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The cAMP Responsive Element Binding Protein (CREB) as a Participant in the Mammalian UVC Response and as a Target for Repression by the Activated Glucocorticoid Receptor

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THE cAMP RESPONSIVE ELEMENT BINDING PROTEIN (CREB) AS A PARTICIPANT IN THE MAMMALIAN UVC RESPONSE AND AS A TARGET FOR REPRESSION BY THE ACTIVATED GLUCOCORTICOID RECEPTOR*

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SUMMARY

Exposure of mammalian cells to short-wavelength ultraviolet radiation (UVC) induces dramatic changes in gene expression (UVC response). To date, only few transcription factors are known to mediate the UVC response: AP-1 (Fos/Jun), NF-κB, TCF/Elk1, and Jun/ATF-2. In this thesis, I present evidence that a ubiquitous transcriptional activator, the cAMP Responsive Element Binding Protein (CREB), is also involved in mediating the UVC response. UVC irradiation causes phosphorylation of CREB at serine residue 133, an event which is invariantly required for the transactivation potential of CREB. In contrast to previous views, CREB and the proximal cAMP Responsive Element (CRE) in the promotor region of the proto-oncogene c-*fos* appear to play an important role in the UVC-induced transcriptional activation of this gene. A novel serine/threonine protein kinase with a molecular weight of 108 kD is responsible for this UVC-induced CREB phosphorylation. Furthermore, I show that, in HeLa cells, several growth factors and pro-inflammatory cytokines (Epidermal Growth Factor, basic Fibroblast Growth Factor, Interleukin-1α, Tumor Necrosis Factor-α, and a multi-component, UVC-induced, secreted activity, known as Extracellular Protein Synthesis Inducing Factor, EPIF) induce the same phosphorylation of CREB. Previous studies have shown that UVC mimics the action of some of these growth factors by activating their receptors at the cell surface. It was, therefore, proposed that UVC induces gene expression through the signal transduction routes activated by these growth factors. By using several independent approaches, however, I show here that this model does not apply to the UVC-initiated signal transduction to CREB. The ability of the cells to mobilize their intracellular Ca^{2+} stores, on the other hand, is absolutely required for the UVC signaling to CREB and to the UVC-inducible p42 MAP kinase, since depletion of these stores abolishes the UVC responsiveness of both these endpoints. I present data, however, which argue against the assumption that free cytosolic calcium is an induced "second messenger" in the UVC-initiated signal transduction. Finally, the effects of UVC on the CREB-related transcriptional activator, ATF-1, were also investigated. In many respects, the mechanisms of UVC-induced signal transduction to CREB, described here, also appear to apply to this protein.

In the second part of my thesis, I present data indicating that CREB is a target for repression by the activated glucocorticoid receptor in the regulation of the transcriptional induction of the c-*fos* gene by cAMP. In numerous cell types, elevated cytosolic cAMP levels differentially regulate the expression of several AP-1 family members: they induce the transcription of c-*fos* and *jun*B genes, but strongly repress c-*jun*. I describe here that glucocortioids antagonize both the positive and the negative effects of cAMP, thus providing a novel level of regulation of AP-1 expression. I have further investigated the mechanisms through which glucocorticoid- and cAMP-dependent signal transduction pathways interfere with one another to regulate the activity of the c-*fos* promotor. The repression of the cAMP-induced c-*fos* expression by glucocorticoids is an intrinsic property of the ligand-activated glucocorticoid receptor (GR) and is mediated through the major cAMP Responsive Element (CRE) in the c-*fos* promotor. This suggests that the repression occurs via interference of GR and the transcription

factor operating through this CRE, namely CREB. The interference is, most likely, a direct or third partner-mediated protein-protein interaction of GR and CREB. Data presented here indicate that this interference does not require binding of GR to DNA and also does not prevent CREB from binding to its recognition sequence in the c-*fos* promotor. Furthermore, glucocorticoids do not affect the ability of the cAMP-dependent Protein Kinase A (PKA) to activate CREB via phosphorylation at serine residue 133. They also do not interfere with the function of the co-activator of CREB, the CREB Binding Protein (CBP). Taken together, these observations favour a mechanism of repression in which glucocorticoids down-modulate the transctivation potential of CREB via GR-induced conformational changes in the CREB molecule which, in turn, are likely to repress the formation of a functional CREB-CBP-basal transcriptional machinery complex.

DER TRANSKRIPTIONSFAKTOR CREB ALS EIN VERMITTLER DER SÄUGER-UVC-ANTWORT UND ALS EIN ZIEL FÜR DIE REPRESSION DURCH DEN AKTIVIERTEN GLUCOCORTICOIDREZEPTOR

ZUSAMMENFASSUNG

Die Exposition von Säugerzellen mit kurzwelliger UV-Strahlung (UVC) induziert dramatische Veränderung in der Genexpression (UVC-Antwort). Bis jetzt sind nur wenige Transkriptionsfaktoren bekannt, die die UV-Antwort vermitteln: AP-1 (Fos, Jun), NF-κB, TCF/ELK1 und Jun/ATF-2. In dieser Arbeit zeige ich, daß ein ubiquitärer Transkriptionsfaktor, das an das auf cAMP-reagierende Element bindende Protein (CREB), ebenfalls an der Vermittlung der UVC-Antwort beteiligt ist. UVC-Bestrahlung von Zellen führt zur Phosphorylierung von CREB am Serin 133, eine unbedingte Vorraussetzung für die Transaktivierungsfähigkeit von CREB. Phosphoryliertes CREB und das in der Promoterregion des Protoonkogens c-fos proximal gelegene auf cAMP-reagierende Element (CRE) spielen eine wichtige Rolle bei der UV-induzierten transkriptionellen Aktivierung dieses Gens. Eine neue Serin/Threonin-Proteinkinase mit einem Molekulargewicht von 108 kD ist für die UV-induzierte CREB-Phosphorylierung verantwortlich. Weiterhin zeige ich, daß in HeLa-Zellen verschiedene Wachstumsfaktoren und entzündungsfördernde Cytokine (epidermaler Wachstumsfaktor, basischer Fibroblastenwachstumsfaktor, Interleukin-1α, Tumornekrosisfaktor α und eine multifaktorielle UV-induzierte sezernierte Aktivität, bekannt als „Extrazellulärer Proteinsynthese-Induzierender Faktor", EPIF) dieselbe Phophorylierung an CREB induzieren. Frühere Untersuchungen haben gezeigt, daß UVC in der Wirkung einigen Wachstumsfaktoren ähnelt, indem es deren Rezeptoren an der Zelloberfläche aktiviert. Es wurde deshalb postuliert, daß UVC über die gleichen Signaltransduktionswege Gene induzieren kann wie diese Wachstumsfaktoren. Unter Verwendung verschiedener unabhängiger experimenteller Strategien kann ich hier jedoch zeigen, daß dieses Modell nicht für die UVC-initiierte Signaltransduktion zum CREB-Protein zutrifft. Dahingegen ist die Fähigkeit der Zellen, ihre intrazellulären Calciumspeicher zu mobilisieren, unbedingt für die UVC-Signalisierung zu CREB und zur UVC-induzierbaren P42 MAP-Kinase notwendig; Entleerung dieser Speicher verhindert die Reaktion von CREB und P42 MAP-Kinase auf UVC. UVC induziert jedoch nicht eine Zunahme an freiem cytosolischen Calcium.

Zusätzlich wurden ebenso die UVC-Effekte auf den CREB-verwandten Transkriptionsfaktor ATF-1 untersucht. In vielerlei Hinsicht scheinen die hier beschriebenen Mechanismen der UVC-induzierten Signaltransduktion zu CREB auch auf dieses Protein zuzutreffen.

Im zweiten Teil meiner Arbeit zeige ich Ergebnisse, die darauf schließen lassen, daß CREB ein Ziel für die Repression des durch cAMP induzierten cfos-Gens durch den aktivierten Glucocorticoidrezeptor (GR) darstellt. In zahlreichen Zelltypen regulieren erhöhte cytosolische cAMP-Spiegel die Expression verschiedener AP-1 Familienmitglieder auf unterschiedliche Art: Sie induzieren die Transkription des c-fos und des junB Gens, reprimieren jedoch c-jun stark. Ich beschreibe hier, daß Glucocorticoide sowohl die positiven als auch die negativen Effekte von cAMP antagonisieren, was eine neue Dimension der Regulation der AP-1 Expression eröffnet. Weiterhin habe ich die Mechanismen untersucht, durch welche sich die Glucocorticoid- und cAMP-abhängigen Signaltransduktionswege gegenseitig bei der Regulation der c-fos Promoteraktivität beeinflussen. Die Repression der cAMP-induzierten c-fos Expression durch Glucocorticoide ist eine eigenständige Fähigkeit des Liganden-aktivierten GR und wird durch das am stärksten auf cAMP-reagierende Element (CRE) im c-fos Promoter vermittelt. Dies läßt vermuten, daß die Repression durch die Interferenz des GR mit dem an diesem CRE aktiven Transkriptionsfaktor, nämlich CREB, bewirkt wird. Diese Wechselwirkung ist höchstwahrscheinlich eine direkte oder eine durch einen dritten Partner vermittelte Protein-Protein-Interaktion des GR mit CREB. Die hier dargestellten Ergebnisse lassen darauf schließen, daß diese Interferenz weder die DNA-Bindung des GR erfordert, noch die Bindung von CREB an seine Zielsequenz im c-fos Promoter verhindert. Weiterhin beeinflussen Glucocorticoide nicht die Fähigkeit der cAMP-abhängigen Proteinkinase A (PKA), CREB durch Phosphorylierung am Serin 133 zu aktivieren. Sie interferieren auch nicht mit der Funktion des Coaktivators von CREB, dem CREB-bindenden Protein (CBP). Zusammenfassend machen diese Beobachtungen einen Repressionsmechanismus wahrscheinlich, durch welchen Glucocorticoide das Transaktivierungspotential von CREB dadurch erniedrigen, daß der GR Konformationsänderungen im CREB-Molekül induziert. Diese könnten ihrerseits die Bildung eines funktionellen Komplexes aus CREB-CBP und den transkribierenden Enzymen verhindern.

CONTENTS

SUMMARY

1. INTRODUCTION **1**

PART ONE **1**

1.1 The cAMP Responsive Element Binding Protein (CREB) and its relatives as mediators of the nuclear response to increased cAMP levels and other extracellular signals 1

1.1.1. The nuclear response to cAMP 1

1.1.2. The modular structure of CREB and the mechanisms by which its transactivation potential is regulated 6

1.1.3. CREB is a member of a large family of transcriptional activators - the complexity of the nuclear response to cAMP 9

1.1.4. The physiological role of CREB 11

1.2. The mammalian nuclear response to short-wavelength ultraviolet radiation (UVC response) 12

1.2.1. Ultraviolet radiation - some definitions 12

1.2.2. The effects of the solar ultraviolet radiation (UVR) on animal organisms and cells 12

1.2.3. Ultraviolet radiation as an extracellular signal - UVR-induced gene expression (UV response) 14

1.2.4. The UVC response - what is its biological relevance? 15

PART TWO **17**

1.3. Glucocorticoid-induced gene repression: a novel property of the glucocorticoid receptor 17

1.4. Molecular mechanisms of glucocorticoid-induced gene repression: action of transcription factors without binding to DNA - cross-modulation of transcription factor activities via protein-protein interactions 18

2. SPECIFIC AIMS **20**

3. RESULTS (PART ONE): THE cAMP RESPONSIVE ELEMENT BINDING PROTEIN (CREB) AS A PARTICIPANT IN THE MAMMALIAN UVC RESPONSE **22**

3.1. Multiple extracellular signal transduction pathways converge at CREB 22

3.1.1. UVC induces phosphorylation of CREB at the essential serine residue 133 22
3.1.2. Epidermal growth factor, interleukin-1α and basic fibroblast growth factor induce serine 133 phosphorylation of CREB in HeLa cells 26
3.2. The role of the -60 CRE and of CREB serine 133 phosphorylation in the UVC-induced transcription of the c-*fos* gene 28
3.2.1. CREB participates in the UV-induced transcriptional activation of the c-*fos* gene through the proximal cAMP responsive element (-60 CRE) of the c-*fos* promotor 28
3.2.2. UVC and cAMP, although they both cause CREB phosphorylation at serine 133, differentially regulate the transactivation potential of CREB: the possible role of the co-activator of CREB, CREB Binding Protein (CBP) 32
3.3. Signal transduction pathway(s) leading to UVC-induced CREB phosphorylation 35
3.3.1. Down-modulation of signaling cascades - a tool to explore common components of signal transduction pathways 35
3.3.2. Down-modulation of the adenylyl cyclase-dependent signal transduction pathway does not interfere with UVC-induced CREB phosphorylation 37
3.3.3. UVC, although it feeds into certain growth factor-dependent signal transduction pathways to activate p42 MAP kinase, uses different routes to induce CREB phosphorylation 39
3.3.3.(i). Down-modulation of growth factor-dependent signaling cascades hampers the UVC responsiveness of p42 MAP kinase but not of CREB 40
3.3.3.(ii). Suramin interferes with extracellular protein synthesis inducing factor (EPIF)- and TNF-α-, but not with UVC-induced CREB phosphorylation 46
3.3.3.(iii). The tyrosine kinase inhibitor tyrphostin-51 inhibits the EGF-induced, but not the UVC-induced CREB phosphorylation 48
3.3.3.(iv). Third stimulus interference: TPA pre-treatment abolishes the responsiveness of CREB to a second stimulation with EGF, but not with UVC 49
3.3.4. The UVC-induced signal transduction to both CREB and p42 MAP kinase is Ca^{2+}-dependent 51
3.3.4.(i). Elevation of the cytosolic Ca^{2+} levels in HeLa cells causes both phosphorylation of CREB at serine 133 and p42 MAP kinase activation 52
3.3.4.(ii). Depletion of the intracellular Ca^{2+} stores prevents UVC signaling to CREB and to p42 MAP kinase 53
3.3.4.(iii). UVC does not induce elevation of the free cytosolic calcium: an argument against the "second messenger" hypothesis 57
3.3.5. Identification of the UVC-inducible CREB kinase 61

