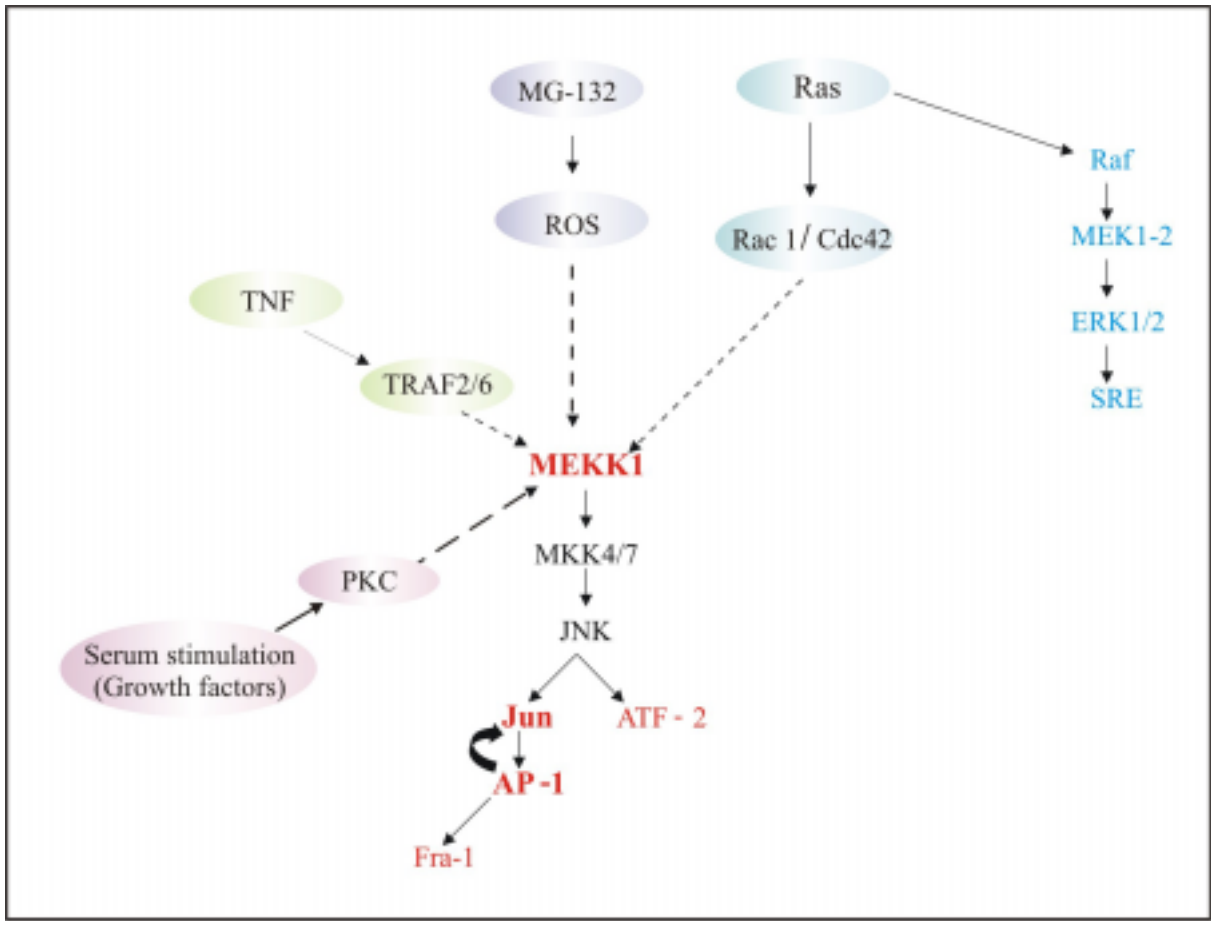


Figure 32 JNK pathway for different MEKK1 activating stimuli.

HeLa and HeLa RARβ clones were transiently transfected with Ha-ras^{mt} or MEKK1Δ expression vectors and after 48h nuclear or total extracts were prepared.
Figure 33 shows AP-1 binding reconstitution after Ha-ras^{mt} or MEKK1Δ transfection. As was anticipated, AP-1 binding was increased after transfection: Three fold for Ha-ras^{mt} protein and nearly 5 fold for MEKK1Δ. The Oct-1 binding activity, used as a specificity control, was not modulated in its binding.

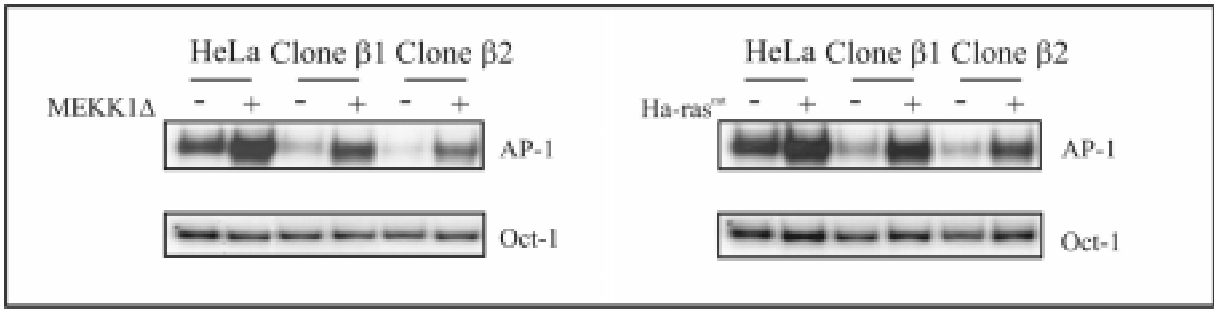


Figure 33 AP-1 EMSA from cells transient transfected with mutant Ha-ras or MEKK1Δ. Nuclear extracts were prepared 48h post-transfection. Two micrograms of the protein was incubated with the ^{32}P-labeled AP-1 or Oct-1 oligo. After 30min of room temperature incubation the 5,5% polyacrylamide gels were loaded.

To test the effect of transient transfection with Ha-ras^{mt} and MEKK1Δ on c-Jun stabilization and messenger RNA induction Western blots and semi-quantitative RT-PCRs were performed. The results showed an increase in the c-Jun at RNA level for Ha-ras^{mt} and MEKK1Δ transfected

cells. However, c-Jun phosphorylation in the RARβ clones was only detected after MEKK1Δ transfection. Additionally, Fra-1 as an endogenous reporter gene for AP-1 activity, showed induction at RNA and protein level in cells transiently transfected with both Ha-ras^{mt} and MEKK1Δ (Figure 34 panels A and B). The unchanged expression of *c-fos* in the transfected cells showed that the increase of *c-jun* and *fra-1* was specific and did not represent a general effect on all AP-1 family members (Figure 34 panel A). Note that the RARβ expression appeared to be unaffected and the AP-1 binding reconstitution and c-Jun accumulation did neither changed the RARβ RNA expression nor protein content (Figure 34 panels A and B).

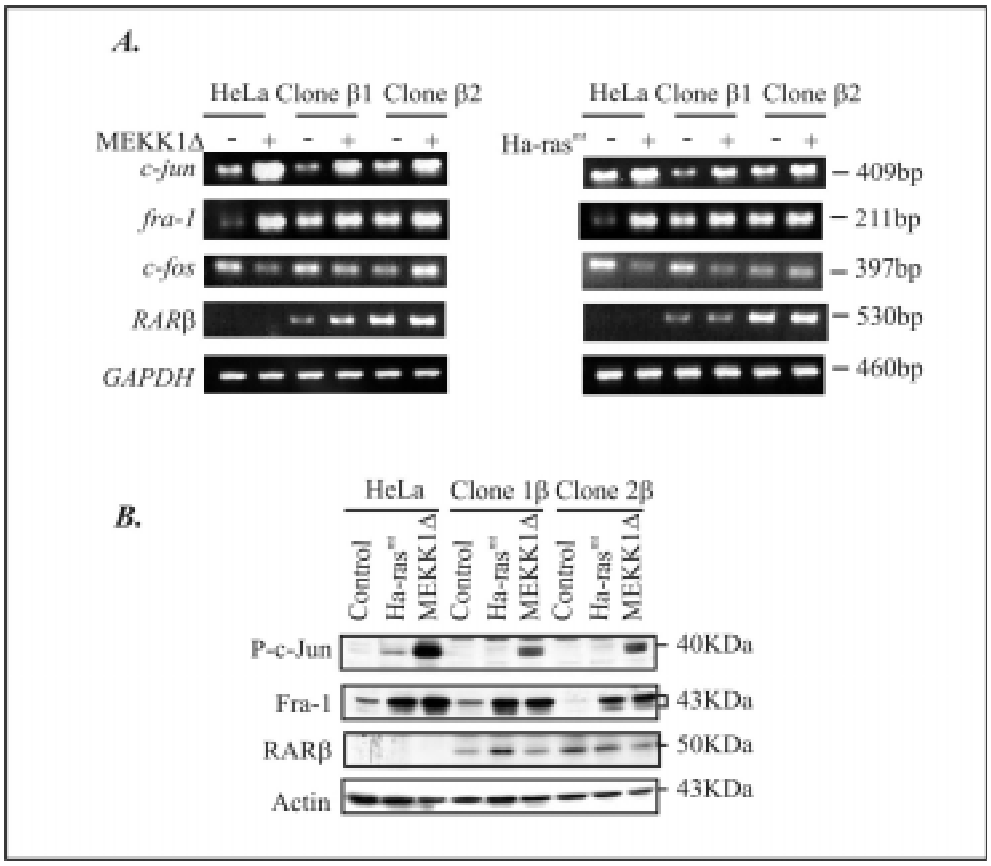


Figure 34 Expression of some AP-1 family members after Ha-ras^{mt} or MEKK1Δ transfection. ***A***. Expression of AP-1 family members and RARβ receptor. Total RNA was reverse-transcribed and PCR reaction was performed with specific primers for *c-jun*, *fra-1*, *c-fos*, *RARβ* and *GAPDH* as internal control. ***B***. Western blot analysis of 25μg of nuclear protein from cells transfected with Ha-ras^{mt} and MEKK1Δ. Forty-eight hours post-transfection nuclear extracts were prepared. After electrotransfer, the filters were incubated with the c-Jun, Fra-1 and RARβ antibodies. Anti-actin antibody shows equal loading and transfer.

Since c-Jun accumulation was undetectable in the HeLa RARβ clones after transient transfection with Ha-ras^{mt}, and because Fra-1 was increased, an EMSA supershift was performed (Figure 35). As expected c-Fos content was unchanged in the HeLa or HeLa RARβ clones under control or transient transfected conditions, instead is possible to suggest that the c-Fos content was even reduced due to the increase in the AP-1 complex after transfection. In the case of c-Jun and Fra-1 it is possible to observe a clear increase in the incorporation of these proteins in the AP-1 complex after transfection with Ha-ras^{mt} or MEKK1Δ. The PhosphorImager quantitation shows an increase of Fra-1 in the AP-1 complex of 3 times for HeLa parental cells and 1.5 times for each clone (the AP-1 complex of the clones has nearly 2 times more Fra-1 than HeLa under

control conditions see section 3.1.2.1). In the case of c-Jun the increment was nearly 3 times for Ha-ras^{mt} (however the protein was undetectable in Western blot) and 5 times for MEKK1Δ. Basically is possible to say that c-Jun/c-Jun homodimers and c-Jun/Fra-1 heterodimers formed the AP-1 complex after Ha-ras^{mt} and MEKK1Δ transfection.

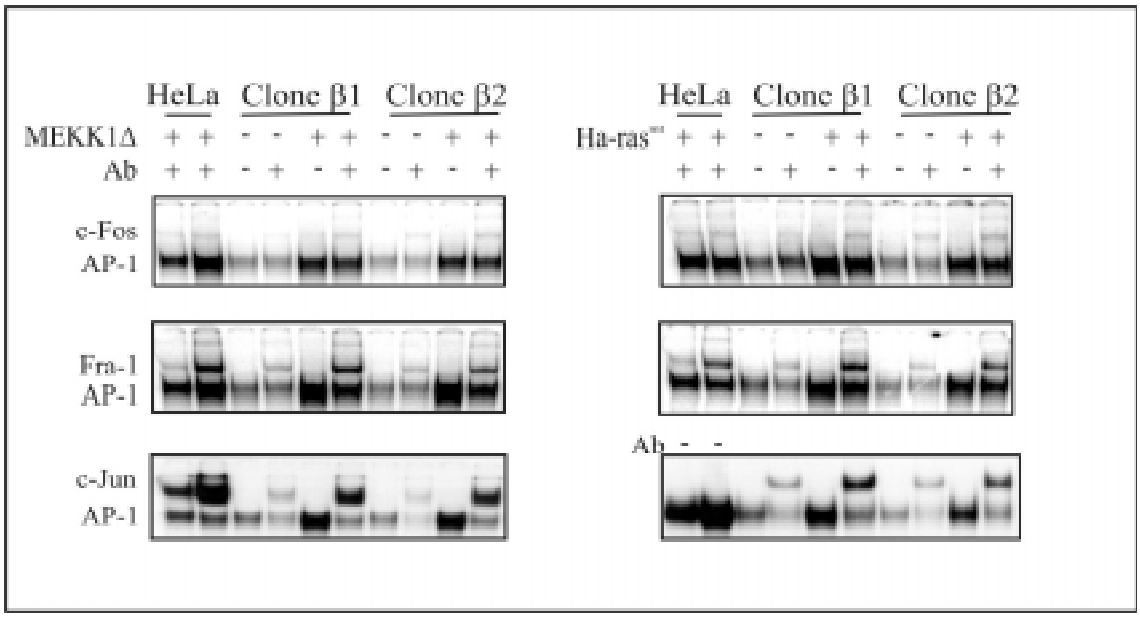


Figura 35 AP-1 incorporation of c-Fos, Fra-1 and c-Jun after transient transfection with Ha-ras^{mt} and MEKK1Δ. Two micrograms of nuclear extracts were mixed with ^{32}P-labeled AP-1 recognition sequence. After 30min of room temperature, 1μg of the corresponding antibody was added and the incubation was prolonged for 1h. The binding reaction was loaded on a 5.5% polyacrylamide native gel.

I had shown that activators of Jun-N-terminal kinase (JNK) pathway allow the reconstitution of AP-1 binding, but the c-Jun protein level depends on the strength and duration of the induction. In order to check if MEKK1Δ is a better inductor of JNK in the HeLa RARβ clones, leading to a more active AP-1 complex due to c-Jun stabilization by phosphorylation, *c-jun* gene induction and protein accumulation, a reporter gene assay was performed. Luciferase under the control of TPA response element was co-transfected with the SV-40-β-galactosidase plasmid and the Ha-ras^{mt} or MEKK1Δ vectors. Figure 36 shows a comparison between the normalized TRE-Luc fold induction in cells transfected with Ha-ras^{mt}, MEKK1Δ and control TRE-Luc activity. The results show that MEKK1Δ is a stronger activator of AP-1 activity than Ha-ras^{mt} (nearly 3 times more TRE-Luc activity for MEKK1Δ transfected cells in comparison with Ha-ras^{mt} transfected cells as is shown in the figure 36).

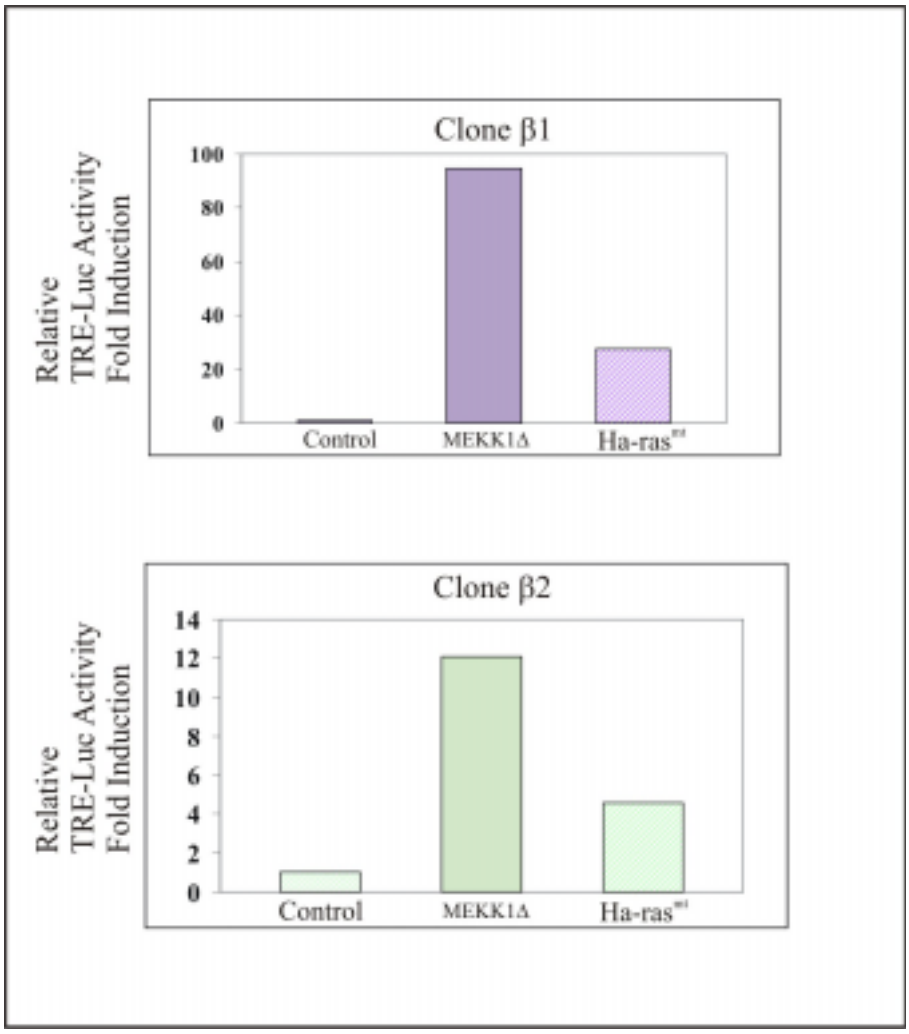


Figure 36 TRE-Luc induction in cells transfected with Ha-ras^{mt} or MEKK1Δ. Semi-confluent cells were triple transfected with TRE-Luc, SV-40-β-galactosidase and Ha-ras^{mt} or MEKK1Δ. Forty-eight hours after transfection, the cells were harvested and extracts were assay for luciferase activity. Each value was normalized according to the β-galactosidase activity and compared with the controls. The controls were normalized and arbitrarily set to 1. Experiments were performed by duplicate.

Finally, an activity test (Figure 37) showed the strongest activation of JNK after MEKK1Δ transfection in HeLa and HeLa RARβ clones, reflected in the c-Jun fusion protein phosphorylation.

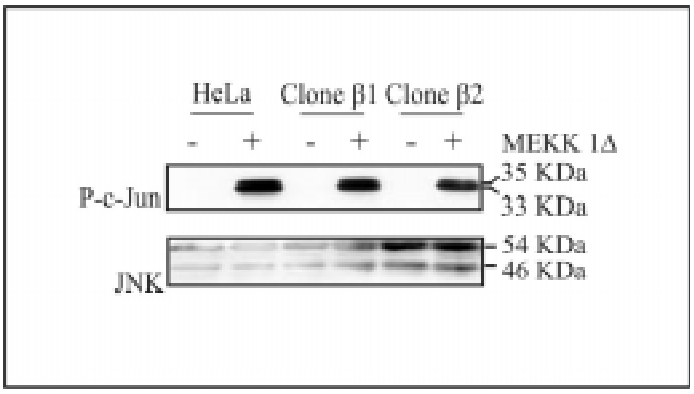


Figure 37 JNK activity 48h after transient transfection with MEKK1Δ. JNK from the cell extracts was pulled down using c-Jun fusion protein beads followed by the kinase reaction in the presence of the ATP. The JNK activity was determined by Western blot using a phospho-c-Jun antibody specific for the c-Jun fusion protein. Equivalent loading and protein transfer were confirmed by incubating the filters with an anti total JNK antibody, (-) refers to untransfected controls.

3.1.4 Role of RARβ on protein degradation

The alteration in the rate of proteasomal degradation by RARs is a mechanism described in the control of some proteins of the cell cycle. The treatment of cancer cells with *all-trans*-retinoic acid inhibits the expression and activity of cyclin D1, cyclin E, CDK2 and CDK4, increased p27 levels and shifted Rb to a hypophosphorylated state (Langenfeld *et al*., 1997, Sueoka *et al*., 1999; Dow *et al*., 2001). It has been postulated that regulation of many of these proteins is a post-translational mechanism that involves the ubiquitin-proteasome pathway . In order to check if the down regulation of c-Jun could be coupled to the post-translational regulation of some of these cell cycle proteins in the HeLa RARβ clones, Western blots were performed. The results shown in figure 38 show a slightly lower cyclin D1, when HeLa and the RARβ clones were compared.

All the other proteins analyzed remain at comparable levels, except p53 levels that showed a slight up regulation and the cyclin dependent kinase (CDK) inhibitors p21 and p27, which were strongly up regulated in the RARβ clones when compared to the HeLa parental cells.

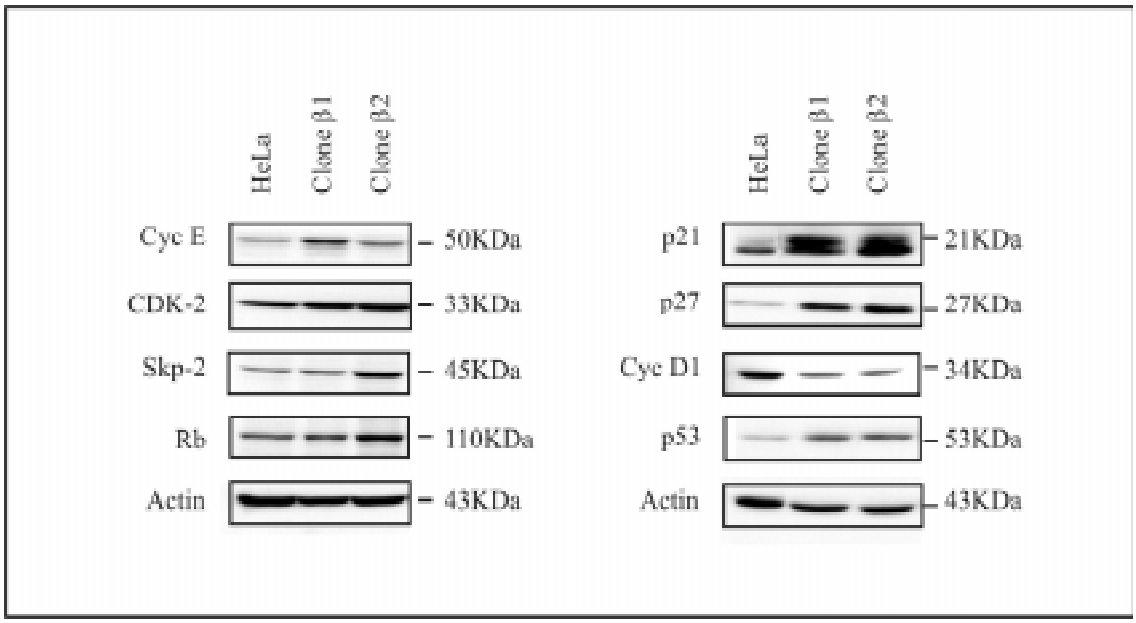


Figure 38 Effect of the constitutive expression of retinoic acid receptor β on cell cycle regulatory proteins. Protein extracts were prepared from HeLa and HeLa RARβ clones under control culture conditions. Twenty-five micrograms of protein were loaded per lane in a 10% SDS-polyacrylamide gel. After electrotransfer the filters were incubated with specific antibodies against cyclin E (CycE), cyclin dependent kinase 2 (CDK-2), S-phase kinase associated protein 2 (Skp-2), Retinoblastoma (Rb), p21, p27, Cyclin D1 (CycD1) and p53. Equal loading and transfer was monitored by incubation of the membranes with an anti-actin antibody. The molecular weight of the proteins is indicated. Total extracts CycD1, CDK-2, Skp-2, Rb and p27. Nuclear extracts CycE, p21 and p53.

The induction of the cyclin-CDK inhibitors as well as the reduction in the cyclin D1 is probably responsible for the slower growth rate of the RARβ clones in comparison with the HeLa parental cells (Figure 39). As expected, the clones have a slower growth than the parental cells without evidence of cell death.

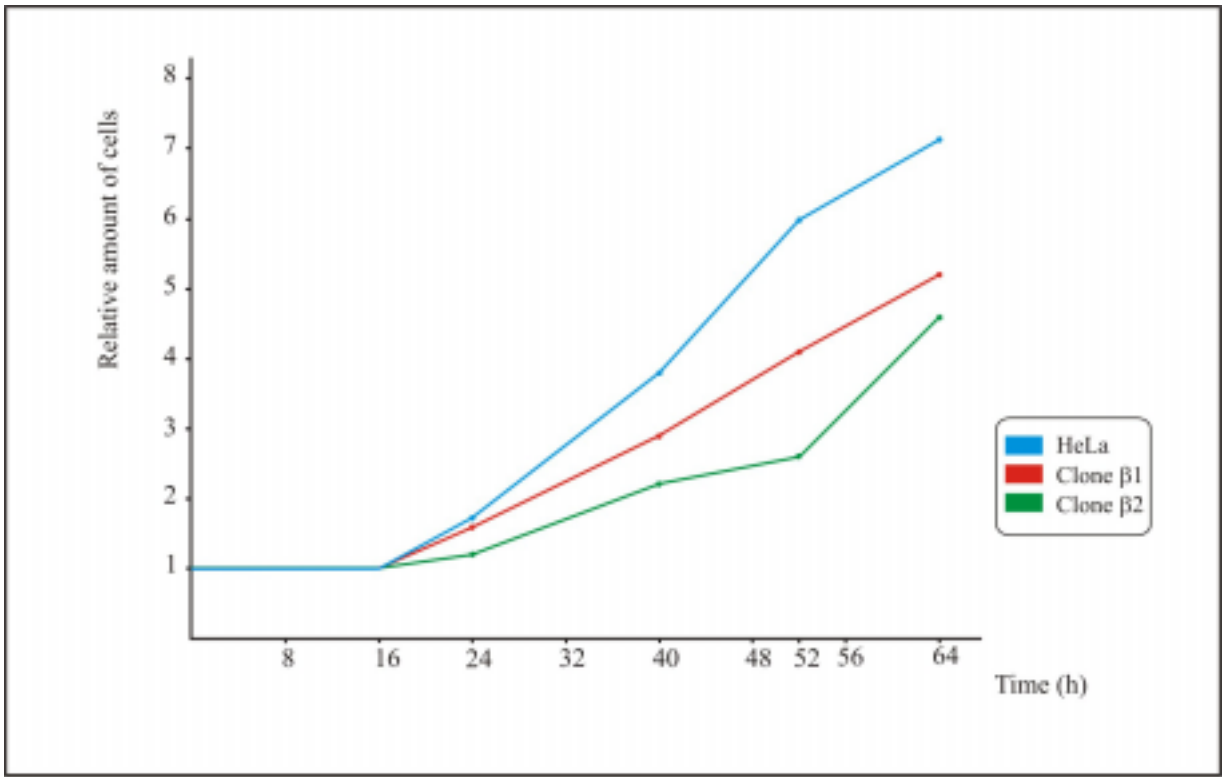


Figure 39 Cell growth curve for HeLa and HeLa RARβ clones. Cells were plated at 1,5x10^6 in 60cm^2 dishes. Cells were trypsinized and counted. HeLa cells that growth much more faster where counted by duplicate and the average was calculated.

These results are in favor of a degradation pathway specifically directed against c-Jun, because the post-translational down regulation of cell cycle regulators exhibits different tendencies in comparison with c-Jun in the RARβ clones. However it was shown that retinoic acid receptors can modulate the proteasome machinery leading to the stabilization of some proteins and the degradation of the others. The results presented in the section 3.1.3 strong suggest that proteasomal targeting plays a role in the c-Jun degradation and that degradation is stimulated by RARβ.

3.1.4.1 Role of POH1 in c-Jun stabilization in HeLa RARβ clones

Recently, has been reported that a regulatory subunit of the proteasome, POH1 (Human Pad1 homolog), can stabilize c-Jun and is important for AP-1 activation (Spataro *et al*., 1997; Nabhan *et al*., 2001). A link between RARs signalization and POH1 activity has not been described, but inhibition of this kind of proteasomal subunits can lead to increasing rate of c-Jun protein degradation in HeLa RARβ clones.

Human Pad1 is an enzyme found in the lid of the regulatory part of the proteasome complex. It is a de-ubiquitinating enzyme that efficiently removes the ubiquitin. The proteins to be degraded must enter the proteasome's cylinder through a narrow opening end. It is likely that the poly-ubiquitin chain must be removed to allow the whole protein to be fed through the opening and hydrolysed (Wilkinson, 2002).

How this protein contributes to c-Jun stabilization is presently not understood. But it seems likely that the stabilization of c-Jun is caused by an intrinsic activity of the POH1, which does not require integration of the protein into the proteasome lid. Under POH1 overexpressing conditions the half life of the c-Jun protein has been shown to increase by more than 1 hour. It is

possible that POH1 functions as a c-Jun de-ubiquitinase but is not clear how this activity is selective towards c-Jun. By targeting c-Jun and modulating its stability, POH1 represents a major mechanism for regulation the transcriptional activity of AP-1 (Spataro *et al*., 1997; Nabhan *et al*., 2001).

In order to test if POH1 has some relevance in the degradation phenomenon that leads to c-Jun down regulation in HeLa RARβ clones, the steady-state content of *poh1* was analyzed at the RNA level under control and MEKK1Δ or Ha-ras^{mt} transient transfection conditions.

As depicted in figure 40 panel A no differences are observed in the *poh1* mRNA expression between HeLa and HeLa RARβ clones under control or stimulated conditions. Figure 40 panel B shows a Western blot for the POH1 protein. The results show that the protein is in the cytoplasmic fraction where 70% of the proteasome machinery is located. Additionally it is clear that the proteasome inhibitor MG-132 did not increase the protein level in the cells.