4. RESULTS (PART TWO): CREB AND THE PROXIMAL c-*fos* CRE AS TARGETS FOR REPRESSION BY THE ACTIVATED GLUCOCORTICOID RECEPTOR....66
4.1. Antagonistic effects of cAMP and glucocorticoids on the expression of AP-1 family members....66
4.1.1. Glucocorticoids interfere with the expression of a set of AP-1 family members regulated by cAMP....66
4.1.2. Glucocorticoids specifically repress c-*fos* transcription induced by cAMP....67
4.2. Mechanism of the glucocorticoid-induced repression of the cAMP-induced c-*fos* transcriptional activation....69
4.2.1. The repression of cAMP-induced c-*fos* expression by glucocorticoids is mediated through the major CRE in the c-*fos* promotor....69
4.2.2. The repression of cAMP-induced c-*fos* transcription by glucocorticoids is mediated through the interference of the activated glucocorticoid receptor and CREB....70
4.2.3. The activated GR represses the c-*fos* promotor without binding to DNA....72
4.2.4. Glucocorticoids do not alter the pattern of occupation of the c-*fos* CRE *in vivo*....74
4.2.5. Glucocorticoids do not prevent PKA-mediated phosphorylation of CREB at serine 133....75
4.2.6. Glucocorticoids do not inhibit the transactivation potential of a GAL4-CBP chimeric protein....77

5. DISCUSSION....80

6. MATERIALS AND METHODS....98
6.1. General methods....98
Phenol/chlorophorm extraction of nucleic acids....98
Ethanol (or 2-propanol) precipitation of nucleic acids....98
Determination of nucleic acid concentration....98
Restriction endonuclease digestion of DNA....98
Size separation of nucleic acids by agarose gel electrophoresis....98
Isolation of DNA fragments from agarose gels....99
Radioactive labeling of DNA....99
Preparation of nuclear extracts....99
Preparation of poly A^+ RNA....100
Transformation of bacteria....100
Large scale plasmid preparation from bacteria....101
6.2. Cell culture and transfections....101
Cell lines....101

Cell culture....102
Transient transfection of cells....102
Treatment of cells....103
6.3. Analytical methods....104
Analysis of gene expression via chloramphenicol acetyltransferase (CAT) reporter gene assay....104
Plasmids used....105
Genomic footprinting....106
Northern blot hybridization....108
DNA probes used in Northern blot hybridization....108
Immunoblot analysis of proteins (Western blot)....109
Antibodies used....110
"In gel" kinase assay....110
Immunofluorescence....110
Free cytosolic Ca^{2+} measurement....111

7. ABBREVIATIONS....112

8. REFERENCES....116

1. INTRODUCTION

The *positive* and *negative* regulation of gene expression is one of the most complex and expanding fields of molecular biology. At the level of transcription, this regulation is mediated by sequence-specific DNA-binding proteins known as *transcription factors.* Depending on the signals it receives (e.g. phosphorylation/dephosphorylation events or interactions with other proteins), a transcription factor may itself be subject to positive and negative regulation which, in turn, may influence the expression of its target genes. In this thesis, I describe such a dual *modus operandi* for an ubiquitous transcription factor, the cAMP Responsive Element Binding Protein (CREB), exemplified by the regulation of the proto-oncogene c-*fos*. The transactivation potential of CREB is enhanced by an adverse environmental agent, short-wavelength ultraviolet radiation (UVC), but it is a target for repression by glucocorticoid hormones. Furthermore, UVC and glucocorticoids affect CREB-dependent transcription via different mechanisms, whose elucidation is a major goal of this thesis. Before starting with this rather specific matter, I would like to introduce the main topics of transcriptional regulation this work will deal with.

PART ONE

CREB AS A PARTICIPANT IN THE MAMMALIAN UVC RESPONSE

1.1 CREB AND ITS RELATIVES AS MEDIATORS OF THE NUCLEAR RESPONSE TO INCREASED cAMP LEVELS AND OTHER EXTRACELLULAR SIGNALS

1.1.1. The Nuclear Response to cAMP

Although examples of cell-to-cell signaling are found in unicellular eucaryotes (e.g. in budding yeast), it is the multicellular organism which developed complicated mechanisms for cell communication. Cells in higher animals, for instance, are programmed through the genetic information they contain to make numerous challenging decisions during the development: whether to live or to die, to proliferate or to differentiate, to synthesize macromolecules required for proliferation or to stay quiescent, to migrate or to remain stationary. In all these cases, however, the ability of the cell to realize its genetic potential and to take an appropriate decision depends on the set of extracellular signals it receives. These signals may originate from another *signaling* cell or be components of the abiotic environment of the cell or multicellular organism. Very often they act in various combinations to determine a complex final response. Cells communicate with each other by means of diverse signaling molecules, among them proteins, small peptides, amino acids and their derivatives, nucleotides, steroids, retinoids, fatty acid derivatives, and even dissolved gases such as nitric monoxide and carbon monoxide.

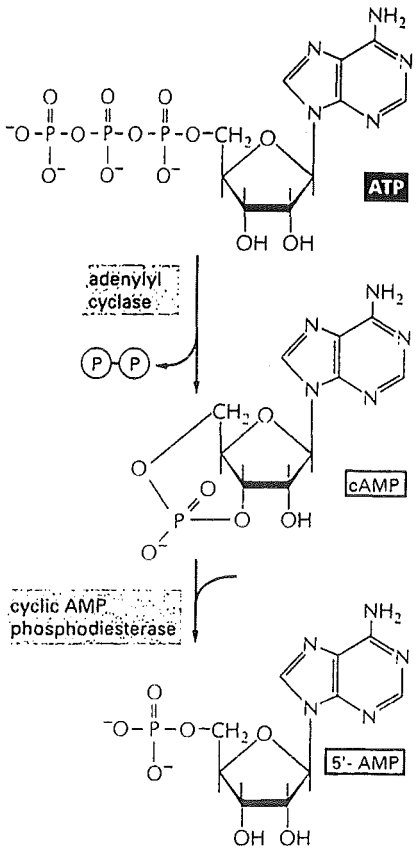


Fig. 1: The synthesis and degradation of cyclic 3′,5′-cyclic adenosine monophosphate (cAMP). The irreversibility of the reaction catalysed by adenylyl cyclase is achieved by a rapid hydrolysis of the released pyrophosphate. The degradation of cAMP by the cAMP phosphodiesterase is one of the mechanisms which allow the attenuation of the cAMP-mediated extracellular signaling. (From Alberts et al., 1994).

The specificity of the signal recognition (and thus the correct response) is always achieved by means of cellular proteins (*receptors*) which bind with high affinity the signaling molecule (a *ligand*). The binding of the ligand to its receptor leads to receptor activation and to generation of a cascade of intracellular signals that alter the behaviour of the cell. With some exceptions (e.g. steroid hormones and retinoids which are relatively small and hydrophobic molecules or dissolved gases such as nitric oxide) most of the extracellular signaling molecules do not enter the cell and bind to transmembrane receptor proteins on the target-cell surface. Thus, in order to achieve an adequate response, the cell faces the need to "translate" the primary extracellular signal into a chain of intracellular signaling events. In many cases this is accomplished through alterations in the concentration of certain small intracellular molecules, which are induced by the ligand-bound receptor. These intracellular molecules, referred to as "*second messengers*", initiate the further intracellular transmission of the signal. This rather complicated mechanism for the extracellular signal to deliver its "message" across the plasma membrane, while physically remaining outside the cell during the signaling event, has several important advantages. First, when required, a multitude of extracellular signals can converge to a limited number of second messenger molecules, thus achieving a similar or identical and potentiated response. Second, by varying the set of receptors presented on the cell surface, two different cell types can respond specifically to different extracellular signals though using the

same second messenger. Third, one ligand-bound receptor may induce the synthesis (or release from special cellular stores) of many second messenger molecules, thus allowing amplification of the signal. Two second messengers that are widely used by different extracellular signaling molecules in almost every animal cell, are Ca^{2+} and *3',5'-cyclic adenosine monophosphate (cAMP).*

In animal cells, cAMP (**fig. 1**) mediates the action of a large variety of extracellular signals including hormones, growth factors and neurotransmitters, and regulates many biochemical and physiological processes such as carbohydrate, lipid, protein, and nucleic acid metabolism, synaptic transmission, and ion channel function. **Table I** lists some of the hormone-induced cellular responses mediated by cAMP (Alberts et al., 1994).

Table I

Some Hormone-Induced Cellular Responses Mediated by cAMP		
Hormone	**Target Tissue**	**Major Response**
Thyroid stimulating hormone (TSH)	Thyroid gland	Thyroid hormone synthesis and secretion
Adrenocorticotropic hormone (ACTH)	Adrenal cortex	Cortisol secretion
Luteinizing hormone (LH)	Ovary	Progesterone secretion
Adrenaline (Epinephrine)	Muscle	Glycogen breakdown
Parathormone	Bone	Bone resorption
Adrenaline (Epinephrine)	Heart	Increase in heart rate and force of contraction
Glucagon	Liver	Glycogen breakdown
Vasopressin	Kidney	Water resorption
Adrenaline, ACTH, glucagon, TSH	Fat	Triglyceride breakdown

In addition to its short-term metabolic actions (e.g. glycogen breakdown in muscle or liver cells, see Table I), cAMP has been found to propagate changes in the genetic program of the cell. Genes have been identified, for instance, whose transcription is induced by extracellular

stimuli increasing the cytosolic concentrations of cAMP. A typical example is the gene coding for the 14-amino acid peptide hormone somatostatin. This hormone was initially isolated from the hypothalamus, but later was also found in the gastrointestinal tract, in the pancreas, and in some pancreatic tumors (Desbuquois, 1990 and references therein). Somatostatin secretion is increased by agents whose action is mediated by cAMP, including the vasoactive intestinal peptide (VIP), glucagon, and epinephrine (adrenaline). Interestingly, VIP expression is also positively regulated by cAMP (Montminy et al., 1986 and references therein). This increase of both somatostatin and VIP expression in response to cAMP appears to occur at the transcriptional level and to depend on the presence of an 8-nucleotide palindrome sequence, 5′-TGACGTCA-3′, in the promotor regions of the somatostatin and VIP genes (Montminy et al., 1986; Tsukada et al., 1987). Because of its ability to mediate the transcriptional activation of genes in response to cAMP, this promotor element was termed *cAMP Responsive Element* (*CRE*, Montminy et al., 1986). The promotors of many other genes, whose transcription was found to be stimulated by increased cytoplasmic cAMP levels, contain the same motif, or at least its invariant core sequence 5′-TGACGT-3′. These include the fibronectin gene (Dean et al., 1990), the proto-oncogene c-*fos* (Sassone-Corsi et al., 1988; Fisch et al., 1989; Berkowitz et al., 1989), the phosphoenolpyruvate carboxykinase gene (Short et al., 1986), the choryonic gonadotropin α-subunit gene (Silver et al., 1987), the proenkephalin gene (Comb et al., 1986), and the tyrosine aminotransferase gene (Boshart et al., 1990). The finding of the CRE and of the first transcriptional activator binding to the somatostatin CRE, namely the *cAMP Responsive Element Binding Protein* (*CREB*, Montminy & Bilezikjian, 1987) provided a major breakthrough in understanding cAMP signaling to the nucleus.

How does the binding of a hormone to its receptor on the cell surface result in an intracellular increase in the cAMP concentration? Furthermore, how does this increase result in an enhanced promotor activity of the target genes? **Figure 2** delineates the major steps involved in the transmission of an extracellular signal to CREB. All the signaling molecules acting via cAMP bind to specific receptor proteins which belong to the class of "seven-transmembrane-domain receptors", known also as "trimeric G protein-coupled receptors" (reviewed by Strader et al., 1994). The G proteins (reviewed by Conklin & Bourne, 1993; Neer, 1995) are made up of three polypeptides: an α subunit that binds and hydrolyses GTP, a β subunit, and a γ subunit. When GDP is bound, the α subunit associates with the βγ dimeric complex to form an inactive heterotrimer that binds the receptor. Conformational changes in the receptor upon ligand binding induce conformational changes in the α subunit as well, in a way that it loses the affinity to GDP which is then replaced by GTP. Once GTP is bound, the α subunit dissociates both from the receptor and from βγ. This leads to the activation of plasma membrane-associated enzymes known as adenylyl cyclases (reviewed by Tang & Gilman, 1992; Choi et al., 1993) that convert ATP to cAMP.

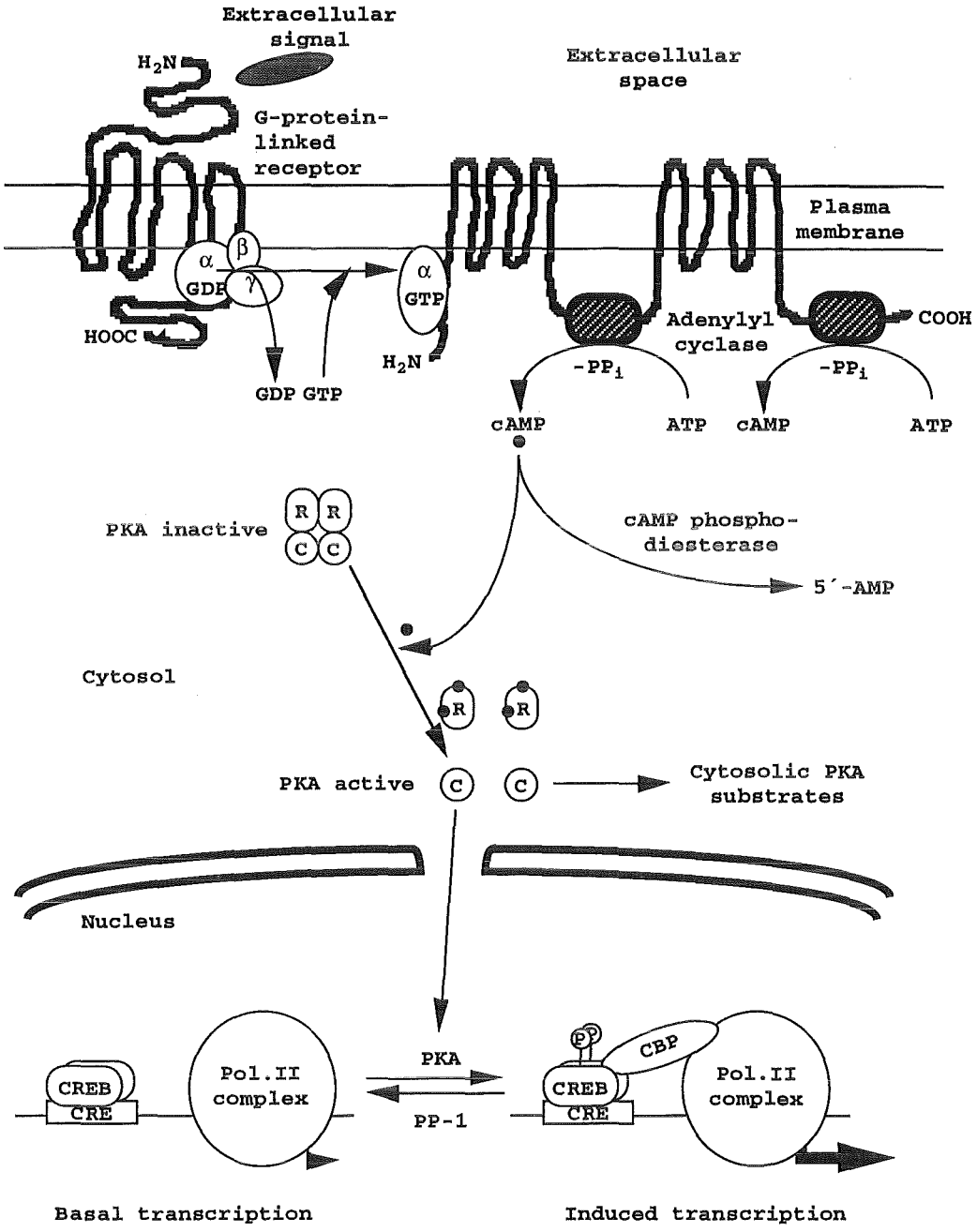


Fig. 2: A schematic representation of the cAMP-mediated nuclear response to extracellular signaling through G protein-coupled receptors (see explanations in the text). α, β, γ - α, β, and γ subunits of the trimeric G protein complex; R - regulatory (cAMP-binding) subunit of the cAMP-dependent protein kinase (PKA); C - catalytic subunit of PKA; PP-1 - protein phosphatase-1, responsible for the dephosphorylation of CREB and for the attenuation of the CREB-dependent transcription; CBP - CREB binding protein, the co-activator of CREB.

Interestingly, the further signal transmission from cAMP to CREB appeared to include only one step, namely the cAMP-dependent protein kinase (PKA, reviewed by Taylor et al., 1990). This enzyme is thought to account for all the effects of cAMP in most animal cells and catalyses the transfer of the terminal phosphate group from ATP to specific serines or threonines of its target substrates. In non-stimulated cells, PKA is inactive and is found in the cytosol and

in Golgi apparatus anchoring sites as a tetrameric complex of two catalytic subunits and two regulatory subunits that can bind cAMP. Upon cAMP binding, the conformation of the regulatory subunits changes causing them to dissociate from the complex. The released catalytic subunits are thereby activated and can phosphorylate cytosolic substrates, such as the crucial enzyme in the glycogen breakdown in skeletal muscle cells, phosphorylase kinase. A population of activated PKA catalytic subunits migrates to the nucleus (Nigg et al., 1985), and their appearance there correlates with the induced transcription through cAMP responsive elements (Riabovol et al., 1988). Consistently, Gonzales & Montminy (1989) identified a single serine residue (serine 133) of CREB as a direct target for the catalytic subunit of PKA. Phosphorylation of CREB at this serine appeared to be an obligatory prerequisite for the CREB-dependent transcriptional activation of gene expression after cAMP stimulation (Gonzales & Montminy, 1989; the mechanisms of CREB activation are discussed in **1.1.2**).

Consistent with the pleiotropic biological effects of cAMP, other cAMP-inducible promotor elements that are not homologous to CREs have been discovered which bind transcription factors different from CREB. For instance, the DNA motif 5´-CCGCCCGCG-3´ in the promotor of the human metallothionein IIA gene binds the transcription factor AP-2 and confers cAMP inducibility to the gene (Imagawa et al., 1987). The transcription factor rNFIL-6 recognizes the cAMP inducible motif 5´-ATTAGGACAT-3´ present within the multifunctional regulatory region of the c-*fos* promotor known as the Serum Responsive Element (SRE) (Metz & Ziff, 1991). An uncharacterized activator protein distinct from CREB operates through the cAMP-inducible element located in the transcribed, but not translated region of the c-*fos* gene (+18/+38, Härtig et al., 1991). Despite the increasing number of cAMP responsive promotor elements and cAMP-activated transcription factors, there is evidence based on transgenic animal models that CREB is the major mediator of the transcriptional effects of cAMP in certain tissues and organs (discussed in **1.1.4**).

1.1.2. The Modular Structure of CREB and the Mechanisms by Which Its Transactivation Potential Is Regulated

The intron/exon structure of the gene encoding CREB and the modular structure of the CREB protein are shown in **fig. 3, A** and **B** (Lee and Masson, 1993). CREB (341 amino acids, 43 kD, Montminy & Bilezikjian, 1987) belongs to the *basic region/leucine zipper* (bZip) group of transcriptional activators which, in higher eucaryotes, includes also the Jun and Fos families of transcription factors (reviewed by Angel and Karin, 1991). The basic region (the name reflects the abundance of positively charged amino acids in this region) represents the DNA-binding domain that allows specific recognition of CREs. Immediately downstream of it lies the C-terminus of the molecule that forms an extensive amphipathic α helix in which every seventh amino acid is a leucine. Due to this arrangement, the leucine side chains (4 in the case of CREB) protrude from one side of the helix and form a hydrophobic surface. Two such helices from two protein molecules can associate in a coiled-coil conformation (leucine zipper), thus allowing protein dimerisation (Landschulz et al., 1988).

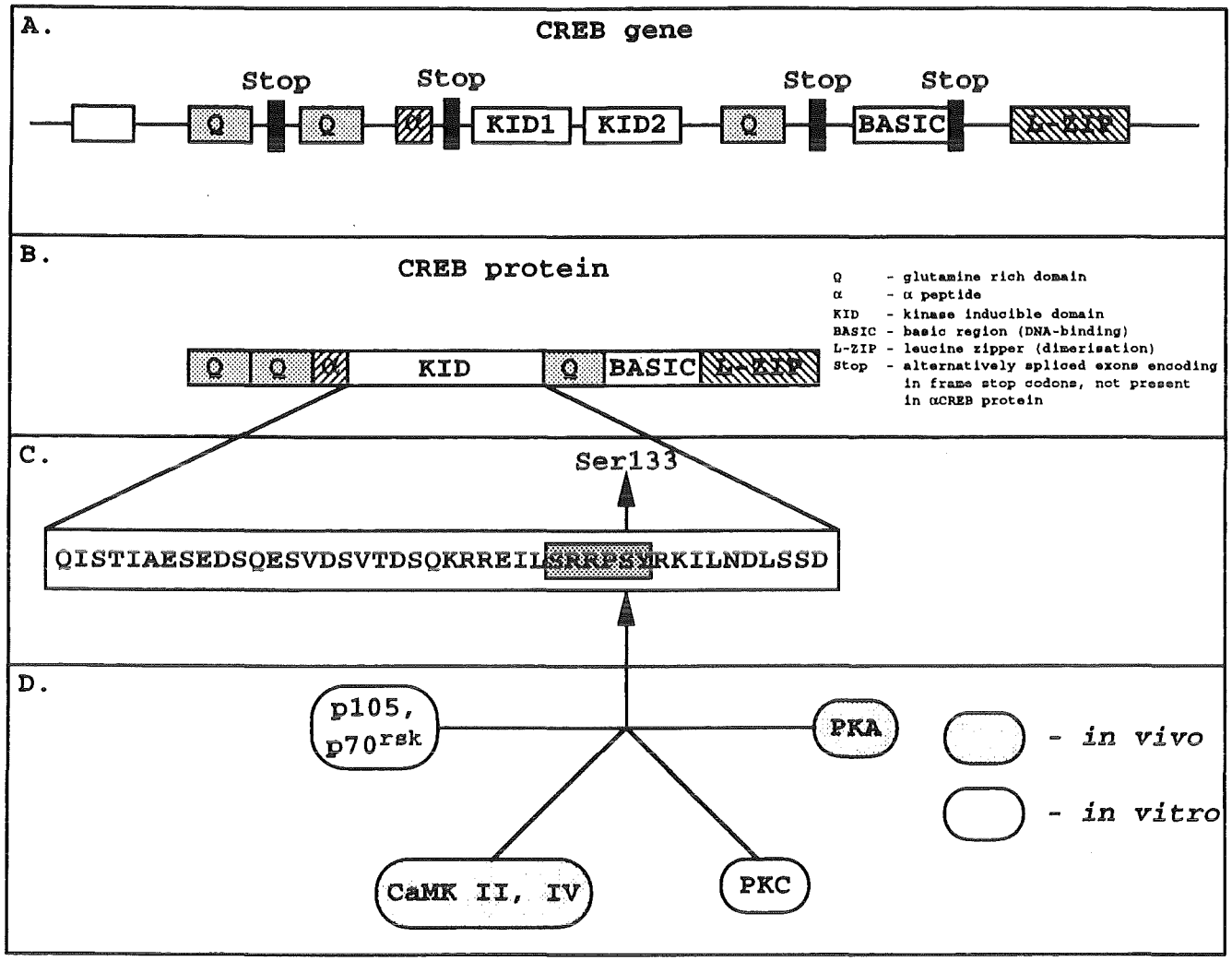


Fig. 3: The modular structure of CREB and the regulation of its transactivation properties through phosphorylation.

A. Schematic representation of the CREB gene. The black boxes represent short exons introducing stop codons in some of the splice variants of the CREB mRNA. The functions of the CREB proteins encoded by these variants remain unclear (Lee & Masson, 1993).

B. The modular structure of CREB protein. The naturally occuring splice variant ΔCREB lacks the α peptide and is a weaker transactivator.

C. The kinase-inducible domain (KID). The dotted box shows the PKA phosphoacceptor site containing the serine residue 133.

D. Some of the serine/threonine protein kinases found to phosphorylate serine 133 *in vivo* and/or *in vitro*.

The transactivation portion of CREB is located N-terminally of the bZip region and is subdivided into three functionally different domains: the glutamine-rich regions (encoded by three exons), the α peptide (18 amino acids encoded by one exon), and the kinase-inducible domain (KID, encoded by two exons). The exact role of the glutamine rich domains remains to be clarified but it is speculated that they are dispensable for the transactivation function of CREB if multiple CREB molecules are bound to the same promotor with several CREs but may be required for transcriptional activation of natural promotors (Lee and Masson, 1993).

The α peptide is encoded by a separate exon and is not present in a naturally occurring splice variant called ΔCREB (Yamamoto et al., 1990, the splicing forms of CREB are discussed in **1.1.3**). Although both αCREB and ΔCREB are transcriptional activators, the α-form is several times more potent (Yamamoto et al., 1990). Interestingly, the α peptide can be placed in different regions of CREB without a dramatic loss of activity, indicating an autonomous function (Yamamoto et al., 1990).