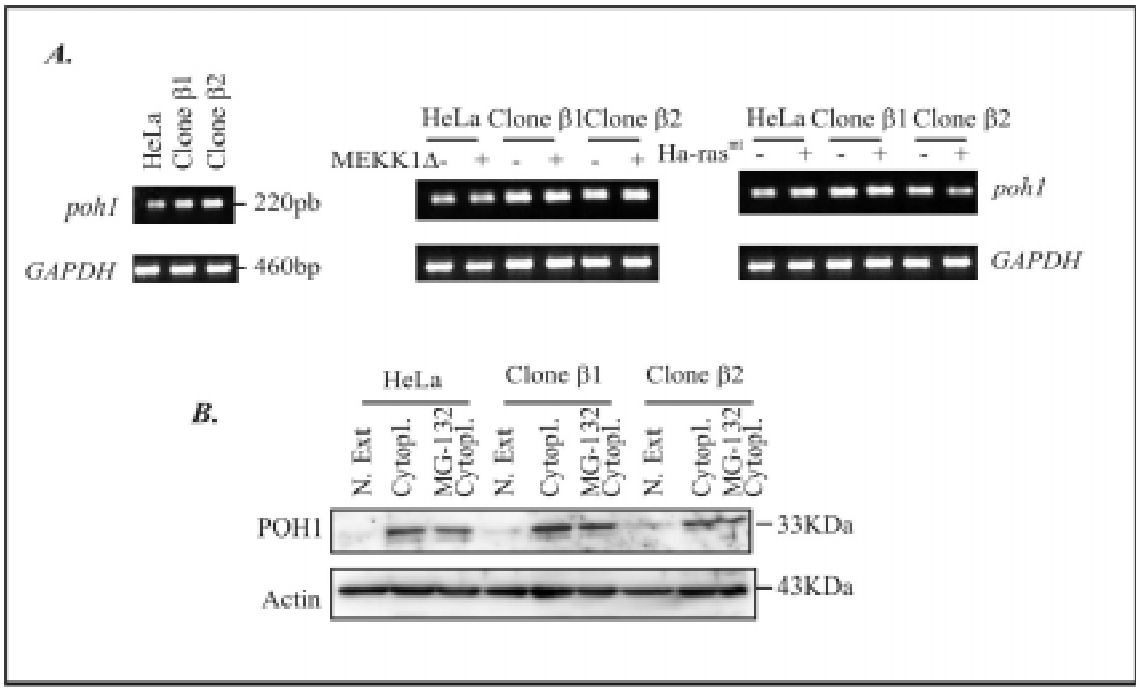


Figure 40 POH1 expression at RNA and protein level in HeLa and HeLa RARβ clones. ***A***. Semiquantitative RT-PCR for *poh1* mRNA expression. One microgram of RNA was used in the RT reaction. For PCR, 1μl of the RT reaction was amplified with the specific pair of primers. GAPDH was used as internal control. (+) refers to Ha-ras^{mt} or MEKK1Δ transfected cells. ***B***. Western blot to detect POH1 protein expression. POH1 polyclonal antibody was kindly provided by Dr. Norbury, Oxford University. Anti-actin antibody confirm equal loading and transfer.

The cytoplasmic content of the POH1 protein is comparable between HeLa and HeLa RARβ clones, which argue against quantitative differences of POH1 to explain the presence or absence of c-Jun. However, it is possible that RARβ or a gene target interact with the POH1 to inhibit its function. To evaluate this possibility, transient transfection experiments with the POH1 expression vector were performed. The results in figure 41 panel A show an increase of c-Jun in the AP-1 complex in the clones and in the parental cells as was determined by EMSA super shift assay. By PhosphorImager quantitation was detected a 1.3-1.6 fold increase of c-Jun in the AP-1 complex. This effect was specific for AP-1 transcription factor because no changes were observed in Oct-1 binding. However, the c-Jun content in Western

blot was barely detected even after POH1 transient transfection (Figure 41 panel B). To check if the c-Jun incorporated in the AP-1 complex leads to more active AP-1 transcription factor, Fra-1 expression was determined by Western blot. The induction of Fra-1 protein was discrete but reflex a differential AP-1 activity, suggesting that the increment of AP-1 by POH1 transient transfection results in a slight more active transcription factor.

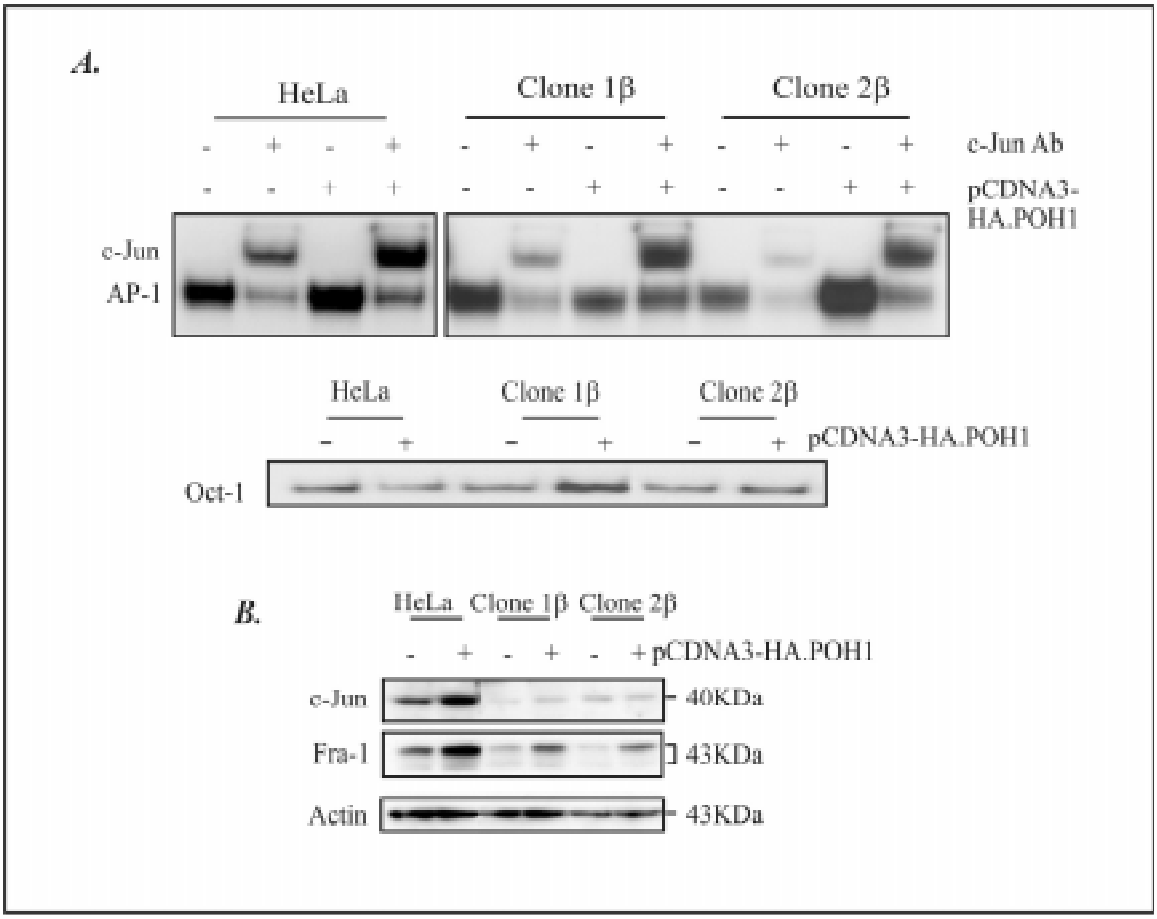


Figure 41 POH1 transient transfection in HeLa and HeLa RARβ clones. ***A***. EMSA super shift analysis of cells transfected with 2μg of pCDNA3-HA.POH1 expression vector. Two micrograms of nuclear extract were incubated with the radioactive label AP-1 or Oct-1 recognition sequence. After 30 minutes at room temperature, 1μg of anti phospho-c-Jun antibody was added to the AP-1 samples and the incubation was prolonged for an additional hour. Note that larger exposure time was used for HeLa RARβ clones that is the reason for the lack of differences in the AP-1 content between HeLa and the clones. ***B***. Twenty-five micrograms from the same nuclear extracts were used in the Western blot analysis of c-Jun and Fra-1 protein expression.

3.2 Effect of RARβ expression on HPV-18 oncogenes E6/E7

3.2.1 HPV-18 oncogenes E6/E7 expression in HeLa RARβ clones

The treatment of HPV-18 positive cells with retinoic acid down regulates oncogene expression (Bartsch *et al.*, 1992) and induces growth arrest (Si *et al*., 1996), but the mechanism by which this reduction is achieved has not been analyzed so far. It is as well established that the constitutive expression of RARβ receptor in HeLa cells leads to retardation of the tumor growth in nude mice (Geisen *et al*., 2000 and lab. results). As was showed in the previous section HeLa RARβ clones grow slowly in culture and cointain higher levels of p53 protein in comparison with HeLa parental cells (Figures 38 and 39). To test whether this observations are correlated with the E6/E7 oncogene expression, the RNA and protein content of the oncogenes was determined in the HeLa RARβ clones.

The results in the figure 42 show a reduction of the oncogene expression on both levels in the RARβ clones compared to HeLa parental cells. The reduction was detected in the absence of exogenously added ligand, suggesting that this is basically due to constitutive RARβ expression in the clones. As control HeLa cells treated with pharmacological doses of *all-trans*-retinoic acid (atRA) also exhibit a down regulation of oncogene expression at mRNA and protein levels. Nevertheless, in this case the reduction can not be assign only to RARβ because the atRA treatment activated all retinoic acid receptors.

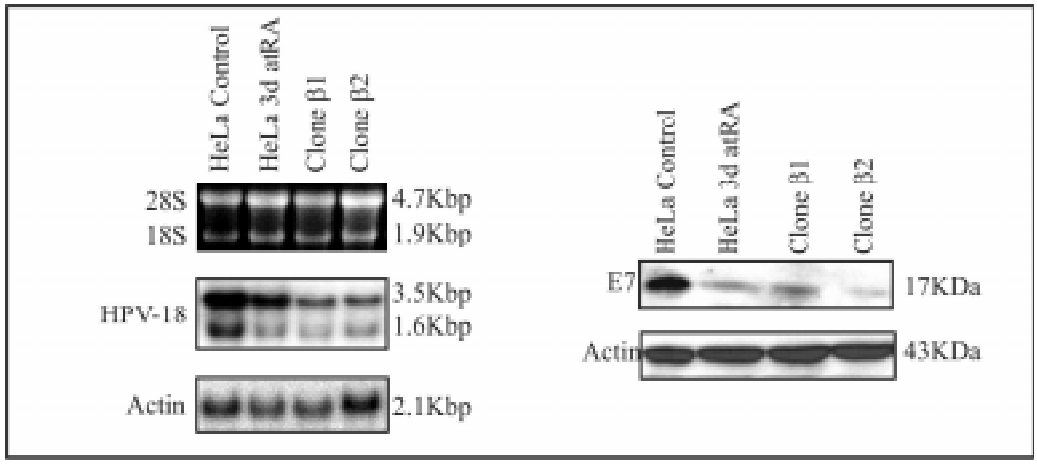


Figure 42 HPV-18 oncogene expression in HeLa and HeLa RARβ clones. ***A***. Detection of mRNA levels of HPV-18 E6/E7 by Northern blot. RNA quality is shown by RNA gel and equal loading (5μg) is shown by actin hybridization. ***B***. Western blot for E7 protein. Thirty micrograms of cytoplasmic protein was electrophoresed on a 12%-SDS polyacrylamide gel. Filter was incubated with an anti-E7 antibody, and subsequently incubated with an anti-actin antibody used to confirm equal protein loading.

In order to test whether HeLa RARβ clones treated with pharmacological doses of atRA show further reduction in the oncogene expression, they were cultured 3 days in the presence of 10μM retinoic acid. Figure 43 shows that retinoic acid leads to further reduction of the HPV-18 expression in the clone RARβ2, but the oncogene expression is unchanged for the clone RARβ1.

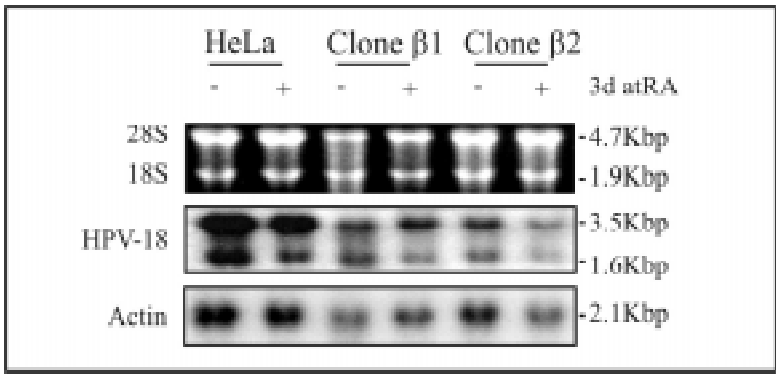


Figure 43 E6/E7 oncogene mRNA expression in HeLa RARβ clones treated with *all-trans*-retinoic acid. After treatment of the cells for 3 days with atRA, RNA was extracted and electrophoresed. After transfer, blots were incubated with HPV-18 probe. RNA quality is shown in the RNA gel and equal loading was monitored by actin hybridization.

Down regulation in the HPV-18 oncogenes may contribute to the slow proliferation rate of the RARβ clones.

The transcription factor AP-1, plays an important role in determining the efficiency of HPV expression (Offord *et al*., 1990; Butz and Hoppe-Seyler, 1993). Constitutive expression of RARβ receptor in HeLa cells diminishes the AP-1 content on these cells (Figure 18). It was therefore important to analyze if the AP-1 binding to the upstream regulatory region of HPV-18 was also reduced in comparison with other cellular proteins controlling HPV transcription.

3.2.2 Analysis of the upstream regulatory region of HPV-18: Protein-DNA interaction at the enhancer and promoter elements in the HeLa RARβ clones

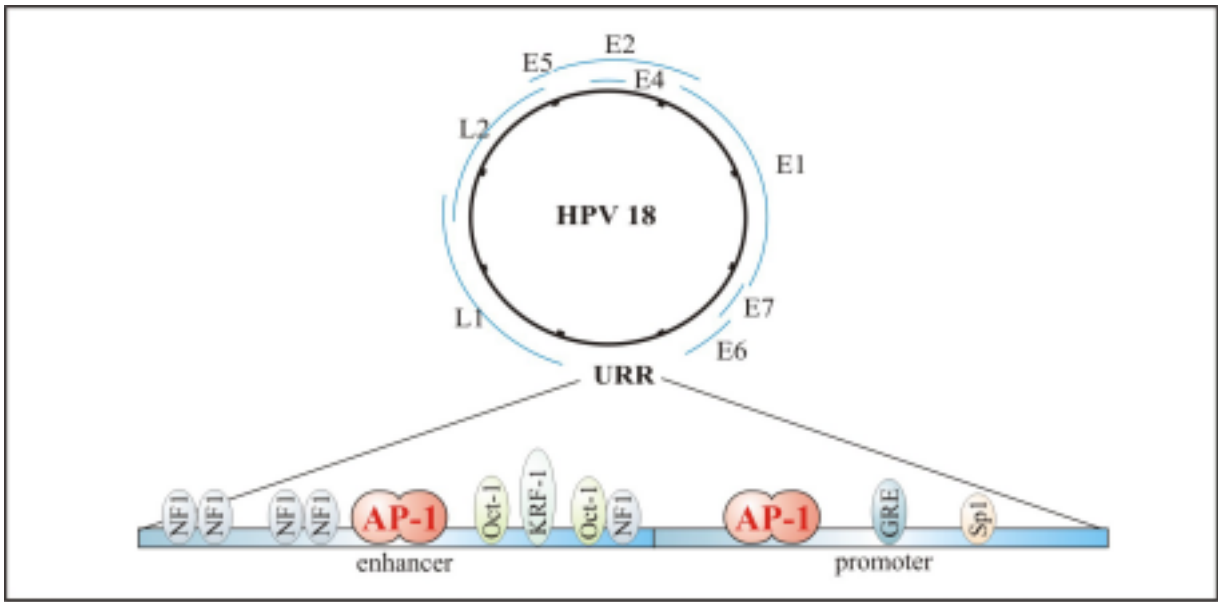


Figure 44 Schematic overview of HPV-18 Upstream regulatory region. NF-1, Nuclear factor 1; AP-1, Activator protein 1; Oct-1, Octamer binding transcription factor 1; KRF-1, Epithelial cell-specific transcription factor 1; GRE, Glucocorticoid response element; Sp-1, SV40 promoter binding protein.

Oligonucleotides carrying the binding sequence for the transcription factors presented in the figure 44 were synthesized and electrophoretic mobility shift assay were performed. Figure 45 panel A shows

a comparison between the binding of AP-1 to the TRE sequences of collagenase 1 consensus, HPV-18 enhancer and HPV-18 promoter. The reduction in the AP-1 binding is clear in all three sequences. PhosphoImager analysis showed that the binding to the enhancer and promoter sequences is reduced around 70-75% in comparison with HeLa parental cells. The panels B and C of the figure 45 show that binding of other transcription factors was not reduced, indicating that RARβ overexpression selectively reduced AP-1.

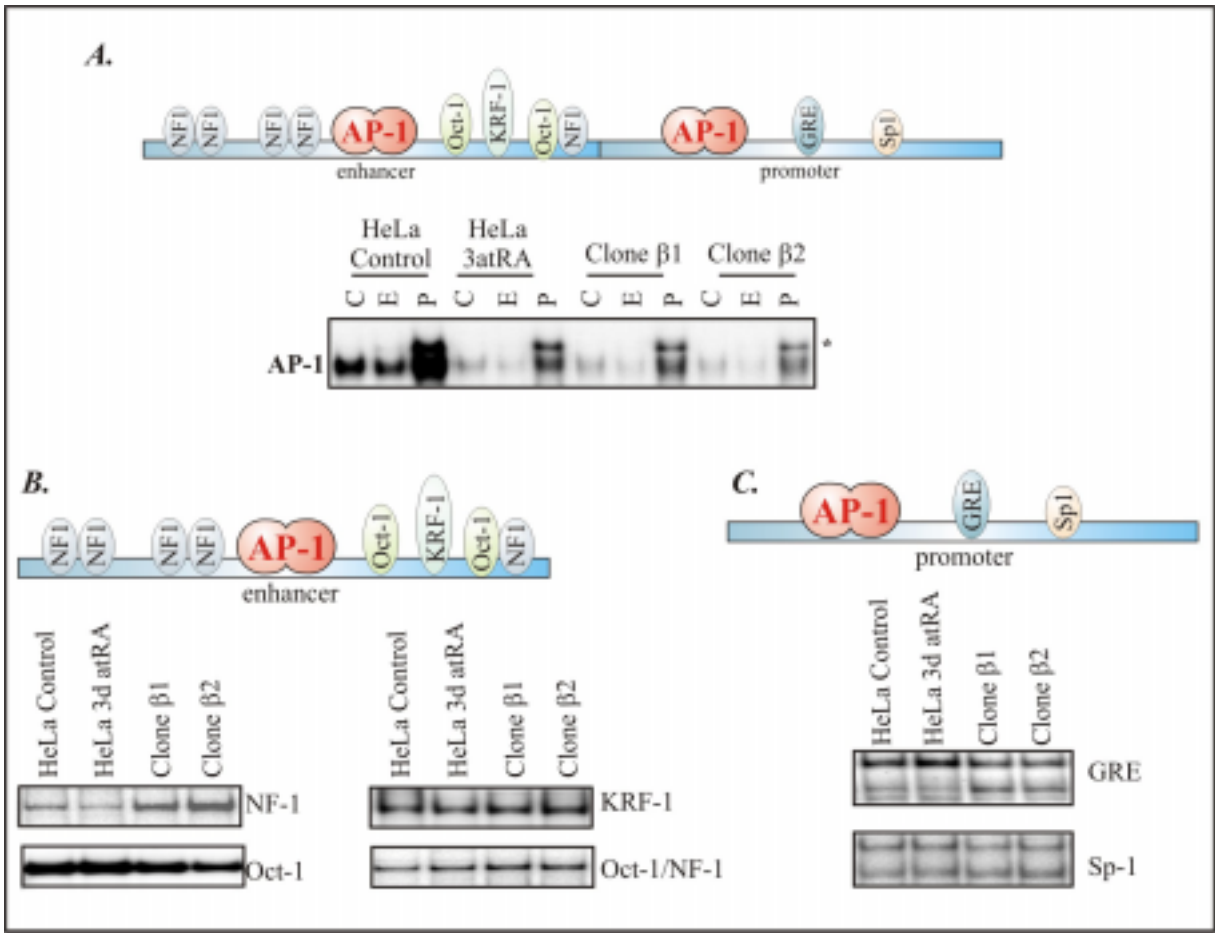


Figure 45 Protein-DNA interaction for the different regulatory elements of the HPV-18 URR. EMSA using ^{32}P-labeled oligonucleotides. Nuclear extracts from HeLa untreated cells, HeLa treated 3 days with atRA or HeLa RARβ clones were analyzed. ***A***. Comparison of AP-1 binding to the TRE sequences of collagenase 1 (Consensus=C), HPV-18 enhancer (E) and HPV-18 promoter (P). ***B***. Analysis of the transcription factors, which bind to the enhancer of the HPV-18 URR. ***C***. Analysis of the transcription factors, which bind to the promoter of the HPV-18 URR.

AP-1 binding reduction correlated with the down regulation of the oncogene expression in the HeLa RARβ clones. These data are additionally supporting by the reduction of the AP-1 binding in HeLa cells treated with retinoic acid that as well exhibited less HPV-18 E6/E7 expression.

3.2.3 AP-1 binding reconstitution and HPV-18 expression

Results described above showed that different treatments could abrogate the RARβ effect and stabilize/accumulate c-Jun leading to AP-1 binding reconstitution. Additionally, it was demonstrated that AP-1 increment is functional by a transient reporter TRE-Luciferase assay or testing the increment of the endogenous marker gene Fra-1.

Postulating that HPV-18 E6/E7 oncogene expression depends on AP-1 and observing a reduction in HPV transcription in the clones which contain less AP-1, I tested whether AP-1 binding reconstitution would lead to HPV transcriptional up regulation of the E6/E7 oncogenes.

RNA samples from every treatment which increased AP-1 were analyzed by Northern blot to check the HPV-18 E6/E7 expression. The different strategies used were exogenous c-Jun over-expression, tumor necrosis factor α treatment, MG-132 proteasome inhibitor treatment, serum starvation and stimulation, MEKK1Δ over-expression, Ha-ras^{mt} over-expression and c-Jun stabilization by transient transfection with POH1.

The RNA samples belong to the same experiments in which an AP-1 binding reconstitution was observed (Figures 24, 25, 28, 30, 33, 41). The results are shown in the figure 46.

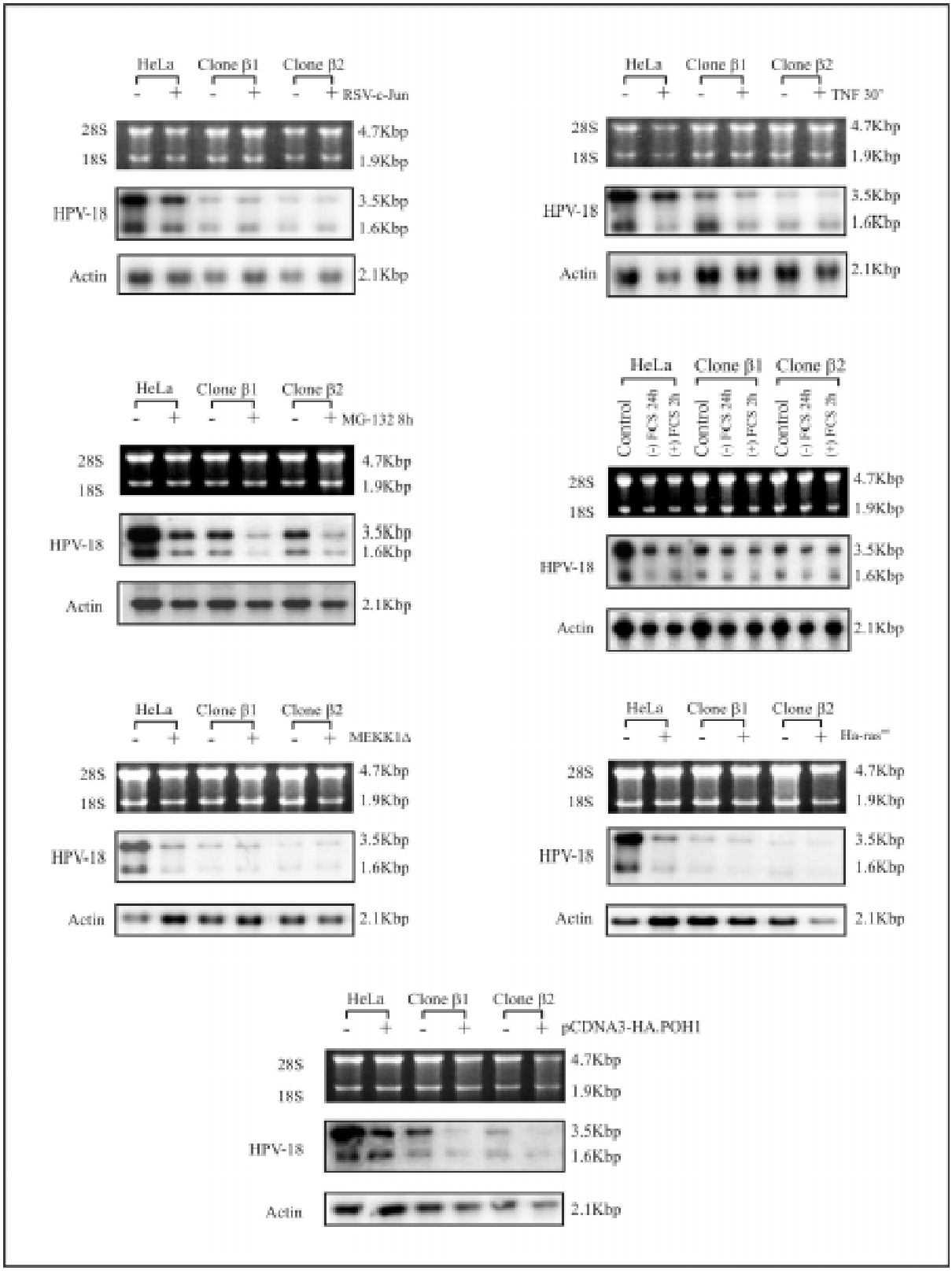


Figure 46 Northern blot analyses of HPV-18 expression after different treatments for AP-1 reconstitution. Cells were harvested 48h after transient transfection. The blots were hybridized sequentially with an HPV-18 probe and actin probe to monitored equal RNA loading and transfer.

Surprisingly HPV-18 oncogene mRNA expression was not induced and in contrast some of the treatments even reduced mRNA levels in HeLa parental cells.
To investigate the reason why HPV expression was not increased, I tested whether AP-1 binding to the specific AP-1 sequences of the HPV-18 URR was reconstituted. The results depicted in figure 47 show that after MEKK1Δ or Ha-ras^{mt} transient transfection AP-1 binding at the enhancer and the promoter sequences was restored. Therefore the unresponsiveness of HPV-18 URR can not be due to an inability of AP-1 to bind to the specific URR sequences after its induction. Oct-1 binding activity shows the selective effect over AP-1.

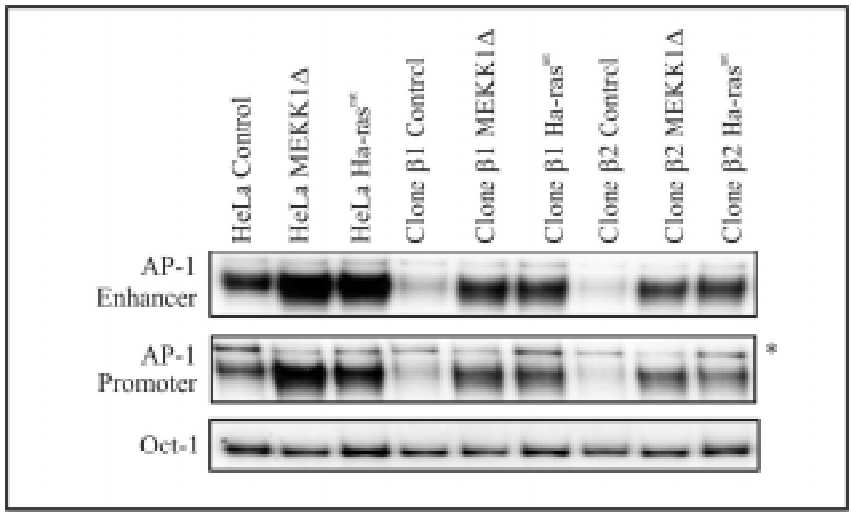


Figure 47 DNA binding activity of the AP-1 reconstituted at the enhancer and promoter of the URR HPV-18. Electrophoresis mobility shift assay. Two micrograms of nuclear protein from MEKK1Δ or Ha-ras^{mt} transient transfected cells were mixed with labeled oligonucleotide.

In order to check if unresponsiveness could be also demonstrated for other promoters that contain AP-1 binding sites, classical AP-1 regulated genes were analyzed at mRNA level.
The regulation of the metalloproteinase (MMPs) genes has been described to be strongly dependent on the AP-1 binding sites present in their promoters (Figure 48 panel A) (Benbow and Brinckerhoff, 1997; Westermarck and Kähäri, 1999; Angel *et al.*, 2001). To analyze whether the expression of three different metalloproteinases were induced after transient transfection with MEKK1Δ or Ha-ras^{mt}, RT-PCR was performed. The results presented in the figure 48 panel B show that the collagenase 1 (MMP-1) and stromyelin (MMP-3) were not expressed under control conditions in HeLa or HeLa RARβ clones but were induced after transient transfection with MEKK1Δ or Ha-ras^{mt}. However in the case of the clone RARβ2 the induction after Ha-ras^{mt} was very weak. In contrast the collagenase 3 (MMP-13) gene was constitutively expressed and after MEKK1Δ or Ha-ras^{mt} transfection no changes in its expression were observed.