The transactivation functions of CREB depend invariantly on the presence of the kinase-inducible domain. This serine-rich part of the molecule (spanning the amino acid sequence 101-144, **fig. 3 C**) bears phosphoacceptor sites for various serine/threonine kinases (casein kinase II, PKA, protein kinase C, reviewed by Lalli & Sassone-Corsi, 1994) and is heavily phosphorylated at several serine residues *in vivo* (Lee et al., 1990). A single serine within this domain, namely serine 133, appears to be obligatory for the transactivation properties of CREB - to date, substitution of this residue with alanine, aspartic acid or glutamic acid is the only single amino acid exchange known to completely abolish the transactivation by CREB (Gonzales & Montminy, 1989; Lee et al., 1990). Serine 133 is the target for the cAMP-activated catalytic subunit of PKA (Gonzales & Montminy, 1989). The failure of the aspartic acid or glutamic acid exchanges to substitute for serine 133 indicates that the function of the serine 133 phosphorylation is different from just providing a negative charge (Gonzales & Montminy, 1989). How does the PKA-mediated serine 133 phosphorylation of CREB activate the transcription of cAMP-dependent genes? An answer became possible when the co-activator protein of CREB (*CREB binding protein, CBP*) was found (Chrivia et al., 1993). CBP "bridges" CREB to the TFIIB factor of the basal transcriptional machinery and allows the signal from the cAMP-addressed CREB to reach the polymerase II complex, resulting in enhanced transcription. Consistently, CBP can bind only CREB phosphorylated at serine 133 and does not bind CREB point mutants with substituted serine 133 (Chrivia et al., 1993; Kwok et al., 1994). A schematic presentation of the interaction between phosphorylated CREB and CBP and its role in addressing the transcriptional machinery is shown in the lower part of **fig. 2**.

The effect of serine 133 phosphorylation on DNA binding by CREB remains controversial. Both constitutive, phosphorylation-independent, and phosphorylation-induced occupation of CREs have been observed using the genomic (*in vivo*) footprinting technique. An example for the constitutive DNA binding of CREB is the CRE of the c-*fos* promotor (Runkel et al., 1991; M. Iordanov, unpublished). Phosphorylation-dependent DNA binding has been reported for the CRE of the tyrosine aminotransferase gene (Nichols et al., 1992). Nichols et al. (1992) speculate that high affinity (symmetrical) CREs bind CREB independently of the phosphorylation state, whereas low affinity (asymmetrical) CREs bind predominantly CREB phosphorylated at serine 133. The validity of this hypothesis is challenged by the fact that the c-*fos* CRE which is constitutively occupied, is not symmetrical (5´-TGACGTTT-3´ in the case of the human c-*fos* gene and 5´-TGACGTAG-3´ in the case of its murine homologue).

In addition to PKA, several other serine/threonine kinases phosphorylate serine 133 of CREB, at least in *in vitro* assays (**fig. 3 D**). Among the kinases whose role for the serine 133 phosphorylation has been demonstrated *in vivo* are the calcium/calmodulin-dependent kinases type II and IV (Sheng et al., 1991; Dash et al., 1991; Matthews et al., 1994; Sun et al., 1994). Three growth factor-regulated serine/threonine kinases have been implicated in the serine 133 phosphorylation, namely the $p70^{S6K}$ in COS cells (de Groot et al., 1994), $p90^{RSK}$ in human melanocytes (Böhm et al., 1995), and a novel, nerve growth factor-activated p105 kinase in PC12 pheochromocytoma cells (Ginty et al., 1994). The increasing number of CREB serine 133 phosphorylating kinases broadens the putative spectrum of extracellular signal transduction cascades which address CREB. An important outcome of these studies is that all the extracellular stimuli which lead to phosphorylation of CREB at serine 133 via kinases other than PKA are able to induce the transactivation potential of CREB at least in the context of a complex promotor, such as the promotor of c-*fos* (Sheng et al., 1991; Ginty et al., 1994). Thus, a protein which was regarded mostly as a mediator of the nuclear response to cAMP appears to be involved in numerous other nuclear responses, including that to the mitogenic signaling through growth factors.

1.1.3. CREB Is a Member of a Large Family of Transcriptional Activators - the Complexity of the Nuclear Response to cAMP

CREB is an activator that binds to, and directs transcription from, CREs (Montminy & Bilezikjian, 1987). Hurst & Jones (1987) also purified a factor which binds to cAMP- and adenovirus E1A immediate early gene product responsive elements. They termed this factor Activating Transcription Factor (ATF) speculating that it is similar or probably identical to CREB. It appeared later (Hurst et al., 1991) that this factor is a product of a CREB-related gene which was cloned by Hai et al., 1989 and called ATF-1. To date, eight different ATF cDNA clones have been isolated, each of them derived from a separate gene (Hai et al., 1989). They all manifest a high degree of homology with CREB in their basic region/leucine zipper, though displaying divergence in other parts of the protein. However, an involvement of a certain ATF family member in mediating the cAMP response could be shown only for ATF-1 (Hurst et al., 1991). In addition, ATF-1 proved to be a much weaker stimulator of cAMP-induced transcription than CREB (Hurst et al., 1991).

Another gene encoding a cAMP-dependent transcriptional regulator is CREM (CRE Modulator, Foulkes et al., 1991), a splice variant of which, CREMτ, is expressed at high levels in germ cells and seems to play an important role in the spermatocyte development (Lalli & Sassone-Corsi, 1994 and references therein).

All three proteins, CREB, ATF-1, and CREMτ, display high degrees of homology in their kinase-inducible domains (KIDs) and contain an invariant serine residue for PKA phosphorylation (serine 133 in CREB, serine 63 in ATF-1, and serine 117 in CREMτ, Lee and Masson, 1993; Lalli & Sassone-Corsi, 1994 and references therein).

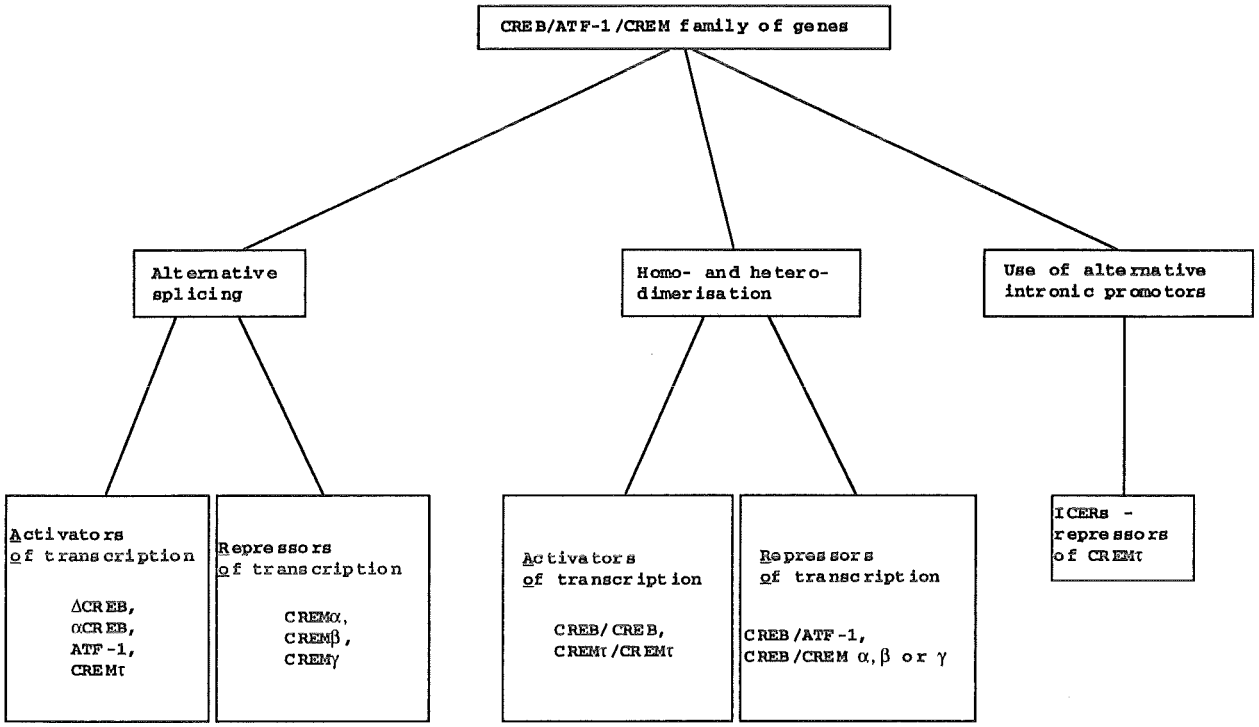


Fig. 4: The complexity of the nuclear response to cAMP. The products of the CREB, ATF-1, and CREM genes act as either activators or repressors of transcription. This is achieved through three different mechanisms: alternative splicing, choice of the dimerization partner, and use of alternative intronic promotors (shown for the CREM gene).

cAMP can cause not only increased but also decreased expression of genes. As for the gene induction, these negative effects of cAMP appear to be, at least in part, explained by the function of cAMP-responsive transcriptional regulators. The complexity of the nuclear response to cAMP (transcriptional activation vs. transcriptional repression or a tissue-specific cAMP response) is generated at three levels, summarized in **fig. 4**. The genes constituting the CREB family, namely CREB, ATF-1, and CREM, encode either activators or repressors of transcription. Whether a transcriptional regulator acts as an activator or repressor depends on the following factors:

(i) alternative splicing, generating proteins with different properties (e.g. different DNA-binding activity, as demonstrated for the CREM splice variants α, β, γ, and τ, Foulkes et al., 1991).

(ii) the ability to form homo- and heterodimeric combinations (e.g. CREB/CREB, a strong activator vs. CREB/ATF-1, a repressor of CREB homodimer activity, Ellis et al., 1995).

(iii) use of alternative intronic promotors. This level of regulation has been found so far only in the case of CREM. A cAMP-inducible intronic promotor within the CREM gene directs transcription of short RNA species which code for small proteins consisting of only basic regions and leucine zippers without transactivation domains. These transcriptional repressors are called ICERs (Inducible cAMP Early Repressors) and are thought to play a role in the

transcriptional attenuation of the cAMP response by occupying the CREs and thus blocking the access of activators of transcription such as CREMτ (Molina et al., 1993; Lalli & Sassone-Corsi, 1994).

1.1.4. The Physiological Role of CREB

CREB is likely to participate in many regulatory processes in both the embryo and the adult organism. The role of CREB *in vivo* has mainly been studied and proved in neuroendocrine processes. Struthers et al. (1992) created transgenic mice overexpressing a dominant negative CREB mutant (CREBM1, bearing a serine 133 to alanine 133 substitution) under the control of the rat growth hormone promotor. This promotor directs transcription exclusively in the somatotroph cells of the anterior lobe of the pituitary. For the pituitary somatotrophs, cAMP serves as a mitogenic signal (Struders et al., 1992 and references therein) and for this reason they are a suitable system to study the role of CREB *in vivo*. The transgenic mice displayed a severe phenotype: dwarfism, atrophic anterior lobe of the pituitary (but normal posterior and intermediate lobes), and absence of somatotroph cells, indicating that the dominant negative CREB mutant strongly interfered with the ontogeny of the pituitary somatotrophs and with some further developmental steps dependent on the function of those cells.

In view of this observation it was somehow unexpected that homozygous transgenic mice with their CREB gene inactivated by homologous recombination (CREB$^-$ mice) did not show any growth or developmental retardation and displayed normal pituitary ontogeny (Hummler et al., 1994). Interestingly, in all tissues of the CREB$^-$ mice studied, a 2 to 3-fold upregulation of CREM gene expression has been observed. The expression of ATF-1 was not upregulated, but the protein was highly expressed in almost all tissues from both CREB$^-$ and wild type mice. For these reasons, a compensatory mechanism within the CREB/ATF family has been postulated in which CREB, CREM and ATF-1 can take over each other's function in the absence of one of the proteins (Hummler et al., 1994). Since the CREB dominant negative mutant (CREBM1) used by Struthers et al. (1992) possibly forms abortive heterodimers with ATF-1 and/or CREM, thus interfering with the action of all three proteins, this could be one explanation for the strong phenotype of the CREBM1 overexpressing mice.

The CREB$^-$ mice, however, provided evidence for a specific CREB function, namely its implication in mammalian long-term memory (LTM) (Bourtchuladze et al., 1994). This kind of memory was found to depend on *de novo* protein synthesis in contrast to the short-term memory (STM) which can occur also in the absence of protein synthesis (reviewed by Matthies, 1989). Studies with fear conditioning revealed that the CREB$^-$ mice have normal short-term memory but deficient long-term memory (Bourtchuladze et al., 1994). Interestingly, analogous results (affected LTM and normal STM) were obtained with *Drosophila* mutants bearing a dominant negative CREB transgene (Yin et al., 1994). Finally, the same group reported a potentiation of the LTM in *Drosophila* mutants overexpressing an activator form of CREB (Yin et al., 1995).

Hence, although the exact physiological functions of CREB and its relatives are far from being completely understood, it is now clear that this family of transcription factors plays important roles in several processes spanning from growth regulation, both during and after embryonic development, up to memory consolidation.