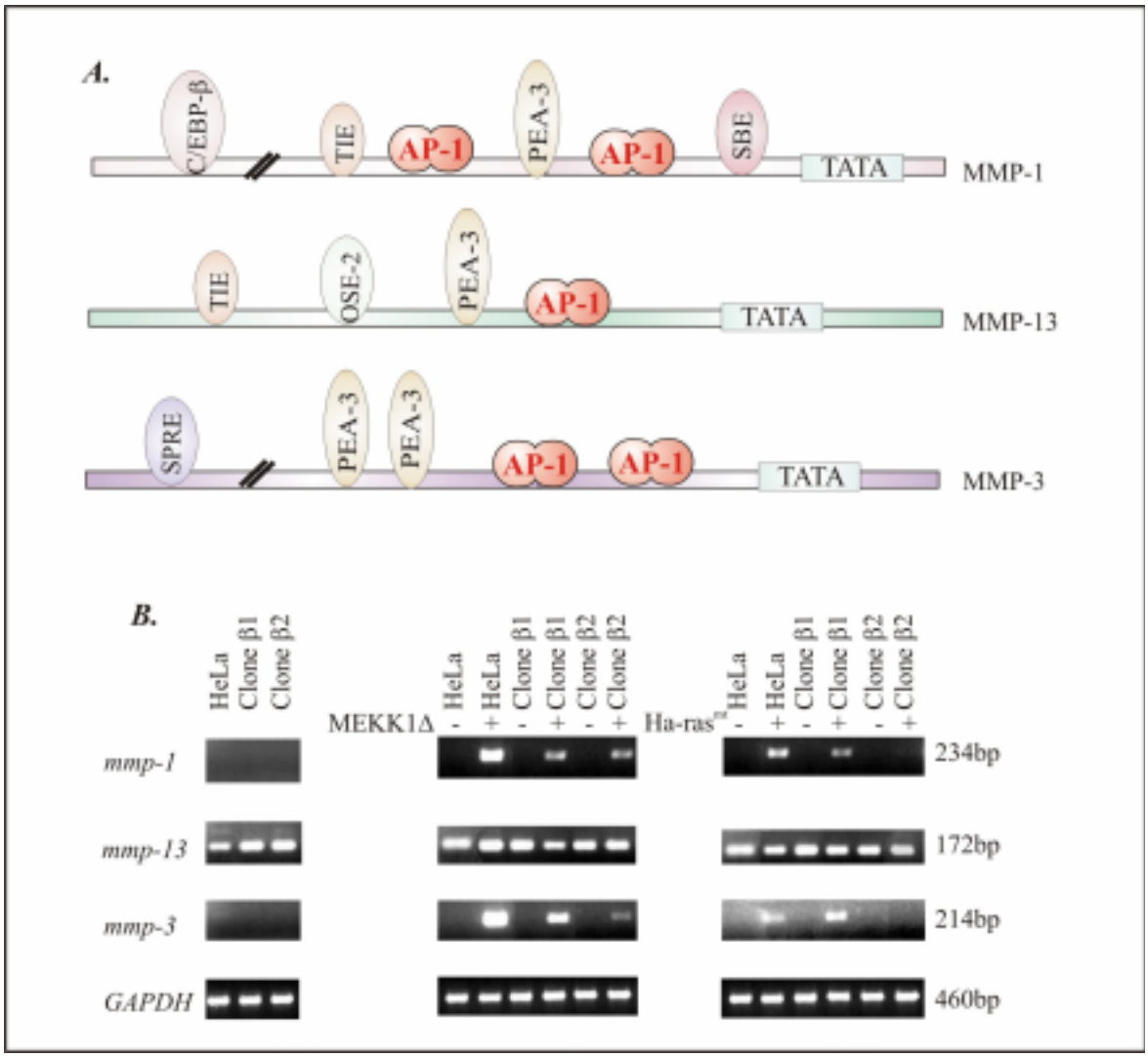


Figure 48 Metalloproteinases induction after AP-1 reconstitution. ***A***. Schematic overview of the metalloproteinases promoter region. C/EBP-β, CCAAT/enhancer binding protein β; TIE, TGF-β inhibitory element; PEA-3, Polyomavirus enhancer A-binding protein 3; SBE, STAT binding element; OSE-2, Osteoblast-specific element 2; SPRE, Stromelysin-1 PDGF-responsive element. ***B***. mRNA expression of metalloproteinases *mmp-1*, *mmp-13* and *mmp-3* under control conditions and after transient transfection with MEKK1Δ or Ha-ras^{mt} was detected by semi-quantitative RT-PCR. The products were separated on 2% agarose gels.

Next I tested the possibility that HPV-18 unresponsiveness could be due to a different composition of the AP-1 after c-Jun induction and AP-1 binding reconstitution. This new composition can alter the AP-1 function over the HPV regulatory region. Additionally the results presented in the previous chapter indicated that in all cases were AP-1 binding was restored, Fra-1 expression was also increased at mRNA and protein levels (Figures 30 panel B, 34, 35 and 41 panel B).
The increase of Fra-1 in the AP-1 complexes can lead to less *trans*-activator capability of AP-1 (Suzuki *et al*., 1991; Yoshioka *et al*., 1995) or can recruit a repressor that diminishes HPV-18 expression. However, this is not a general effect because other AP-1 driven promoters can be induced (e.g. the metalloproteinases MMP-1 and MMP-3).
All this evidence prompted us to verify the negative role of Fra-1 in HPV-18 E6/E7 expression in HeLa and HeLa RARβ clones.

3.2.4 Role of Fra-1 as negative regulator of the HPV-18 expression

3.2.4.1 Antisense silencing of Fra-1 during AP-1 binding reconstitution by MEKK1Δ in HeLa RARβ clones

In the first approach to confirm the Fra-1 inhibitory effect over the HPV-18 promoter after the AP-1 binding reconstitution, I used an antisense construct to try to silence Fra-1 expression after the transient transfection with MEKK1Δ. The aim was to increase the AP-1 content, inducing c-Jun, without concomitant incorporation of Fra-1 in the AP-1 complex.

The pCI-neo Fra-1 AS which carried the cDNA of *fra-1* gene in antisense orientation under the control of the human cytomegalovirus (CMV) promoter, was used in transient co-transfections with MEKK1Δ. The ratio of pCI-neo Fra-1 AS:MEKK1Δ was 3:1, to ensure that all cells that receive MEKK1Δ have also incorporated the *fra-1* antisense construct

The results are shown in the figure 49. The antisense did not work neither in HeLa nor in HeLa RARβ clones and Fra-1 induction was not diminished after pCI-neo Fra-1 AS transfection. The Fra-1 induction and c-Jun phosphorylation revealed successful transfection, because MEKK1Δ induces both effects.

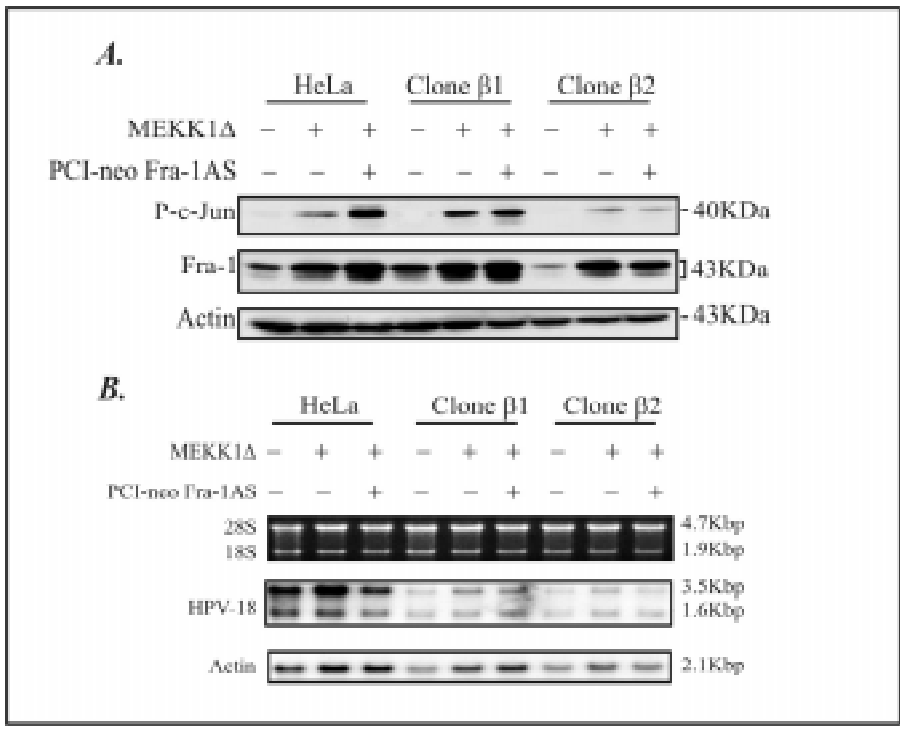


Figure 49 Fra-1 antisense and MEKK1Δ co-transfection in HeLa and HeLa RARβ clones. ***A***. Western blot analysis of 25μg of nuclear extracts from transient transfected cells. After electrotransfer, the blots were incubated with phospho-c-Jun and Fra-1 antibodies. Equal loading was confirmed with an actin-specific antibody. ***B***. HPV-18 expression after transfection was monitored by Northern blot. After transfer, the filter was hybridized with an HPV-18 specific probe. RNA loading and transfer was monitored by hybridization with an actin probe.

Since the transient expression of Fra-1 antisense construct was unsuccessful, the next strategy was the stable expression of *fra-1* cDNA in HeLa cells using amphotropic retroviral vectors.

3.2.4.2 Retroviral infection of HeLa cells to reach stable and constitutive expression of Fra-1

In order to test if Fra-1 over-expression is enough to reduce HPV transcription, HeLa cells were infected with retroviral vectors carrying the cDNA of *fra-1* gene (Figure 50).

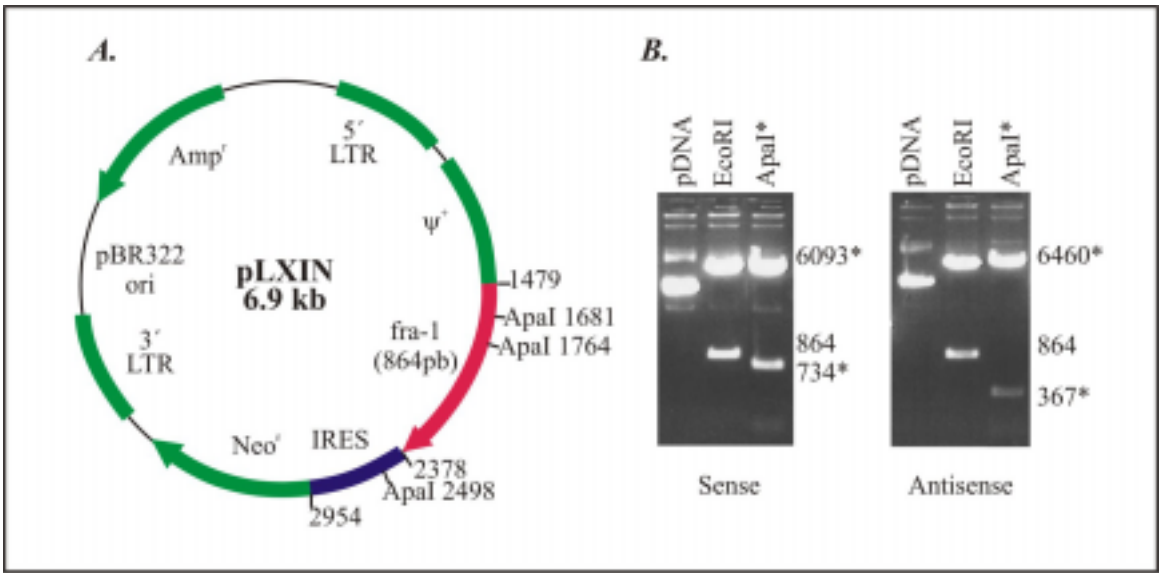


Figure 50 Restriction analysis of the *fra-1* retroviral plasmid. ***A***. The Fra-1 cDNA was cloned in the HpaI site (1479) of the pLXIN vector. ***B***. The orientation of the insert was determined by restriction analysis with ApaI.

The sense and empty vectors were used to transfect the packaging cell line PT67 (Figure 51). Twenty-six and forty-eight hours post-transfection supernatants from the virus producing cells were collected, the virus containing medium was filtered and stored at –70°C.

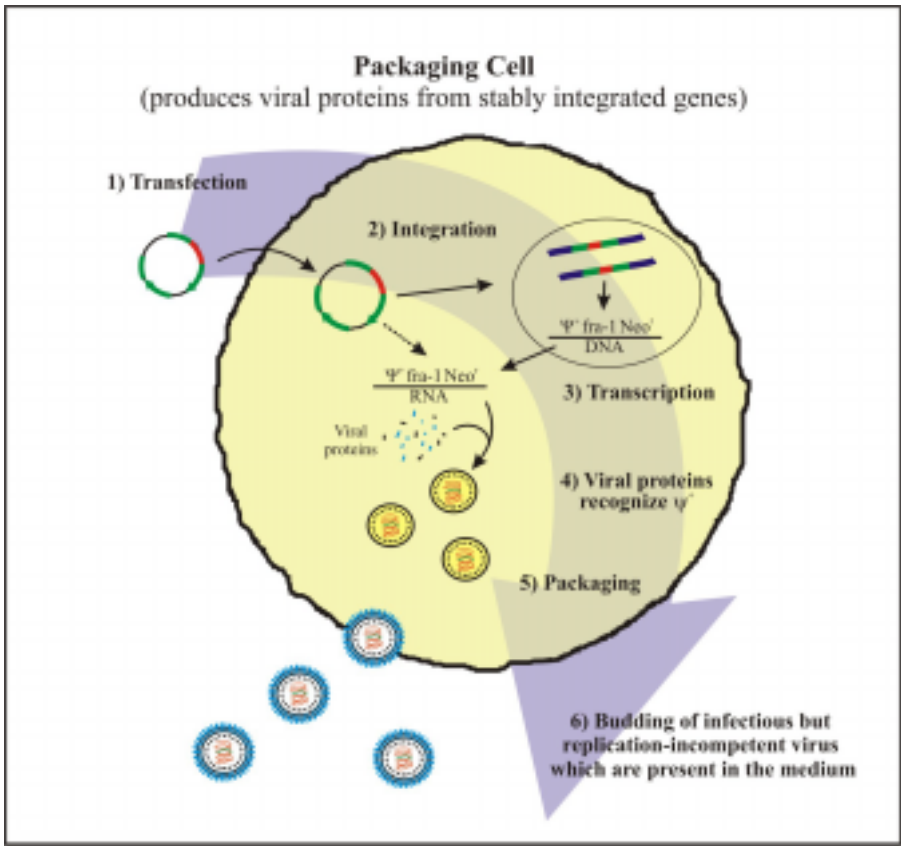


Figure 51 Packaging cell line scheme.

HeLa cells were infected with the virus-containing medium and after 24 hours, the cells were selected in the presence of geneticin (G-418). After 7 days resistant clones were pooled and RNA and nuclear extracts were prepared.

Figure 52 panel A shows the c-Jun and Fra-1 protein expression levels. After retroviral infection Fra-1 was overexpressed while c-Jun was not altered. In the panel B, the Fra-1 incorporation in the AP-1 complex was analyzed. After PhosphorImager quantification an increment of 10 fold in Fra-1 incorporation in the AP-1 complex was determined. However in the panel C, a comparison in the HPV-18 expression between HeLa cells infected with Fra-1 and HeLa cells infected with the empty vector shows that oncogene expression was not reduced by Fra-1 over-expression in the HeLa cells.

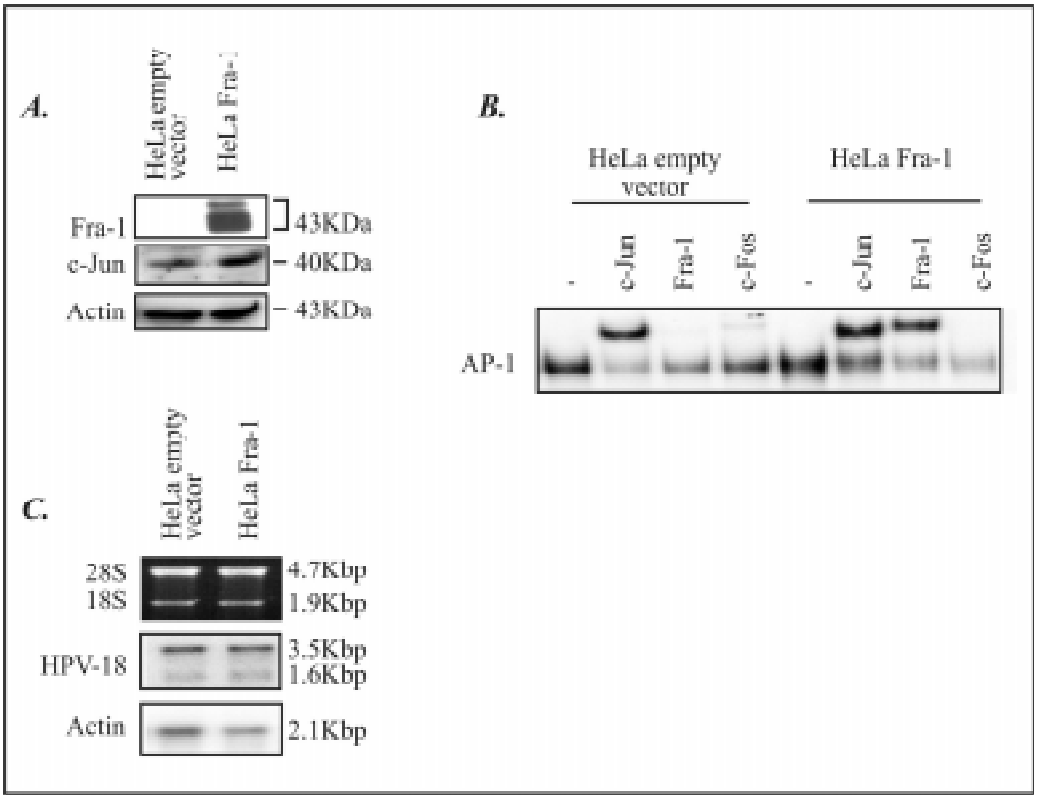


Figure 52 Analysis of HeLa cells that constitutively express *fra-1* gene. ***A***. Immunoblot analysis of nuclear extracts separated in SDS-10% polyacrilamide gel following by electrotransfer and incubation with specific antibodies. Equal loading and protein transfer was confirmed with an anti-actin antibody. ***B***. EMSA super shift analysis of the c-Jun, Fra-1 and c-Fos. Two micrograms of nuclear extract was mixed with AP-1 binding oligonucleotide and incubated 30min at room temperature. Subsequently 1μg of the specific antibody was added following by a 60min incubation at 4°C. ***C***. HPV-18 expression was determined by Northern blot analysis. Five micrograms of RNA was loaded in 1% agarose gel. After transfer the blot was hybridized with an HPV-18 specific probe. Transfer was controlled by hybridization with an actin probe.

From the results I concluded that the increased incorporation of Fra-1 into the AP-1 complex which is observed after constitutive and stable expression of the *fra-1* cDNA is not sufficient to reduce HPV-18 oncogene expression.

IV. DISCUSSION

4.1 RARβ *trans*-repression of AP-1 transcription factor

4.1.1 RARβ *trans*-repression of AP-1 transcription factor

HeLa cells do not express detectable levels of RARβ both on RNA and protein level. Nevertheless RARβ can be induced by pharmacological doses of retinoic acid without alterations in the expression of RARα (Figure 16).

Stable transfection of HeLa cells with retinoic acid receptor β leads to a constitutive expression of the nuclear receptor (Figure 16) which result in a slower growth rate in comparison with HeLa parental cells (Figure 39). The expression of RARα was not alter by transgene expression. RARβ was functional as shown by RARE-Luciferase expression experiments (Figure 17).

In order to analyze the effect of constitutive RARβ expression on AP-1 activity, its amount in HeLa RARβ clones was determined. The results of the figure 18 showed that AP-1 is highly reduced, reaching only between 20-30% of the HeLa parental cells AP-1 content. The RARβ-mediated AP-1 *trans*-repression has been well documented in other cancer models (Chen *et al*., 1995; Yang *et al*., 1997; Li *et al*., 1999; Li *et al*, 1998a; Li *et al*., 1998b; Wu *et al*., 2002).

In the case of HeLa parental cells treated with atRA, the AP-1 content is approximately 40% of the HeLa untreated cells (Figure 18). However is important to note that retinoic acid treatment of HeLa cells induces RARβ expression and simultaneously also activates all retinoic acid receptors. Hence, the AP-1 effect after retinoic acid treatment might be due to more than one mechanism. In contrast in the RARβ clones, AP-1 reduction appears to be mainly due to the RARβ constitutive expression.

As in other cancer models in HeLa cells AP-1 trans-repression by retinoic acid receptor β acts and might be a mechanism to control cell proliferation.

4.1.2 Mechanism of AP-1 down regulation

The mechanistic basis of the anti-AP-1 activity by retinoic acid receptors has remained elusive and the role of the specific retinoic acid receptors isotypes (α, β, γ) or the accessory protein interactions has not been addressed (Altucci and Gronemeyer, 2001). Some of the hypotheses tested to date to explain RARs/AP-1 *trans*-repression will be briefly described below (Figure 53).

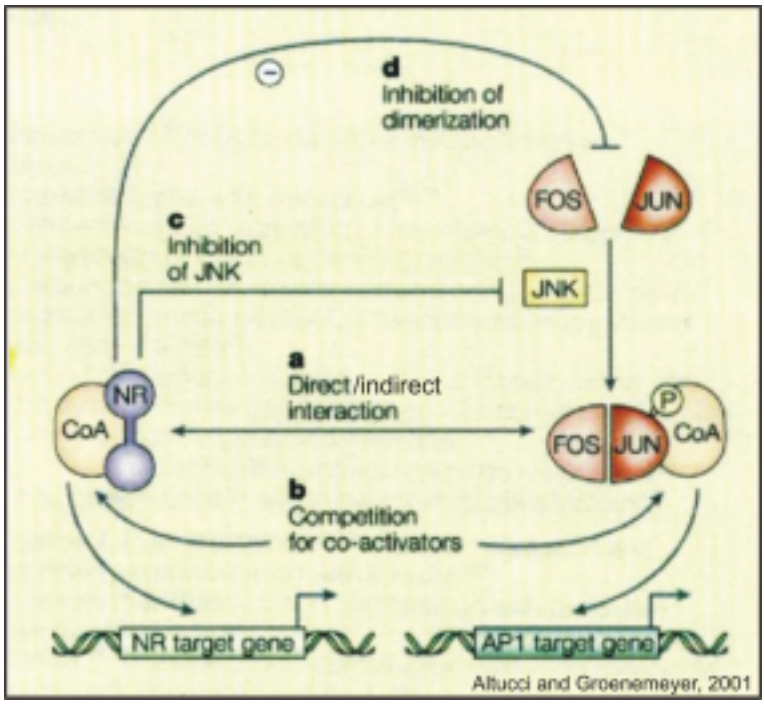


Figure 53: Several mechanisms, which need not be mutually exclusive, may account for the RARs/AP-1 *trans*-repression. a). Direct o indirect interaction that generate abortive DNA complexes. b). Competition for limiting amounts of a common co-activator (Co-A), such as CBP o p300. c). Inhibition of Jun amino terminal kinase (JNK) signalling pathway which prevent c-Jun activation. d). Jun-Fos dimerization disruption by ligand-activated RARs.

i. *Competition for a common coactivator:* Competition for limiting amount of the coactivators between liganded RARs and AP-1. The competition results in the activation of only one pathway (Kamei *et al.,* 1996).

ii. *Blockage in the dimerization:* interference between liganded RARs and Jun/Jun homodimerization and Jun/Fos heterodimerization, preventing the formation of AP-1 complexes. This effect can be direct or involve an unknown intermediate factor (Zhou *et al.,* 1999).

iii. *Down-regulation of c-Fos expression and/or activity:* transcriptional interference of the nuclear receptors with the serum response element in the promoter of *c-fos*, inhibits its expression leading to AP-1 reduced activity. Additionally, it has been reported that retinoids inhibit the transcriptional *trans*-activation of *c-fos* (Talmage and Listerud, 1994; Perez *et al.,* 1994).

iv. *Induction of Fra-1 expression:* since Fra-1 lacks a *trans*-activation function, the retinoid mediated induction of *fra-1* might function as a negative regulator of AP-1 activity (Kaiser *et al.,* 1999).

v. *Direct or third protein-mediated binding between c-Jun and RARs or c-Fos and RARs leads to sequestration of the AP-1 complexes and titrate them out.* However, the interaction ap-

pears to be weak because the complexes can not be detected in the absence of chemical cross-linking agents. Additionally, the RAR binding can generate an unproductive complex RAR/AP-1 at the TRE sequence that eventually can induce DNA bends, association with co-repressors or conformational changes in the AP-1 dimer reducing its *trans*-activation potential (Schüle *et al.,* 1991; Pfahl, 1993; Schroen and Brinckerhoff, 1996).

vi. *Direct or indirect inhibition of Jun N-terminal kinase activation in the cytoplasm,* the direct inhibition could be possible due to an interaction between the nuclear receptor and the enzyme or could interact through intermediate proteins in the frame of multi-protein complexes where JNK may be associated. There also remains the possibility that some up stream component is the primary target of the activated nuclear receptor and not the JNK itself, being the consequence the lack of JNK activation (Gonzalez *et al.,* 1999; Gonzalez *et al.,* 2000). Additionally, it has been postulated that the JNK inhibition can be mediated by the retinoic acid transcriptional induction of a phosphatase responsible for the JNK dephosphorylation and inactivation. One candidate is the MAPK phosphatase 1, MKP-1, which increased in abundance with retinoic acid treatment. The JNK inhibition consequently prevents c-Jun phosphorylation resulting in a negative regulation of c-Jun transcriptional activity and *c-jun* mRNA expression and increases the c-Jun protein degradation (Lee *et al.,* 1999).

vii. *Inhibition of c-Jun protein induction:* this event can occurs either through inhibition of c-Jun translation and/or accelerated degradation of c-Jun. Is postulated that retinoic acid treatment might stimulate c-Jun dephosphorylation promoting its degradation but the inhibition of c-Jun protein induction requires prolonged pre-treatment with retinoic acid (Fisher *et al.,* 1998). On the other hand, it has been suggested that retinoic acid mediates the activation of the glycogen synthase kinase 3, which potentially could inhibit AP-1 activity through c-Jun C-terminal phosphorylation, impairing the DNA binding of the AP-1 complexes. Moreover, for other transcription factors phosphorylation by GSK-3 can lead to targeting to the proteasome for degradation (Benkoussa *et al.,* 2002).

In order to clarify the mechanism by which AP-1 *trans*-repression is achieved in HeLa cervical cancer cells, the most relevant AP-1 family members were analyzed at RNA and protein levels (Figure 19). It is remarkable that c-Jun protein was almost undetectable in the RARβ clones, nevertheless *c-jun* mRNA was present. These results are according to the hypothesis vii above, although the sole overexpression of the RARβ without a pre-treatment with all-*trans*-retinoic acid, is enough to induced the c-Jun degradation.

Interestingly, the c-Jun reduction is not that strong in the HeLa cells after 3 days of retinoic acid treatment. This could be explained because the atRA treatment can activated all nuclear receptors and probably more than one mechanism is involved in the AP-1 *trans*-repression under such conditions. The way by which retinoic acid affects the AP-1 pathways may vary from cell type to cell type. For example, retinoic acid inhibits *c-jun* and *c-fos* expression in synovial fibroblasts. Conversely in vascular

smooth muscle cells, retinoic acid inhibits AP-1 activity without suppressing expression of *c-jun* and *c-fos*. In human bronchial epithelial cells retinoic acid treatment inhibits the growth factor-induced activation of JNK while in human skin, retinoic acid inhibits ultraviolet-triggered accumulation of c-Jun via a post-transcriptional mechanism without affecting the JNK pathway (Moreno-Manzano *et al*., 1999; Fisher *et al*., 1998).
It has been suggested that the mechanism of AP-1 *trans*-repression by RARs can be cell-dependent and/or stimuli-dependent. Likewise, in some cases more than one mechanism can be presented simultaneously (Moreno-Manzano *et al.,* 1999).
In the case of HeLa RARβ clones is possible to dissect the mechanism by which RARβ influences AP-1. In these cells, RARβ-mediated post-transcriptional degradation of c-Jun is mainly responsable for the AP-1 down-regulation. Nevertheless the presence of other mechanisms can not be ruled out.

4.1.2.1 Mechanism of c-Jun down regulation

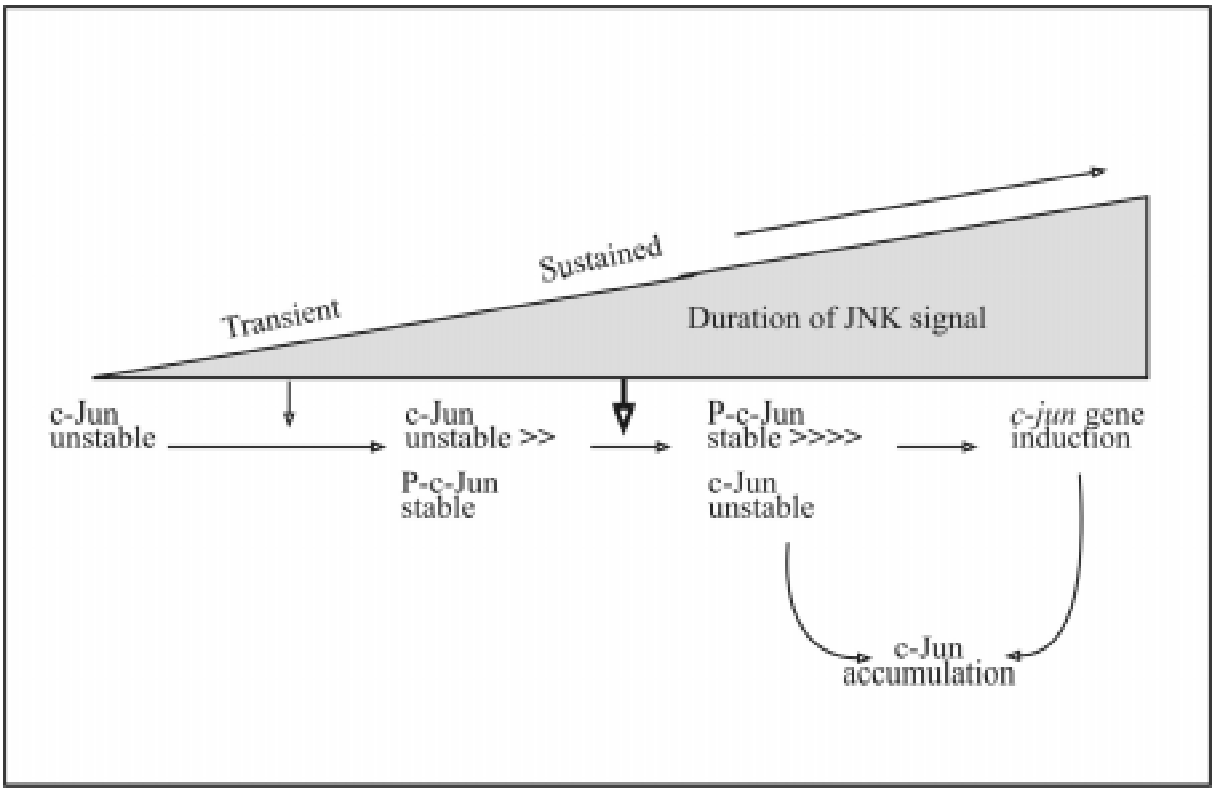
The post-transcriptional degradation of c-Jun could be mediated by alterations in the activity of different proteins responsible for its stabilization or de-stabilization. As was discussed before JNK, MKP-1 and GSK-3, can be involved in the c-Jun degradation either direct or indirectly. In the figure 22 it is possible to compare the expression and activation of these molecules in HeLa and HeLa RARβ clones under control and stimulated conditions. No differences can be observed between HeLa and the RARβ clones. JNK activation is stronger in the clones than in HeLa parental cells. MKP-1 expression is not affected by the constitutive expression of the RARβ and the active GSK-3 is comparable between all cell lines with or without stimulation.
JNK has been postulated as the main regulator of the c-Jun stability (Fuchs *et al*., 1996; Musti *et al*., 1997; Buschmann *et al*., 2000). However my results indicate that c-Jun post-transcriptional down-regulation in the HeLa RARβ clones is JNK independent and JNK activation was not impaired by the over-expression of RARβ even after its activation by atRA treatment (section 3.1.3). The results show that RARβ receptor *per se* is unable to impair the activation of JNK by any stimuli tested. The possible explanation for this is that the mechanism by which atRA inhibits JNK induction is not mediated by RARβ. Additionally, it may be that JNK inhibition by retinoic acid receptor β is stimulus- and cell-type-dependent (Lee *et al*., 1999).