1.2. THE MAMMALIAN NUCLEAR RESPONSE TO SHORT-WAVELENGTH ULTRAVIOLET RADIATION (UVC RESPONSE)

1.2.1. Ultraviolet Radiation - Some Definitions (Madronich, 1993; Josefsson,1993)

Ultraviolet radiation (UVR) is the part of solar electromagnetic radiation which is confined to the wavelength range of 10-400 nm, accounting for less than 9% of the total solar energy output. This wavelength can be broadly divided into extreme UV (10-120 nm), far UV (120-200 nm), vacuum UV (200-240 nm), middle UV or UVC (200-280 nm), UVB (280-315/320 nm), and UVA (315/320-400 nm). The wavelengths shorter than about 280 nm are absorbed almost completely by the earth's atmosphere.

An *irradiation* is the radiant power per unit area integrated over time or the radiant energy per unit area. It is measured in W h m^{-2} or J m^{-2}.

1.2.2. The Effects of Solar Ultraviolet Radiation (UVR) on Animal Organisms and Cells

The majority of the long-term effects of UVR on animals are found to correlate with the ability of UVR to cause damage to DNA and the ability of the challenged organism to handle such damage. A typical example of such a correlation are patients with the syndrome *Xeroderma pigmentosum*, an inherited disease characterized by defects in repairing lesions in DNA (Kraemer & Lee, 1987). Besides their increased sensitivity to light and even short exposures to UVR, these patients develop multiple benign and malignant tumors of the skin. It is calculated that the UVR-dependent risk of melanoma in these patients is raised by a factor of 2000 (Jung, 1986).

In humans, there is substantial epidemiological evidence supporting a strong correlation between solar irradiation of the skin and the frequency of skin cancer. As reviewed by Jung (1992), skin cancers are:

- mostly located in skin areas exposed to sunlight
- more frequent among outdoor workers than indoor workers
- far more frequent among caucasians than among blacks and asians
- dramatically higher in incidence in caucasians at more equatorial latitudes
- known to occur in sun-sensitive phenotypes (skin types I and II) at higher frequency
- known to have increasing incidence in caucasians with exaggerated tanning habits since childhood
- more likely to occur in patients with Xeroderma pigmentosum.

Among the skin cancers whose frequency has been correlated with solar UVR are keratinocyte-derived skin tumors such as the basal cell carcinomas and the squamous cell carcinomas, the melanocyte-borne lentigo maligna, and some forms of malignant melanomas (nodular melanomas and superficial spreading melanomas) (Jung, 1992).

In addition to the direct carcinogenic action on skin stem cells, photocarcinogenesis is promoted by UVR-induced suppression of the immune system (reviewed by Kripke, 1990). In mice, for instance, UVR-induced skin cancers are highly antigenic and are often immunologically rejected when transplanted into syngenic animals. The transplants, however, grow progressively and are not rejected in recipient mice exposed to a short course of UVR before transplantation. In addition, the tumors can be transplanted into sites not exposed to UVR, suggesting that UVR has a systemic suppressive effect on the immune response which determines the rejection of the transplant in non-irradiated mice.

Among the wavelengths within the UV spectrum which are able to reach the earth's surface, the short-wavelength UVB radiation has the highest capacity to damage DNA. A major part of the sensitivity of cells (measured as lethality and/or mutagenesis) to UVR is attributed to UV-induced cyclobutane pyrimidine dimers and to pyrimidine-pyrimidone (6-4) photoproducts (Ananthaswamy & Pierceall, 1990 and references therein).

What are the "intermediate steps" between the UVR-induced DNA damage and the appearance of a transformed and probably malignant cell? Bearing in mind that UVR-damaged DNA is predisposed to undergo point mutations, it is plausible that such mutations in certain genes may be involved in the process of cell transformation. Indeed, point mutations in oncogenes (e.g. K-, Ha-, and N-*ras*) and tumor suppressor genes (e.g. p53) are found in numerous skin cancers (Ananthaswamy & Pierceall, 1990 and references therein). Interestingly, mutations in the p53 tumor suppressor gene have been observed even in healthy skin exposed to chronic solar irradiation (Nakazawa et al., 1994).

Extensive studies on the *Escherichia coli* response to UVR (SOS response) revealed that the appearance and accumulation of UVR-induced mutations in the bacterial genome are not passive processes, but rather require gene products (e.g. *umu*D and *umu*C) whose expression *is actively induced by UVR* . Bacterial strains deficient in *umu*D or *umu*C are nonmutatable by even high doses of UVR as well as by other DNA damaging agents (for a recent review see Friedberg et al., 1995). Thus the question arose whether the UVR-induced mutations in eucaryotes also require induced gene expression. In addition, the UVR-dependent immunosuppression (since it is based on UVR-induced proliferation of T-suppressor lymphocytes, see Kripke, 1990) likely requires gene activation. For these reasons the UVR-induced gene expression in eucaryotes became a widely studied part of the biological action of UVR.

1.2.3. Ultraviolet Radiation as an Extracellular Signal - UVR-Induced Gene Expression (UV Response)

In one respect the cellular effects of ultraviolet radiation in higher eucaryotes resemble strongly those induced by various extracellular signals committed to change the genetic program of the cell: more than 100 genes have been identified so far that do respond to UVR with induced transcription, among them the proto-oncogenes c-*fos*, c-*jun* and different members of their families, stress-associated genes such as metallothionein IIA, the genes coding for several secreted proteases such as collagenase I and plasminogen activator, several growth factor-encoding genes such as the interleukin 1α and basic fibroblast growth factor genes, as well as several viral genomes when present in mammalian cells such as HIV-1 and SV40 (for a more complete list of UV-inducible genes see Herrlich et al., 1992). The genetic (nuclear) reaction of a cell to UVR is termed the *UV response* (reviewed by Herrlich et al., 1992; Herrlich & Rahmsdorf, 1994). Interestingly, the similarity between the extracellular signaling induced by growth factors, tumor promotors, carcinogens and xenobiotics on the one hand and UVR-induced signaling on the other hand is not only restricted to the set of activated genes, but could be observed at the level of transcription factors determining the transcriptional activation of these genes and at the level of signaling cascades leading to the activation of these transcription factors (reviewed by Herrlich et al., 1992; Herrlich & Rahmsdorf, 1994). A typical example is the mitogen- and UVR-induced activation of the transcription factor Elk-1. In cell culture, the activation of this transcription factor by serum and individual growth factors is an important prerequisite for the transcriptional induction of the proto-oncogene c-*fos* through the serum response element (SRE) in the c-*fos* promotor (Zinck et al., 1993). Radler-Pohl et al. (1993) and Sachsenmaier et al. (1994) demonstrated that UVR uses the same signal transduction pathway to induce Elk-1 as the one used by certain growth factors such as the epidermal growth factor (EGF). This signal transduction pathway includes the mitogen-activated protein kinases (p42 and p44 MAP kinases), the p74 Raf-1 kinase, the GDP/GTP-binding protein p21 Ras, and even involves UVR-induced activation of growth factor receptors (Sachsenmaier et al., 1994). Besides Elk-1, there are only three other transcription factors known to date that mediate the UV response: AP-1 (Fos/Jun) (Stein et al., 1989b; Devary et al., 1992; Radler-Pohl et al., 1993), NF-κB (Stein et al., 1989b; Devary et al., 1993), and Jun/ATF-2 (van Dam et al., 1995). As Elk-1, they are all involved in regulating gene expression in response to mitogenic stimuli (e.g. growth factors or pro-inflammatory cytokines).

What is the biological role of the UV response? Is the UVR-induced gene expression beneficial for the cell, which may survive the DNA damage, but may gain an ability to proliferate without constraints? Is it then disastrous for the organism, which would most likely die if the cells gained such an ability? In contrast to procaryotes, in the eucaryotic cell UVR does not appear to induce expression of genes involved in DNA repair. However, pre-exposure of cells to low doses of UVR results in higher cellular resistance to UV-induced DNA damage, a process which depends on ongoing protein synthesis (reviewed by Keyse, 1993). An inhibition of the UVR-induced signal transduction and gene expression by tyrosine kinase blockers

potentiates the cell killing by UVR (Devary et al., 1992). These observations suggest that the UV response increases the potential of the cell to fight against an adverse environmental agent, namely UVR. On the other hand, some of the UVR-induced genes have been implicated in destabilizing the cellular genome. For instance, overexpression of c-Fos, a product of the best studied UVR-inducible gene c-*fos*, increases the frequency of chromosomal aberrations. Furthermore, depletion of cells of c-Fos by antisense c-*fos* oligonucleotides partly suppresses the induction of chromosomal aberrations by UVR (van den Berg et al., 1991). On the other hand, the induction of c-*fos* by UVR may be an important step in the cell survival reaction, since embryonic fibroblasts from c-*fos* knock-out mice (Wang et al., 1992) show increased sensitivity to UVR (Haas & Kaina, 1995; B. Baumann & P. Angel, personal communication). Such a dual role of the UV response, helping the cell to survive a harmful UV irradiation while increasing the probability of genetic changes, should not necessarily be beneficial for the animal organism. In this respect, these effects deserve further intensive investigation.

1.2.4. The UVC Response - What Is Its Biological Relevance?

The UVR used in this work (254 nm) belongs to the short-wavelength UVC. For this reason, the study will deal with a part of the UV response which is termed the UVC response. Is the UVC response biologically relevant, bearing in mind that this part of the UVR (< 280 nm) is efficiently absorbed by the atmospheric ozone? To answer this question one should consider the dependence of most biological effects of UVR on DNA damage which, in turn, depends directly on the wavelength (λ). **Table II** summarizes some of the published data on the action spectrum of UVR. The data shown in the table indicate that not only the mutation rate, but also UVR-induced carcinogenesis and UVR-induced gene expression (the UV response) correlate strongly with the ability of UVR to cause DNA lesions, and that *the biological effects of UVR examined reach maximum values in the UVC part of the spectrum*. The major DNA photoproducts formed at 280-320 nm *in vivo* are the same as those produced more efficiently at 254 nm *in vitro* (Setlow, 1974). Thus, it is quite probable that the naturally occurring biological effects of the solar UVR share the same basic mechanisms with those which would have occurred much more frequently if wavelengths shorter than 280 nm were able to reach the earth's surface. For these reasons, UVC and the UVC-induced photoproducts are probably appropriate *model systems* to study the molecular basis of the biological effects of short-wavelength UVR. Investigating the biological effects of the ultraviolet radiation under conditions where these effects are more pronounced (UVC) helps us to understand the molecular mechanisms of action of the short-wavelength part of the solar radiation reaching the earth's surface.

The UVR dose used in almost all of the experiments described in this thesis (30 J m^{-2}) induces approximately as many cyclobutane dimers in cultured human fibroblasts as would a one-hour exposure of the same cells at 2300 m on a sunny summer day (Klocker et al., 1984).

Table II

Dependence of the Biological Effects of UVR on the wavelength (λ)		
Biological Effect	**Maximum at λ (nm)**	**Reference**
DNA damage (cyclobutane dimer formation)	< 265 nm	Setlow (1974)
Escherichia coli killing	< 265 nm	Setlow (1974)
Mutations in *E. coli*	< 265 nm	Setlow (1974)
Killing of T4 and T6 bacteriophages	< 265 nm	Setlow (1974)
Erythema in mice	<280	Setlow (1974)
Tumor formation in mice	At equal doses, UVC (254 nm) is 35 times more efficient than UVB (295 nm)	Brash (1988)
c-*fos* expression (primary human skin fibroblasts)	265-275 nm	Stein et al. (1989b)
Collagenase I expression (primary human skin fibroblasts)	265-275 nm	Stein et al. (1989b)
HIV-1 CAT expression (HeLa tk^- cells)	265-275 nm	Stein et al. (1989b)
Cell killing (CHO cells)	< 280 nm	Zölzer & Kiefer (1984)

PART TWO

CREB AS A TARGET FOR REPRESSION BY THE ACTIVATED GLUCOCORTICOID RECEPTOR

1.3. GLUCOCORTICOID-INDUCED GENE REPRESSION: A NOVEL PROPERTY OF THE GLUCOCORTICOID RECEPTOR

The glucocorticoid hormone (GH) and its synthetic analogs (e.g. dexamethasone; hereafter GH and its synthetic agonists are referred to as *glucocorticoids*) regulate carbohydrate and amino-acid metabolism, maintain blood pressure, and restrain inflammatory responses (Baxter & Forsham, 1972; Orth et al., 1992). They are known to exert their metabolic activities via induced synthesis of proteins (e.g. enzymes involved in gluconeogenesis in the liver). This positive action of the glucocorticoids depends on an intrinsic property of the glucocorticoid receptor (GR) which, upon activation by ligand binding, acts as a *bona fide* transcription factor and regulates the transcription of the respective genes through specific promotor sequences (Glucocorticoid Responsive Elements, GREs), to which it binds as a homodimer. GR dimerisation requires the C-terminal (second) zinc-finger of the DNA-binding domain characterized by the presence of a specific amino-acid sequence called "the D-loop" (for reviews about the domain organization and the function of GR and of the related steroid hormone receptors see Evans, 1988; Beato, 1989).

Glucocorticoids, however, do not only activate, but also repress the expression of specific genes. The latter property is thought to play a decisive role in the anti-inflammatory and anti-tumor promotion actions of the glucocorticoids (Jonat et al., 1990 and references therein; reviewed by Herrlich & Ponta, 1994). For instance, glucocorticoids inhibit the synthesis of several pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, and this inhibition is, in part, due to transcriptional repression (Beutler et al., 1986; Lee et al., 1988). Furthermore, they block phorbol ester-induced tumor promotion in the mouse skin carcinogenesis system (Belman & Troll, 1972; Scribner & Slaga, 1973). Phorbol ester treatment of mouse skin and of cultured keratinocytes or fibroblasts is accompanied by massive increase in gene expression (reviewed by Rahmsdorf & Herrlich, 1990), among them of the genes for collagenase I and other secreted proteases. These extracellular matrix degrading enzymes are thought to be involved in cell motility, in proliferative responses such as wound healing, and in some chronic destructive diseases. The phorbol ester-induced expression of collagenase I is strongly repressed by glucocorticoids (Jonat et al., 1990 and references therein). Jonat et al., 1990 found that this repression occurs at the transcriptional level and is due to a negative interference between the activated GR and a limiting regulating factor determining the promotor activity of the collagenase I gene, AP-1 (Angel et al., 1987). Further investigations revealed some of the molecular mechanisms underlying this interference.

1.4. MOLECULAR MECHANISMS OF GLUCOCORTICOID-INDUCED GENE REPRESSION: ACTION OF TRANSCRIPTION FACTORS WITHOUT BINDING TO DNA - CROSS-MODULATION OF TRANSCRIPTION FACTOR ACTIVITIES VIA PROTEIN-PROTEIN INTERACTIONS

The "classical" view on transcription factors describes them as sequence-specific *DNA-binding* proteins. In the last few years, however, evidence has accumulated that *bona fide* transcription factors can modulate each other's activity without binding to DNA. A typical example of this new level of transcriptional control is the AP-1-GR mutual interference (Jonat et al., 1990; Yang-Yen et al., 1990; Schüle et al., 1990; for reviews see Cato et al., 1992; Herrlich & Ponta, 1994). It has been shown that repression of an AP-1-dependent promotor (such as the promotor of the collagenase I gene) by glucocorticoids does not involve binding of GR to the promotor, suggesting a mechanism of protein-protein interactions (Jonat et al., 1990). Indeed, AP-1 and GR could be co-immunoprecipitated from cell extracts (Jonat et al., 1990) and interactions between *in vitro* synthesized GR and AP-1 could be detected (Heck, 1993). Antiglucocorticoids (defined as steroid analogs which compete with glucocorticoids for the ligand-binding site of the receptor, but fail to activate GR-dependent gene expression) induce repression, suggesting that gene induction and gene repression are distinct properties of the GR, probably requiring different receptor conformations (Heck et al., 1994). *In vivo* suppression by the GR does not involve removal of AP-1 from its binding site (König et al., 1992). In certain instances, if AP-1 is composed primarily of Jun/Jun homodimers instead of Jun/Fos heterodimers, GR seems to synergize rather than to interfere with AP-1-dependent gene expression (Miner & Yamamoto, 1992; Teurich & Angel, 1995).

Much less is known about the interference of GR with another protein distantly related to the AP-1 family, namely CREB. CREB mediates the action of cAMP on various genes through a cAMP responsive element, the CRE (see **1.1**). Again, positive and negative cross-modulations of CREB and GR activities have been described, depending on the promotor examined and on the cell type. For instance, the α-glycoprotein hormone gene is repressed by glucocorticoids in placental cells (JEG-3 cells), but is activated in baby hamster kidney (BHK) and in CV-1 cells (Chatterjee et al., 1991 and references therein; Stauber et al., 1992). The repression of the α-glycoprotein hormone gene promotor in JEG-3 cells seems to depend primarily on the CRE and on the interference of GR with CREB. A PKA-stimulated GAL4-CREB gene construct driving the expression of a GAL4-dependent reporter is efficiently repressed by the activated GR (Chatterjee et al., 1991). The repression requires the DNA-binding domain of the GR and especially the second zinc-finger (Stauber et al., 1992). Furthermore, the transactivation domain of CREB suffices to serve as a target for GR-mediated repression (Chatterjee et al., 1991). These two observations probably define differences in the mechanisms of interference of GR with AP-1 and CREB, respectively, since dimerisation deficient GR mutants, bearing point-mutations in the "D-loop" of the second zinc-finger, are still able to repress AP-1 activity (Heck et al.,

1994), and, for the repression of AP-1 by GR, the "basic region", but not the transactivation domain of Jun is required (Miner & Yamamoto, 1992; Teurich & Angel, 1995).

The question of whether GR interferes with the CREB-dependent transcription via direct protein-protein interactions is still open. Imai et al. (1993) could show co-immunoprecipitation of CREB with an anti-GR antibody using *in vitro* transcribed and translated proteins. Stauber et al. (1992), however, have used several approaches to detect direct protein-protein interactions between CREB and GR *in vitro*, but failed to do so. For this reason they postulated that the interference might be mediated by a third partner protein.

Recently negative interferences between GR and other transcription factors, different from AP-1 and CREB, have been reported (e.g. GR was found to antagonize the p65 subunit of the transcription factor NF-κB, Ray & Prefontaine, 1994; Caldenhoven et al., 1995; B. Stein, unpublished; S. Heck, unpublished). Furthermore, an important function of the glucocorticoids, namely the glucocorticoid-induced lymphocyte cell death (apoptosis), was ascribed to the negative, but not to the positive transcriptional effects of GR (Helmberg et al., 1995). Thus, the glucocorticoid-dependent repression of gene expression appears to be by no means a "secondary" function of the glucocorticoid receptor, but rather reflects an intrinsic property as important as gene induction.

2. SPECIFIC AIMS

PART ONE

THE *cAMP RESPONSIVE ELEMENT BINDING PROTEIN* (CREB) AS A PARTICIPANT IN THE MAMMALIAN UVC RESPONSE

The principal aim of the first part of this thesis was to explore signal transduction pathways through which ultraviolet radiation induces gene expression. One may expect numerous primary events and possibly numerous pathways to be involved in the cellular response to adverse agents. The only way to define and successfully explore these phenomena is to choose a relevant endpoint (for instance a UVR-activated transcription factor) and to determine the signaling pathway through which UVR activates this endpoint. CREB is a suitable candidate in this regard, since it participates in the transcriptional regulation of immediate early genes (e.g. c-*fos*) and since recent studies have shown that it is a convergence point for numerous extracellular signaling pathways, including some that mediate the nuclear response to certain growth factors and pro-inflammatory cytokines. The goal of this part of my thesis, therefore, was to determine whether UVR induces CREB activation and, if so, what role CREB plays in the cellular response to UVR. More specifically, I addressed the following questions:

1. Does UVR induce post-translational modifications of CREB, known to lead to its activation (e.g. phosphorylation at serine residue 133, see Introduction)?
2. If so, do these post-translational modifications of CREB lead to the induced transcription of genes in a CREB-dependent manner?
2. In the case of a UVR-induced phosphorylation event (the most common post-translational modification of transcription factors), what kinase carries out the phosphorylation?
4. Are the mechanisms of UVR signal transduction to CREB similar to those already described for other UVR-induced transcription factors such as Elk-1 or AP-1?
5. Does the UVR-initiated signal transduction pathway to CREB share common components with signaling pathways induced by other CREB-activating stimuli such as cAMP, Ca^{2+} or growth factors?

PART TWO

CREB AND THE PROXIMAL c-*fos* CRE AS TARGETS FOR REPRESSION BY THE ACTIVATED GLUCOCORTICOID RECEPTOR

The repression of transcription by glucocorticoids is an important function of the glucocorticoid receptor (GR), as it has been implicated in the regulation of numerous cellular responses initiated by glucocorticoids: anti-inflammation, anti-tumor promotion and programmed cell death (apoptosis). In the second part of my thesis I address the problem of the molecular mechanisms underlying this GR-mediated transcriptional repression by investigating the negative interference between GR and CREB. In particular, this part of the thesis will deal with the following questions:

1. How do glucocorticoids interfere with the effects of increased cytosolic cAMP levels on the expression of three cAMP-regulated AP-1 family members (c-*fos*, c-*jun* and *jun*B)?
2. How does the glucocorticoid receptor repress the cAMP-induced, CREB-dependent, c-*fos* transcription?

The questions discussed in both parts of this thesis have so far not been addressed. The results of the studies presented below reveal novel functions and mechanisms of regulation of an ubiquitous transcriptional activator, the cAMP Responsive Element Binding Protein (CREB).

3. RESULTS (PART ONE)

THE *cAMP RESPONSIVE ELEMENT BINDING PROTEIN* (CREB) AS A PARTICIPANT IN THE MAMMALIAN UVC RESPONSE

Mammalian cells exposed to short-wavelength ultraviolet radiation (UVC) display changes in their genetic program, similar to those induced by other adverse environmental agents (e.g. carcinogens and xenobiotics) and by growth factors. These changes occur mostly at the transcriptional level and, collectively, are termed the *UVC response* (reviewed by Herrlich et al., 1992; Herrlich & Rahmsdorf, 1994). The immediate "upstream" event preceding the activation of gene expression in response to UVC is post-translational modifications of pre-existing transcription factors which, in turn, mediate the transcriptional stimulation through UV responsive elements (UREs) in the promotor regions of the target genes. I show here that CREB is a UVC-activated transcription factor and describe the molecular mechanisms of its activation and its functional role in mediating part of the UVC response.

3.1. MULTIPLE EXTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS CONVERGE AT CREB

3.1.1. UVC Induces Phosphorylation of CREB at the Essential Serine Residue 133

Immunofluorescence studies in NIH 3T3 mouse fibroblasts were performed in order to answer the question of whether CREB is a transcription factor addressed by UVC-induced signal transduction cascade(s). For this purpose, I used an affinity purified rabbit antibody which recognizes only CREB phosphorylated at serine residue 133, but does not cross-react with non-phosphorylated CREB. Phosphorylation at serine 133 of CREB by the catalytic subunit of the cAMP-dependent protein kinase A (PKA) is an obligatory prerequisite for the CREB-dependent transcriptional activation of gene expression after cAMP stimulation (Gonzales & Montminy, 1989). To date, substitution of serine 133 by alanine, aspartic acid or glutamic acid is the only single amino acid exchange known to completely abolish the transactivation potential of CREB (Gonzales & Montminy, 1989; Lee et al., 1990; for review see Lee & Masson, 1993). Thus, if CREB were a UVC-activated transcription factor, phosphorylation of serine 133 would be the most promising endpoint to study.

The immunofluorescence pattern with this antibody of untreated (control) or mock-treated (UV-control) NIH 3T3 cells displayed dark-stained nuclei (**fig. 5**), consistent with the non-phosphorylated state of CREB. Treatment of the cells with forskolin, a direct activator of the mammalian adenylyl cyclases (de Souza et al., 1983) for 15 min resulted in a strong positive nuclear staining indicative of CREB phosphorylation at serine 133. The effect was clearly detectable in all cells. Irradiation of the cells with 30 J/m^2 UVC also led to a positive nuclear staining which was indistinguishable in its intensity and ubiquitous appearance from the forskolin-induced CREB phosphorylation. Thus, UVC, in a dose sufficient to induce significant

changes in gene expression (Herrlich et al., 1992), appeared to modify CREB post-translationally through phosphorylation at serine 133.