4.1.2.2 Increase of c-Jun protein leads to AP-1/DNA-binding reconstitution in HeLa RARβ clones

AP-1/DNA-binding reconstitution not always implies c-Jun accumulation to the HeLa parental cells levels. Strong and transient stimuli (such as TNFα) as well as constitutive weak stimuli (such as Ha-ras^{mt} transfection) of the JNK showed AP-1 binding reconstitution in the absence of detectable amount of c-Jun by Western blot. This disparity is maybe produced by a transient phosphorylation of c-Jun, which is preferentially incorporated in the AP-1 complex without accumulation of the protein (Figures 25 and 34). In the other hand, strong and sustained stimuli result in c-Jun protein accumulation as well as AP-1 DNA binding restoration (Figures 27, 30 and 34). Then different stimuli can differentially affect the c-Jun content in HeLa RARβ clones. Transient signals lead to c-Jun degradation but sustained JNK activation (by MG-132, serum stimulation or MEKK1Δ) allows the phosphorylation of more c-Jun molecules.

The phosphorylation of c-Jun increases the expression of *c-jun* mRNA (by an auto-regulatory loop, Steinmuller *et al*., 2001), this then increases the synthesis of c-Jun protein, which can be stabilized by the phosphorylation of the active JNK. This process is not affected by the RARβ expression.
Consequently, it is possible distinguish between the RARβ function in resting and stimulated cells. In resting cells constitutive expression of RARβ leads to an increase of the proteasomal degradation of c-Jun. Possibly RARβ increases degradation of proteins, including c-Jun, by modification or de-regulation of regulatory subunits in the proteasome complex (Boyle, 2001).
Alternatively, RARβ may be less effective in stimulated cells because the sustained activation of JNK and c-Jun phosphorylation can generate a bulk of more stable c-Jun protein. This in addition to the higher rate of *c-jun* mRNA synthesis allow the c-Jun accumulation.

The down regulation of c-Jun is a post-translational phenomenon that involve a proteasomal degradation of the unstable unphosphorylate proteins. The JNK activation is not the target for the RARβ driven labilization of c-Jun because it is possible activate this kinase by all stimuli tested in the presence of RARβ receptor unbound or bound to retinoic acid. After JNK activation, c-Jun becomes phosphorylated and this stabilizes the protein by inhibiting the degradation. All non-phophorylated protein is degraded. Only when a pool of stable proteins is reached the c-jun gene is induced and more c-Jun protein is generated. Under strong and sustain stimulation c-Jun is continuously phosphorylated, and by a feedback mechanism c-Jun is accumulated (model 1).



Model 1: Working model to explain c-Jun response in HeLa RARβ clones in function of JNK signal duration.

4.1.3 Role of RARβ on protein degradation

The mechanisms by which retinoids might modulate ubiquitin-dependent proteolysis are unknown. It is possible that RARs might directly *trans*-activate ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and/or ubiquitin ligase E3 (recognizing specific substrates). Conversely, RARs may disrupt other control mechanisms (Boyle, 2001).

It has been described that retinoic acid can alter the degradation rate of some cell cycle proteins leading to down-regulation of cyclins A, B, D1 and E, CDK-2, CDK-4, CDK-6 and up-regulation of p21 and p27 (Dimberg *et al.,* 2002). The analysis of some of these proteins (Figure 38) showed that in the HeLa RARβ clones only cyclin D1 was affected while p53, p27 and p21 were up regulated.

Cyclin D1 is regulated at transcriptional and post-transcriptional levels (Spinella *et al*., 1999). The human Cyclin D1 gene contains two AP-1 binding sites and c-Jun has been found to induce transcription of Cyclin D1 in transient transfection assays (Shaulian and Karin, 2001). Since HeLa RARβ clones contains significantly less c-Jun and less AP-1 than the parental cells, it is possible that the lower levels of Cyclin D1 are consequence to lower mRNA synthesis. In human bronchial cells treated with retinoic acid, Cyclin D1 degradation depends on the proteasome-ubiquitination pathway. Use of a retinoid receptor selective agonist demonstrated that RARβ receptor but not RARα or RARγ dependent pathways signaled this cyclin degradation (Dragnev *et al*., 2001; Boyle *et al*., 1999). Based on the results from the Western blot it was not possible to clarify which of these mechanisms were presented in the HeLa RARβ clones.

The up regulation of p53 (a negative growth regulator), could correlate with a reduction in HPV-18 E6 expression (Scheffner *et al.,* 1990; Werness *et al.,* 1990; Huibregste *et al.,* 1993) and the absence of c-Jun in the HeLa RARβ clones. It has been described that c-Jun is a negative regulator of both p53 expression and its ability to activate target gene transcription (Shaulian and Karin, 2001). The up regulation of p21 can be partially explained by the p53 up regulation, as p53 is a transcription factor required for p21 expression (Shaulian and Karin, 2001). Additionally it was shown that in the absence of added retinoid, RARβ over-expression in neuroblastoma cells *in vitro* leads to induction of p21 expression mediated by a RARE sequence located in the promoter region of the p21 gene (Cheung *et al*., 1998; Dimberg *et al*., 2002).

p27 up regulation has also been associated with RARβ over-expression in lung cancer cells (Hsu *et al*., 2000) but the mechanism is still unclear being postulated a post-transcriptional protein stabilization without transcriptional modulation (Dimberg *et al*., 2002). This observation suggests a RARβ compromise in the proteasomal regulation.

It can be concluded that the modulation of cell cycle inhibitors, Cyclin D1 and p53 in addition to the AP-1 trans-repression and HPV-18 oncogenes down-regulation (Figure 42) may explain the reduced growth of the HeLa RARβ clones.

4.1.4 Role of POH1 in c-Jun stabilization in HeLa RARβ clones

The analysis of proteasome regulatory proteins involved in c-Jun stability and degradation is difficult due to the fact that many proteasome regulatory sub-units are unknown and in most cases is not clear how the selectivity and specificity of proteasome degradation is conferred. Nevertheless, I analyzed the POH1 protein, a proteasome regulator sub-unit that can confer stability to c-Jun protein (Spataro *et al.,* 1997; Nabhan *et al.,* 2001). RARβ did not affect POH1

mRNA or protein expression (Figure 40) although is still possible that RARβ over-expression can somehow impair POH1 activity. Transient transfection with POH1 expression vector led to incorporation of c-Jun in the AP-1 complex as detected by EMSA supershift. This was a specific effect as Oct-1 binding activity was not affected (Figure 41 panel A). Although c-Jun protein accumulation was not detected by Western blot increased expression of Fra-1 indicated functionality (Figure 41 panel B). The results suggest that the inhibitory capability of the RARβ could be overcome by POH1 transient transfection. POH1 over-expression probably stabilizes some c-Jun molecules increasing strongly their incorporation in the AP-1 complex. In fact is possible that stable transfection of POH1 and constitutive *poh1* gene expression will lead to a higher stabilization of the c-Jun molecules. Alternatively, several proteasome regulator molecules might be involved.

Recently a ubiquitin ligase enzyme huCOP1 (Human constitutive photomorphogenesis protein 1) which interacts specifically with c-Jun protein has been described. COP1 may function as a ubiquitin ligase that directly targets transcription factors for degradation, raising the possibility that ubiquitination of c-Jun by huCOP1 may occur (Bianchi *et al*., 2003). Another constitutive photomorphogenesis protein COP9, has been described as modulator of the c-Jun activity. COP9 increased c-Jun protein content leading to elevated AP-1 transcriptional activity (Naumann *et al*., 1999). Imbalance between these activities could result in c-Jun reduction. Is unknown if the COP proteins are related with the RARβ signalization but dys-regulation of this regulatory proteins may be involve in the c-Jun degradation.

Based on the results presented in the chapter 4.1 it was possible to determine that the labilization of c-Jun protein is driven by RARβ receptor specifically. The mechanisms by which atRA through RARβ exerts its AP-1 repression in HeLa cervical cancer cells is not the same postulated in other cancer cells models (Perez et al., 1994; Kamei et al., 1996; Schroen and Brinckerhoff, 1996; Lee et al., 1999; Zhou et al., 1999; Suzukawa and Colburn, 2002). The results showed that RARβ induced degradation of c-Jun protein, resulting in AP-1 reduction. JNK function is not impaired. The RARβ receptor effect is reversible and under strong and sustained stimulus c-Jun protein is accumulated and AP-1-DNA binding is restored.

4.2 Effect of RARβ expression on HPV-18 oncogenes E6/E7

4.2.1 AP-1 binding reconstitution and HPV-18 expression

Constitutive HPV-18 E6/E7 down-regulation (Figures 42 and 43) correlates with AP-1 *trans*-repression in HeLa cells treated for 3 days with atRA and in the HeLa RARβ clones under control conditions (Figures 18 and 45 panel A). This is consistent with previous reports about the importance of AP-1 transcription factor in HPV-18 oncogene expression (Butz and Hoppe-Seyler, 1993).
The evidence suggest that the mechanism by which retinoic acid negatively regulates HPV-18 E6/E7 expression rely in the AP-1 trans-repression by retinoic acid receptors. Furthermore, in the specific case of the HeLa RARβ clones the results indicate that the mechanism by which retinoic acid receptor β down regulate the oncogene expression is the AP-1 transcription factor reduction due to increased c-Jun degradation.

In contrast with the above results, reconstitution of AP-1 binding failed to up regulate HPV-18 oncogenes E6/E7 expression. For some of the treatments employed in this work the HPV-18 unresponsiveness was not surprising (Figure 46). For example in the case of proteasome inhibitor MG-132 the effect over the cells is highly unspecific and it is possible that inhibitors of HPV-18 expression accumulate with similar kinetics as c-Jun.
Nevertheless specifically in the case of Ha-ras^{mt} over-expression, the result was unexpected. It has been reported that HeLa cells stably transfected with Ha-ras^{mt} exhibit up regulation of HPV-18 expression due to c-Jun/c-Fos induction/activation (Medina-Martinez *et al.,* 1997). Ha-ras^{mt} transfection activates the extracellular signal-regulated kinase (ERK) pathway resulting in c-Fos induction at RNA and protein levels. The c-Fos increment would lead preferentially in c-Fos/c-Jun heterodimers, which are more stable than c-Jun/c-Jun homodimers (Ryseck and Bravo, 1991). The reconstituted AP-1 would mainly content c-Fos/c-Jun complexes and consequently up regulate the HPV-18 expression. In the present work in contrast to the expectations Ha-ras^{mt} transfection did not induce c-Fos expression either at RNA level (Figure 34 panel A) nor at protein level (Figure 35).
One explanation for this disparity is that stable transfectants get cellular modifications that do not occur in transient transfection experiments. Alternatively, c-Fos transcription via Ras/ERK activation might be inhibited. In my Ha-ras^{mt} analysis I found c-Jun and Fra-1 up regulation but an unexpected c-Fos down regulation (Figure 35). The explanation for this observation might be the ability of c-Fos and Fra-1 to down-regulate the *c-fos* promoter. c-Jun up regulation via Ras allows its heterodimerization with c-Fos and Fra-1 but only maintain an elevated level of Fra-1 synthesis due to *c-fos* promoter inhibition. Consequently, in response to Ras-signals the accumulation of Fra-1 is predominant over c-Fos and more c-Jun/Fra-1 complexes will be generated (Figure 54, for review, see Tulchinsky, 2000). This phenomenon was observed in HeLa and HeLa RARβ clones and may explain why AP-1 composition was predominantly c-Jun/c-Jun and c-Jun/Fra-1 in the present work in contrast to c-Jun/c-Fos reported by Medina-Martinez *et al*., 1997.