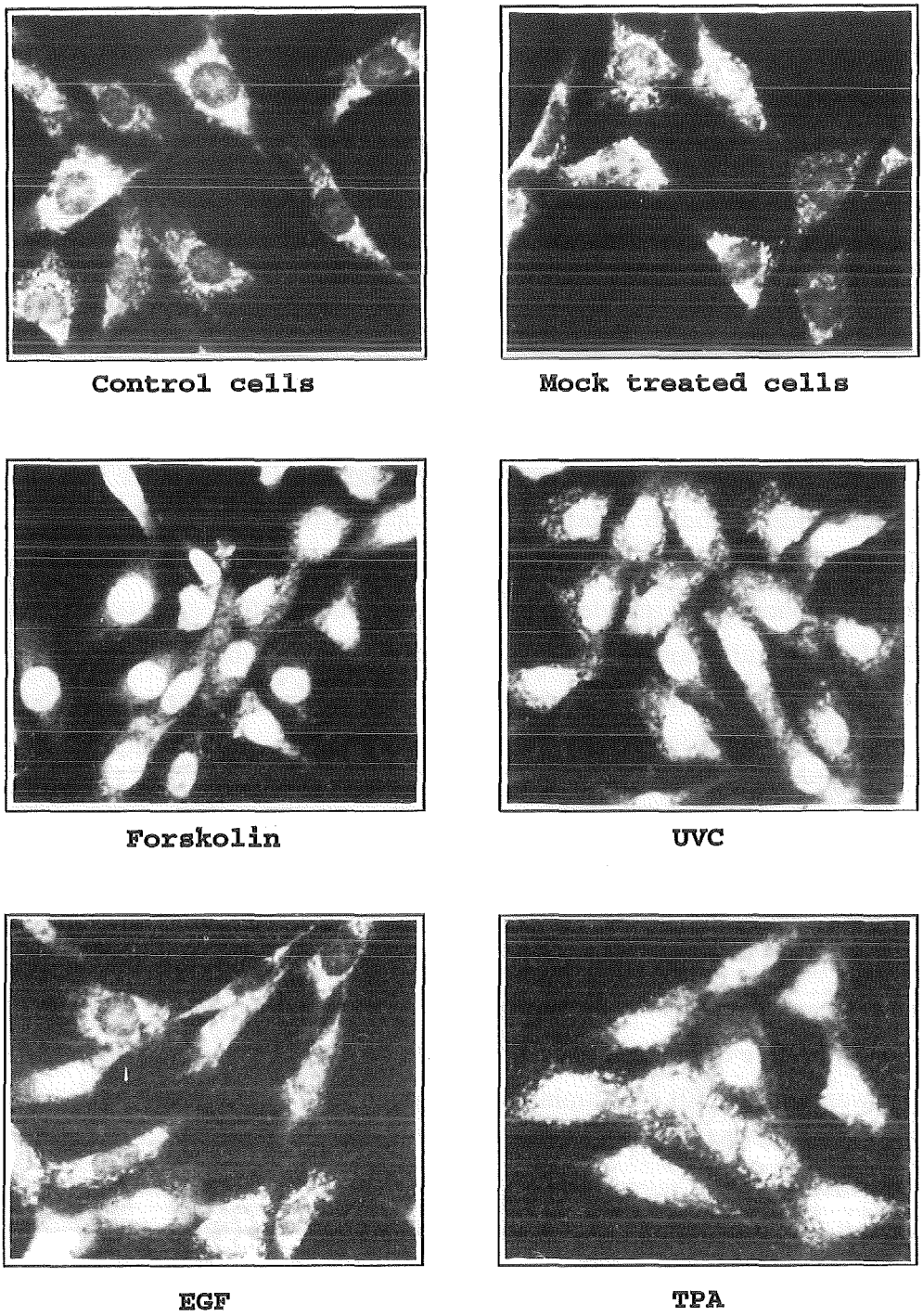


Fig. 5: Forskolin, UVC irradiation, and 12-O-tetradecanoylphorbol acetate (TPA) induce phosphorylation of CREB at serine residue 133 in NIH 3T3 mouse fibroblasts. NIH 3T3 cells were serum starved for 24 hours (DMEM, 0.5% FCS) and then stimulated with forskolin (10 μM), UVC (30 J/m^2), TPA (200 ng/ml), or EGF (20 ng/ml). 15 min later the cells were fixed in 4% *p*-formaldehyde and processed for immunofluorescent analysis as described in Materials and Methods. The antibody used recognizes only CREB phosphorylated at serine 133. The

dark stained nuclei are indicative of the non-phosphorylated state of CREB. The serine 133 phosphorylation is manifested by a positive nuclear staining. Mock treated cells - cells which were processed the same way as the UVC-treated cells, but were not UVC-irradiated: the culture medium was gently removed, the cells were washed with PBS (37^{o}C), and after 21 seconds were given the same medium for 15 min.

UVC shares a similar set of affected transcriptional activators with phorbol ester tumor promotors and numerous growth factors, as shown, for instance, for Fos/Jun (Stein et al., 1989b; Devary et al., 1992; Rahmsdorf et al., 1992; Radler-Pohl et al., 1993) and Elk-1 (Sachsenmaier et al., 1994). In view of this, the ability of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and of epidermal growth factor (EGF) to induce CREB serine 133 phosphorylation was also investigated. Few cells responded to EGF treatment, the majority of them remaining negative. TPA, however, appeared to be an inducer of CREB phosphorylation as potent as forskolin and UVC.

In order to prove that the observed positive nuclear staining in the immunofluorescence analyses was indeed due to phosphorylation of CREB, NIH 3T3 cells were treated with forskolin, UV or EGF for 15 min, lysed in boiling Laemmli buffer (Laemmli, 1970) to avoid possible dephosphorylation and degradation of proteins, and the cell lysates were subjected to SDS-PAGE and immunoblotting analysis, using the antibody directed against CREB phosphorylated at serine 133. **Figure 6** shows that under the experimental conditions used several proteins were recognized by the antibody with apparent molecular weights of 157, 80, 60, 43, and 35 kD. The 157, 80, and 60 kD proteins reacted with the antibody both when derived from untreated and from stimulated cells, and their affinity for the antibody did not change upon stimulation. Thus, these proteins most likely do not account for the effects observed by immunofluorescence, and are possibly responsible for the cytoplasmic background staining in the control cells (fig. 5). In contrast, the 43 and 35 kD proteins were not recognized in the untreated cells and their affinity for the antibody increased dramatically upon forskolin and UV stimulation indicative of induced phosphorylation. The 43 kD band corresponded exactly to the molecular weight of CREB (Montminy & Bilezikjian, 1987), suggesting that this protein band was indeed CREB. The 35 kD band displayed an identical pattern of appearance as CREB, suggesting that a protein related to CREB undergoes similar phosphorylation changes upon forskolin and UV stimulation of 3T3 cells. A member of the CREB family of transcriptional activators, ATF-1 (Hai et al., 1989), contains a consensus phosphoacceptor site for PKA and has 78% homology with CREB in the peptide region used to raise the antibody:

CREB: 123-KRREILSRRPS*YRK-136
ATF-1: 53-KAHGILARRPS*YRK-66

It has a molecular weight of 35 kD (Hai et al., 1989), suggesting that the 35 kD protein was indeed ATF-1. It has been shown in several cell types that the antibody used recognizes

ATF-1 phosphorylated at serine residue 63 which corresponds functionally to serine 133 of CREB (W. Schmid, personal communication). Therefore, these experiments show that the net increase of the nuclear staining in forskolin- and UV-treated NIH 3T3 cells (fig. 5) results from the cAMP- and UV-induced phosphorylation at serine 133 of CREB and serine 63 of ATF-1. Consistent with the weak response of the cells to EGF in the immunofluorescence assay, only a slight enhancement of the CREB phosphorylation (43 kD band) was observed after EGF stimulation in the immunoblot analysis (fig. 6).

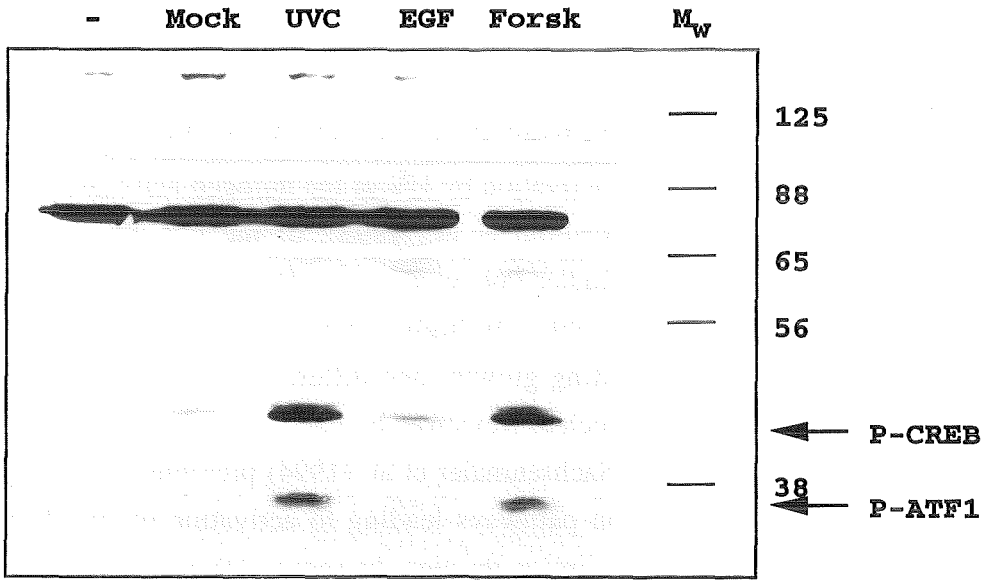


Fig. 6: The net increase of the positive nuclear staining in forskolin and UVC-treated NIH 3T3 cells results from the cAMP- and UVC-induced phosphorylation of CREB and the CREB-related transcriptional activator ATF-1. Confluent NIH 3T3 cells were serum starved for 24 hours and then stimulated with forskolin (10 μM), UVC (30 J/m^2), or EGF (20 ng/ml). 15 min later the cells were quickly lysed in boiling Laemmli buffer to avoid possible dephosphorylation and degradation of the proteins. *Unless otherwise stated, these procedures for growing, serum-starving, and harvesting the cells after treatment apply to all of the experiments where CREB phosphorylation, p42 MAP kinase activation or EGF receptor tyrosine phosphorylation have been examined (figures 7, 9A, 10, 11A, 12-19, and 35).* The cell lysates were resolved in 10% SDS-PAGE, blotted onto a PVDF membrane and subjected to an immunoblot analysis as described in Materials and Methods, using the same antibody as in fig. 5. The bands corresponding to phosphorylated CREB and ATF-1 are indicated. *In order to prove that equal amount of protein has been loaded in all lanes a piece of the gel was always stained by Coomassie Blue (applies to figures 6, 7, 9A, 11A, 12-19 and 35).*

3.1.2. Epidermal Growth Factor, Interleukin-1α and Basic Fibroblast Growth Factor Induce Serine 133 Phosphorylation of CREB in HeLa Cells

In some cell types, CREB was found to be addressed by growth factor-induced signal transduction pathways, in addition to its major function as a mediator of cAMP-induced transcription. For instance, in the PC12 pheochromocytoma cell line, CREB is phosphorylated at serine 133 after treatment of the cells with nerve growth factor (NGF) and participates in the NGF-dependent induction of c-*fos* (Ginty et al., 1994). Kramer et al. (1991) reported on the induced phosphorylation of CREB after stimulation of mink lung CC164 cells with transforming growth factor-β1 (TGF-β1), although the exact phosphorylation site(s) and the functional role of this phosphorylation have not been determined. In many respects the mammalian UVC response resembles growth factor signaling: activation of similar transcription factors is triggered and similar signal transduction cascades are induced (reviewed by Herrlich at al., 1992; Herrlich & Rahmsdorf, 1994). The latter include activation by UV of the mitogen-activated protein kinases p42 MAPK and p44 MAPK (Radler-Pohl et al., 1993), of the p74 Raf-1 kinase, of the GDP/GTP-binding protein p21 Ras (Radler-Pohl et al., 1993; Devary et al., 1992, 1993), and of Src tyrosine kinases (Devary et al., 1992). In higher eucaryotes Src, Ras/Raf complex and MAPKs are key enzymes in transmitting growth- and differentiation-promoting signals from activated membrane receptors to the nucleus (reviewed by Fantl et al., 1993; Avruch et al., 1994; Marshall, 1994; Kazlauskas, 1994). Sachsenmaier et al. (1994) presented data suggesting that the UVC-induced signal transduction pathways leading to activation of p42 MAPK and of Elk-1, and to induction of c-*fos* transcription through the serum responsive element (SRE), are initiated by UV-induced activation of growth factor receptors (GFRs). In HeLa cells, the candidates for UV-activated receptors appear to include the epidermal growth factor receptor (EGF-R), the interleukin-1α receptor (IL-1α-R), and the basic fibroblast growth factor receptors (bFGF-Rs) (Sachsenmaier et al., 1994). With the idea in mind to investigate whether UVC induces phosphorylation of CREB via GFR-dependent routes (see below), the ability of EGF, IL-1α and bFGF to induce CREB phosphorylation was studied in HeLa cells. The rationale to use HeLa cells was that the dependence of the UVC response on growth factor signaling was well established in these cells (Sachsenmaier et al., 1994) and that in NIH 3T3 fibroblasts, EGF induced only marginal CREB phosphorylation. For this purpose, serum-starved HeLa cells were treated with a combination of forskolin and IMX (in order to activate the adenylyl cyclase and to inhibit the cAMP phosphodiesterase, respectively), and either UVC, TPA, EGF, IL-1α or bFGF for 15 min. CREB phosphorylation was monitored by immunoblot assay, using the antibody directed against phosphorylated CREB. Elevation of the cytosolic cAMP level by forskolin and IMX induced strong phosphorylation of two proteins (**fig. 7**):

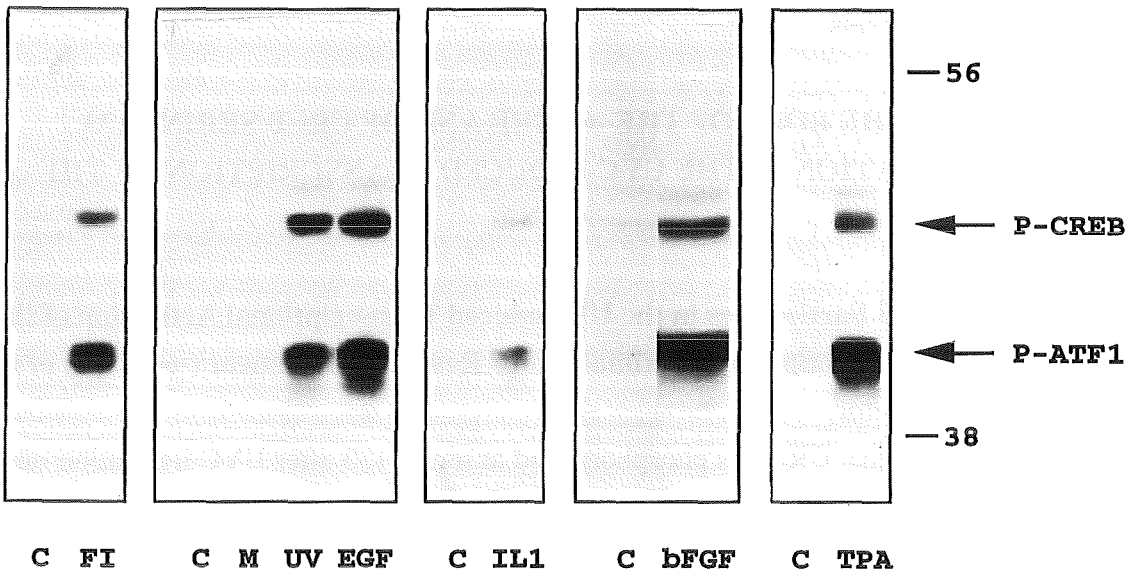


Fig. 7: cAMP, UVC, TPA and several growth factors (epidermal growth factor, interleukin-1α, and basic fibroblast growth factor) induce phosphorylation of CREB at serine 133 in HeLa cells. Immunoblot assays using the antibody directed against CREB phosphorylated at serine 133. Confluent HeLa tk$^-$ cells were serum starved for 24 hours and then stimulated with forskolin and IMX (10 μM and 0.5 mM), UVC (30 J/m^2), EGF (20 ng/ml), IL-1α (2 ng/ml), bFGF (10 ng/ml), and TPA (200 ng/ml) for 15 min. M - mock treated cells. In HeLa cells, CREB and ATF-1 manifest higher apparent molecular weights than in NIH 3T3 cells (47 kD and 40-42 kD respectively, Hurst et al., 1990).