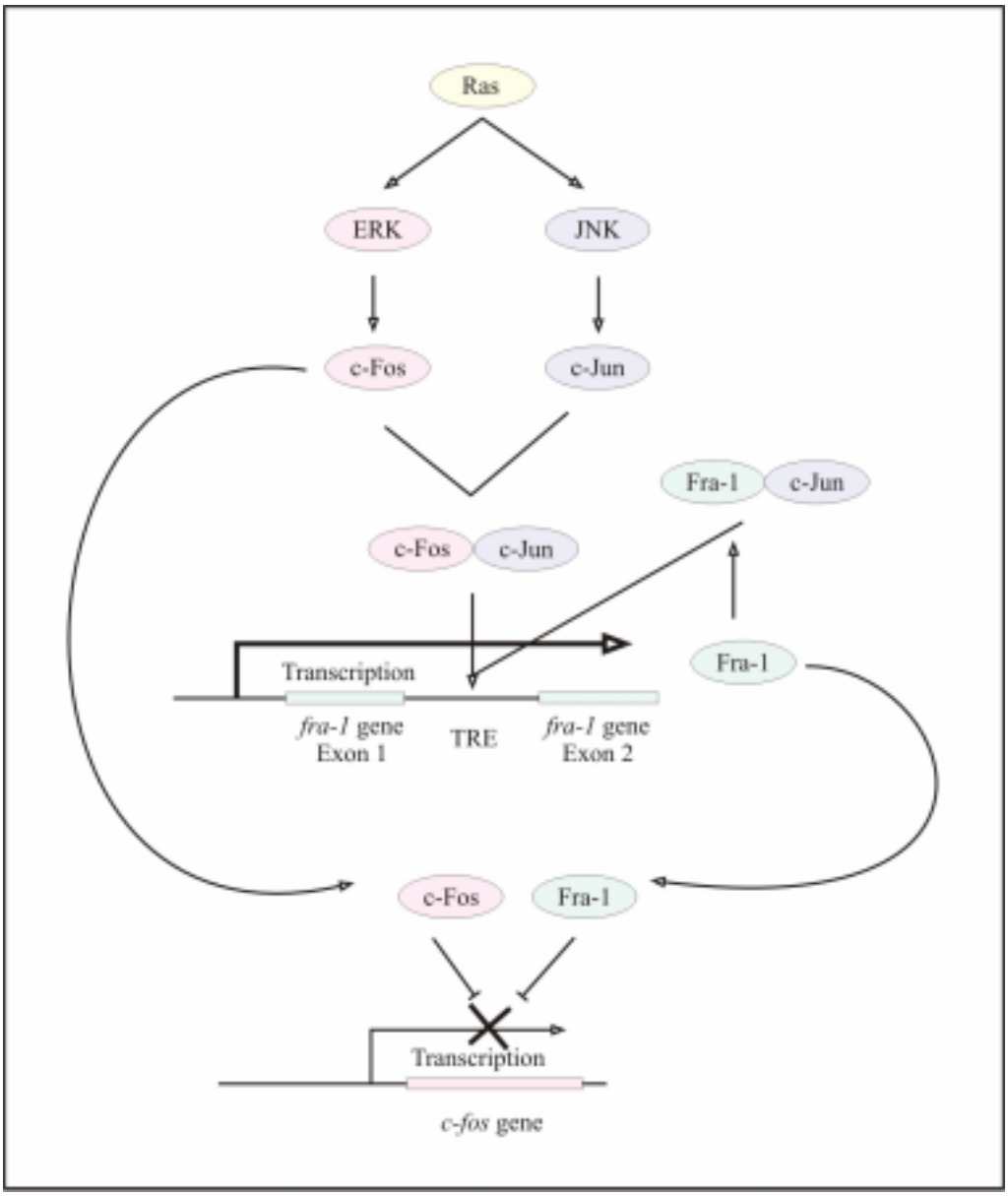


Figure 54: c-Fos negative feedback after Ras stimulation of ERK and JNK pathways

Additionally, the AP-1 differences must account for the differential HPV-18 expression in the both studies, being up regulated when the AP-1 is predominantly c-Jun/c-Fos (Medina-Martinez *et al.,* 1997) and kept down regulated when AP-1 is predominantly c-Jun/c-Jun and c-Jun/Fra-1 (present study).

From the analysis of metalloproteinase gene expression (Figure 48) it is possible to group the AP-1 driven genes in the HeLa RARβ clones in four classes: the non-expressed genes under control conditions that can be up regulated after AP-1 induction (MMP-1 and MMP-3); the genes expressed under control conditions that can be up regulated after AP-1 reconstitution (Fra-1); the genes expressed under control conditions that are not affected after AP-1 elevation (MMP-13) and finally the genes that are down regulated under control conditions and can not be re-induced after AP-1 restoration (HPV-18 E6/E7 oncogenes).

It is described for collagenase 3, (MMP-13) that the dimer c-Jun/Fra-1 regulates its constitutive expression. (Selvamurugan and Patridge, 2000). However for the induction of this gene, an AP-1 composition c-Jun/c-Fos is required. As is shown in the figure 48, the *mmp-13* gene was constitutively expressed under conditions in which c-Fos incorporation in the AP-1 complex was relatively low and the AP-1 composition is predominantly c-Jun/c-Jun and c-Jun/Fra-1 (section 3.1.2.1 and figure 35). On the other hand, the collagenase 1, (MMP-1) promoter is activated and induced by c-Jun/c-Jun homodimers (Westermarck and Kähäri, 1999) as is shown in the figure 48.

The individual Jun and Fos family members exhibit different binding affinities for the AP-1 sites in the MMP promoters (Benbow and Brinckerhoff, 1997).

This observation is according to the demonstration that the composition of AP-1 and the promoter environment (different elements surrounding AP-1 binding site) play a role respect to the differential gene expression (Angel *et al.,* 2001; van Dam and Castellazzi, 2001; figure 55).

AP-1 exhibit differential effects although the same preferential dimer formation (c-Jun/c-Jun, c-Jun/Fra-1). It was possible to detect a c-Jun, Fra-1, MMP-1 and MMP-3 gene induction after AP-1 reconstitution but no HPV-18 oncogene or MMP-13 up-regulation.

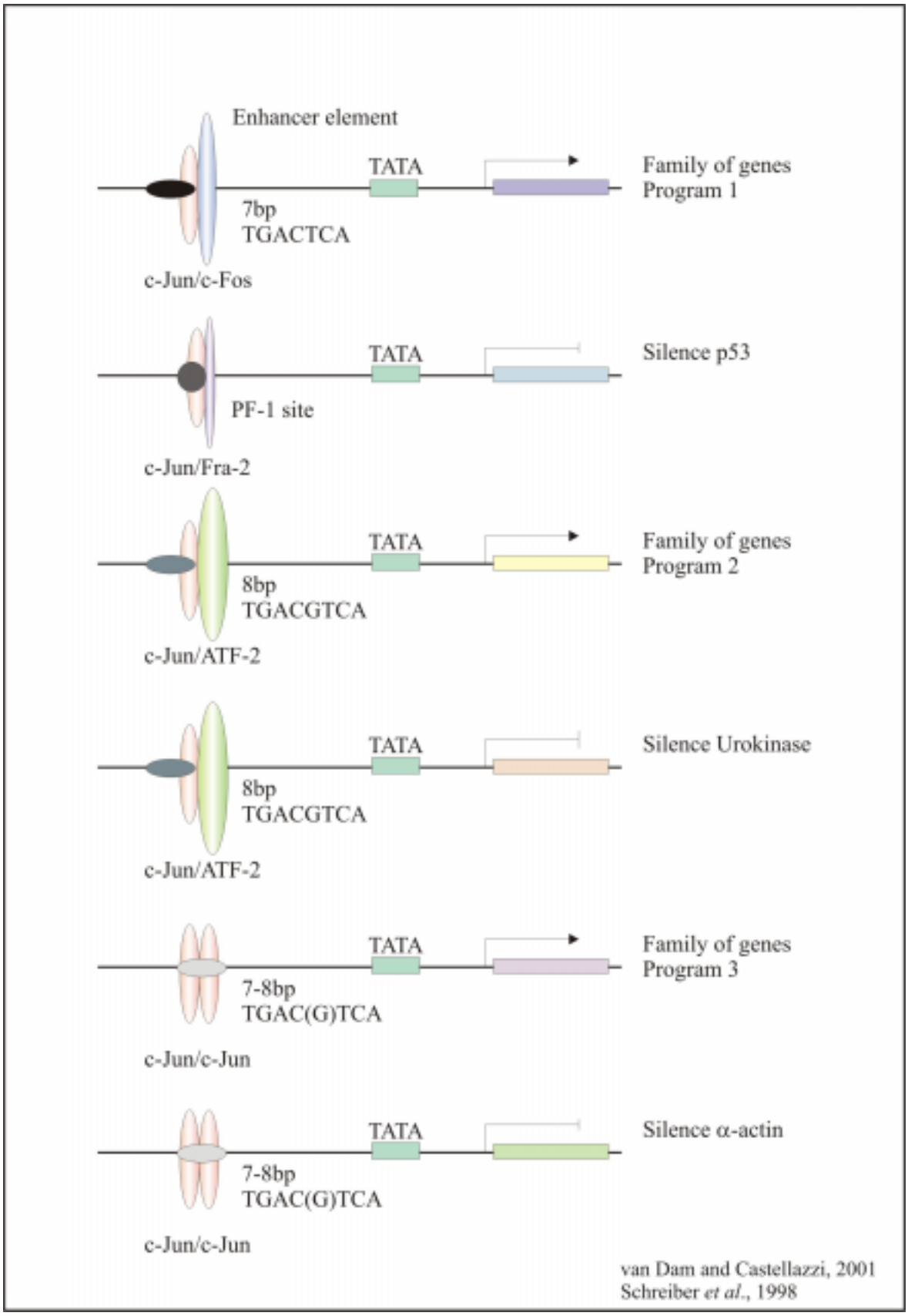


Figure 55 Differences in AP-1 composition and promoter environment regulates positively or negatively different set of genes.

Furthermore, it has been shown that a particular AP-1 composition mediates negative regulation of HPV transcription. Treatment of HPV-16 immortalized human keratinocytes with the antioxidant pyrrolidine-dithiocarbamate (PDTC) selectively reduced the amount of viral mRNA at the level of initiation of transcription. Suppression was accompanied with an enhanced affinity of AP-1 to its cognate binding site within the URR and a preferential association of c-Jun and JunB with Fra-1 instead c-Fos. The suppression of HPV-18 and the higher binding of the AP-1 to the URR were also observed after AP-1 elevation (Figures 46 and 47). The higher incorporation of Fra-1 in the AP-1 complex is shown in figure 35. Elevated levels of Fra-1 compete out the *trans*-activation of AP-1 prototype c-Jun/c-Fos-complexes on AP-1 responsive marker genes (Suzuki *et al.,* 1991; Yoshioka *et al.,* 1995), it was reasonable to assume that viral suppression was mainly due to the reorganization of AP-1 transcription complexes in PDTC treated cells (Rösl *et al.,* 1997) as well as in HeLa RARβ stably transfected clones. In fact it has been demonstrated that AP-1 acts not only as a positive regulator as previously thought, but also functions as a central key element in an intracellular surveillance mechanism negatively controlling HPV transcription and tumorigenicity (Soto *et al.,* 1999).

Moreover, heterodimerization with Fra-1 involved the abrogation of AP-1 activity under certain conditions (Yoshioka *et al.,* 1995). Fra-1 was substantially increased within the AP-1 complex of non-malignant cells and diminished or absent in the malignant counterparts. This suggest that the concentration of Fra-1 within the AP-1 transcription complex might be an important marker to predict *in vivo* growth properties of HPV-positive cells (Soto *et al.,* 2000).

Recently it has been published new evidence of the negative role of the Fra-1 composed AP-1 over the HPV-18 expression. The expression of activated Notch-1 transcription factor in HPV positive cells lead to c-Fos suppression and Fra-1 induction and incorporation in the AP-1 complex. This changes in the intracellular balance of individual AP-1 components result in HPV-18 E6/E7 transcriptional down regulation (Talora *et al.,* 2002).

It is possible to suggest that AP-1 composition after its elevation in HeLa RARβ clones, which is mainly c-Jun/c-Jun and c-Jun/Fra-1, negatively regulates the expression of HPV-18 keeping down the oncogene expression.

4.2.2 Role of Fra-1 as negative regulator of the HPV-18 expression

4.2.2.1 Retroviral infection of HeLa cells to reach stable and constitutive expression of Fra-1

The Fra-1 overexpression in HeLa cells did not modify HPV-18 E6/E7 oncogene expression (Figure 52 panel C). However, is important to notice (Figure 52 panel A) that Fra-1 protein accumulated after HeLa retroviral infection has a faster migration than the protein accumulated by the other treatments (Figures 30, 34, 41, 49). It is possible that phosphorylation may be important in the negative down regulation of HPV-18 or, that phosphorylation can result in recruitment of a third molecule that inhibits HPV-18 expression (Hurd *et al.,* 2002). Alternatively, a third molecule could be expressed, activated or bound to Fra-1/AP-1, only under cell stimulated conditions (Philips *et al.,* 1998; Piu *et al.,* 2001).

It has been shown that tumor cells possess higher phosphatase activity than non- tumorigenic cells. It is possible that this phosphatase activity de-phosphorylates Fra-1 in the HeLa cells stably expressing Fra-1 (only detectable fast migrating forms), leading to an unchanged expression of HPV-18 oncogenes. The inhibition of this phosphatase by stimulated transduction pathways could lead to the Fra-1 phosphorylation (slow migrating forms are detected) which result in the direct or indirect HPV-18 oncogenes down-regulation.
Alternatively, may be important the content of c-Jun in order to allow the formation of c-Jun/Fra-1 complexes. Might be that the up-regulation of Fra-1 did not result in the HPV-18 down regulation because c-Jun, the dimerization partner which is responsible for DNA binding, is not present in enough quantity.
Stable and constitutive Fra-1 overexpression is not sufficient to down regulate HPV-18 and additional modifications of Fra-1 specifically, or AP-1 in general, must be necessary to reduce the oncogene expression.
In conclusion, the complexity and plasticity of the AP-1 transcription factor can not be simplify only by the accumulation of the subunits or the composition of the complex at any given time. Additional effects such as post-translational modifications of the specific subunits or interaction with other proteins during the building of the transcriptional complex can determine the effect of the AP-1 on specific promoter regions given differential responses and these hypothesis open new research perspectives on the AP-1 function and regulation.
The AP-1 down-regulation by retinoic acid receptor beta in HeLa HPV-18 positive cells has consequences at the oncogene expression level but interestingly the re-induction of the HPV-18 expression in HeLa RARβ clones is not easily achieved. This could be a promising advantage in the case of RARβ-based cervical cancer therapy because all changes produced in HeLa RARβ clones after the retinoic acid receptor beta constitutive expression lead to growth arrest and tumor growth suppression.

V. ABSTRACT

The nuclear retinoic acid receptor β2 (RARβ2) gene is epigenetically modified and unable to be significantly induced in many types of cancer, including cervical cancer. RARβ2 can act as a tumor suppressor, since loss of its expression is associated with human tumor progression. One mechanism of RARβ2-mediated growth inhibition is based on its ability to constitutively repress the AP-1 transcription factor, but this mechanism has never been demonstrated in cervical cancer cells.

In this thesis the biological consequences of reconstitute RARβ2 receptor expression in cervical cancer cells was investigated. For this purpose, human papillomavirus HPV-18 positive HeLa cells were stably transfected with RARβ2 cDNA under the control of the β-actin promoter. The characterization of the RARβ2 tranfectants revealed a strongly reduced AP-1 binding to the corresponding specific oligonucleotides, even in the absence of *all-trans*-retinoic acid treatment. In HeLa cells, the AP-1 reduction correlates with diminished HPV-18 oncogene transcription and slower cellular growth. Western blot analysis demonstrated that the only member of the AP-1 family consistently reduced in HeLa RARβ2 clones was c-Jun, despite ongoing gene expression. In order to understand the mechanism by which c-Jun is reduced, and since the phosphorylation of c-Jun is important for protein stabilization, HeLa RARβ2 clones were treated with different c-Jun-N-terminal kinase stimulators. These treatments resulted in a c-Jun increase at RNA and protein levels, and led to a reconstitution of AP-1 binding similar to non-transfected HeLa controls. However, the reconstitution of AP-1 binding levels did not have an inductor effect on the HPV-18 oncogenes, the expression of which has been postulated to be AP-1 dependent. Other classical AP-1 regulated genes such as metalloproteinases were up regulated as expected.

In conclusion, the data of this thesis indicate that RARβ2 induced a destabilization and degradation of c-Jun protein, which causes AP-1 reduction. The mechanism of AP-1 *trans*-repression by RARβ2 in HeLa cells is different from that postulated for other cell systems. RARβ2 constitutive expression lead to reduced cell proliferation of HeLa cells, which can be associated with the RARβ2 tumor suppressor function.

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