The larger protein displayed an apparent molecular weight of 47 kD, while the electrophoretic mobility of the smaller one spanned a region between 40-42 kD. Hurst et al. (1990) have shown that, in HeLa cells, a 47 kD nuclear protein which they named "activating transcription factor-47" (ATF-47, Hurst & Jones, 1987) is identical to CREB. Again in HeLa cells, the same group described a CRE-binding activity in the size range of 40 to 43 kD (Hurst et al., 1990), which later appeared to consist of the product of the ATF-1 gene (Hurst et al., 1991). Thus, in HeLa cells cAMP stimulation caused a dramatic change in the phosphorylation of CREB and of a protein which was most likely ATF-1. UVC treatment, as in NIH 3T3 cells, also led to phosphorylation of the same proteins. This strongly suggests that the UVC-induced phosphorylation of CREB is a ubiquitous rather than cell type restricted phenomenon. EGF, IL-1α, and bFGF also appear to be potent inducers of CREB phosphorylation at serine 133 and of ATF-1 phosphorylation. Again as in NIH 3T3 cells, CREB phosphorylation in HeLa cells was also induced by the phorbol ester TPA.

These results strongly suggest that different signal transduction pathways initiated by diverse extracellular stimuli, such as UV, phorbol esters, inflammatory cytokines, and mitogens, converge to serine residue 133 of CREB. The biological functions of this transcription factor,

therefore, may include activities beyond merely mediating the genetic response to increasing cytoplasmic cAMP levels.

3.2. THE ROLE OF THE -60 CRE AND OF CREB SERINE 133 PHOSPHORYLATION IN THE UVC-INDUCED TRANSCRIPTION OF THE c-*fos* GENE

3.2.1. CREB Participates in the UV-Induced Transcriptional Activation of the c-*fos* Gene Through the Proximal cAMP Responsive Element (-60 CRE) of the c-*fos* Promotor

The finding that CREB is phosphorylated at serine 133 after UVC irradiation raised the question of whether CREB and CREs participate in gene induction by UVC. The proto-oncogene c-*fos* responds to UVC irradiation with induced transcription (Angel et al., 1985), its promotor region contains a major functional CRE (Sassone-Corsi et al., 1988; Fisch et al., 1989; Berkowitz et al., 1989), and the transcription factor binding *in vitro* to this CRE was found to be CREB (Sassone-Corsi et al., 1988; Härtig et al., 1991). Although 5´-deletion analyses and internal mutations of the c-*fos* promotor region pointed to the serum responsive element (SRE, positions -320 to -299) and to a region in the transcribed but not translated part of the c-*fos* gene (positions +18 to +38) as targets for the UVC-induced transcriptional activation of the gene (Büscher et al., 1988), the possible role of the c-*fos* CRE as an UV responsive element had not been investigated. For these reasons, the promotor of c-*fos* appeared to be a suitable model system to study the functional role of CREB phosphorylation by UVC *in vivo*.

Two approaches were chosen to address the role of the c-*fos* CRE and CREB in mediating the UVC response. First, the ability of UVC and cAMP to induce human c-*fos* promotor CAT reporter gene constructs containing or lacking the major CRE at position -60 (-711/+45 *fos* CAT vs. -711/Δ-65/-52/+45 *fos* CAT, Härtig et al., 1991, **fig. 8A**) was analyzed in transient transfection experiments in HeLa cells. Härtig et al. (1991) showed that, in NIH 3T3 cells, deletion of this element in the context of the whole promotor strongly decreases the cAMP inducibility of the gene. If the -60 CRE plays a significant role in the UVC response as well, its deletion should lead to lower UVC-induced transcription despite the presence of an intact SRE and a +18/+38 region in the promotor. Conversely, should the CRE be dispensable for the UV response, differences in the cAMP-, but not in the UV-inducibility of the two promotor constructs, are to be expected.

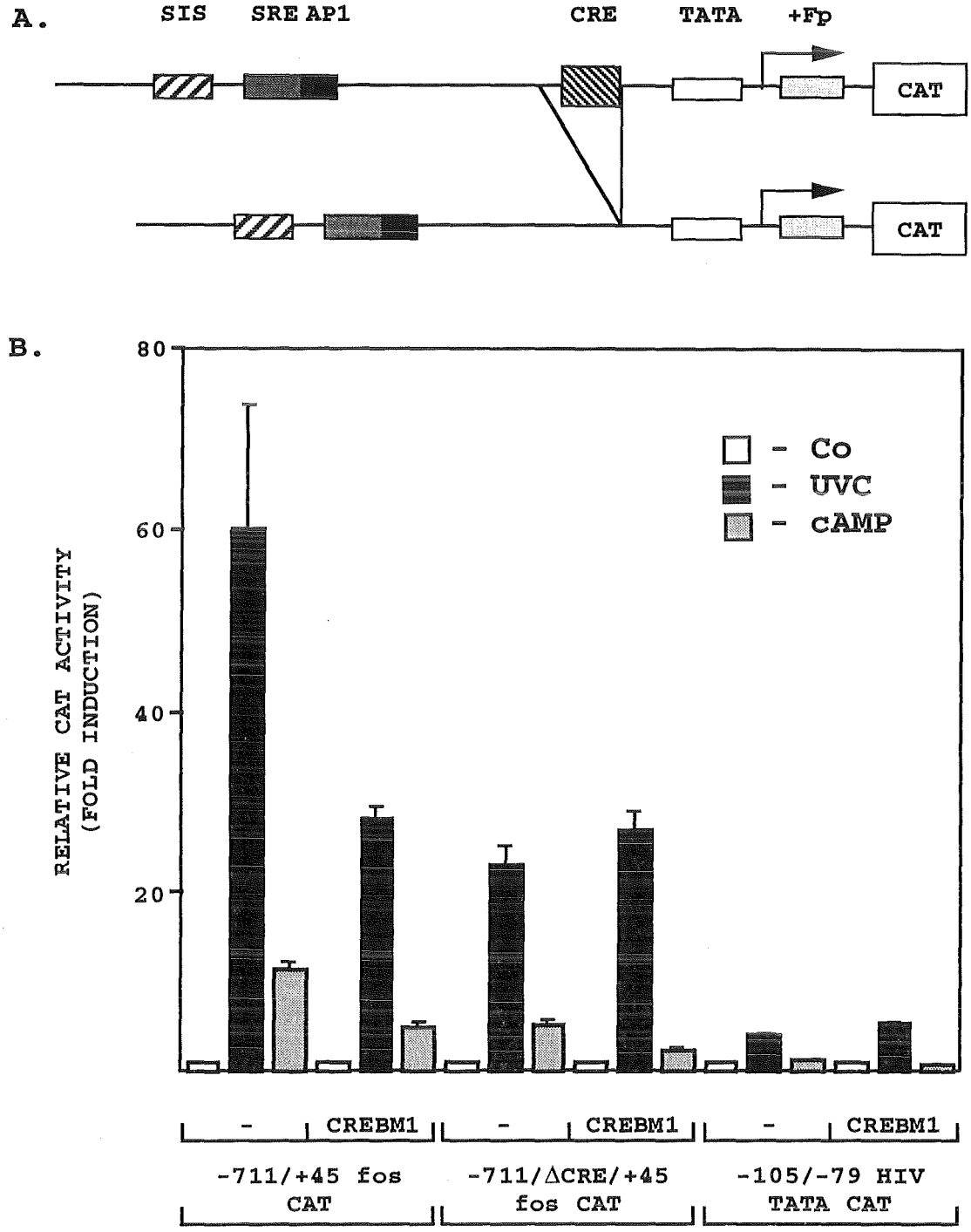


Fig. 8: CREB participates in the UV-induced transcriptional activation of the c-*fos* gene through the proximal CRE of the the c-*fos* promotor.

A. A schematic representation of the -711/+45 *fos* CAT and -711/Δ-65/-52/+45 *fos* CAT (= -711/ΔCRE/+45 *fos* CAT) promotor constructs. SIS - a responsive element participating in the c-*fos* transcriptional induction by conditioned medium from oncogene v-*sis*-transformed cells (v-*sis* encodes an analog of the PDGF β chain) and by PDGF itself (Wagner et al., 1990); SRE - serum responsive element which mediates the transcriptional induction of c-*fos* in response to a plethora of stimuli such as serum, growth factors, pro-inflammatory cytokines, hormones (e.g. insulin), Ca^{2+}, phorbol esters, oxidative stress, protein synthesis inhibitors, and UV (for review see Nordheim et al., 1994); AP-1 - a sequence with yet undefined functions located immediately downstream of the SRE and bearing a

homology to AP-1 and CREB binding sites; CRE - „-60 CRE" (the proximal cAMP responsive element); TATA - TATA element; +Fp - an intragenic enhancer element (+18/+38) participating in the UV and cAMP inducibility of the promotor (Büscher et al., 1988; Härtig et al., 1991).

B. Hela tk$^-$ cells were transfected with 5 µg -711/+45 *fos* CAT or -711/Δ-65/-52/+45 *fos* CAT promotor constructs as described in Materials and Methods. 5 µg RSV0 (-) or a RSV-driven expression vector coding for a dominant negative CREB mutant (CREBM1) were co-transfected where indicated. The cells were serum starved for 24 hours post-transfection and then stimulated with 30 J/m^2 UVC or forskolin and IMX (10 µM and 0.5 mM) for an additional 24 hours. Relative CAT activities (induction factors) were determined as described in Materials and Methods. Results of three independent repetitions (±SEM) are presented.

When transiently transfected into HeLa cells, the promotor construct -711/+45 *fos* CAT showed 11-fold induction after forskolin and IMX treatment, compared to the basal promotor activity in untreated cells (**fig. 8B**). UVC induced the promotor construct 60 times. This corresponds to the UVC- and cAMP-induced endogenous c-*fos* mRNA levels (not shown) and suggests that most, if not all cAMP and UV responsive elements conferring the transcriptional activation of the endogenous c-*fos* gene are present in the promotor construct. Deletion of the -60 CRE reduced the induction by forskolin and IMX to 5-fold, indicating that this element, although not the only one, is a major CRE in the human c-*fos* promotor (see also Fisch et al., 1989; Härtig et al., 1991). This deletion mutant also manifested a significant reduction in its inducibility by UVC (23-fold vs. 60-fold induction of the wild-type promotor). These results clearly implicate the -60 CRE in the response of the c-*fos* promotor to UVC irradiation.

In order to determine if the UVC induction of c-*fos* through the -60 CRE requires CREB phosphorylation at serine 133, a dominant negative CREB mutant (CREBM1, Gonzales & Montminy, 1989) was overexpressed in HeLa cells and its ability to interfere with the UVC and cAMP induction of c-*fos* promotor CAT constructs (-711/+45 *fos* CAT and -711/Δ-65/-52/+45 *fos* CAT) was investigated. CREBM1 contains a single amino acid substitution (serine 133 to alanine 133). It binds to somatostatin CRE with the same affinity as wild type CREB, but is transcriptionally inactive (Gonzales & Montminy, 1989), thus displaying properties of a dominant negative mutant. CREB binding protein (CBP), which acts as a co-activator of CREB by bridging it to the TFIIB factor of the basal transcriptional machinery (Chrivia et al., 1993; Kwok et al., 1994), binds to CREB only if CREB is phosphorylated at serine 133 (Chrivia et al., 1993). Due to the serine 133 to alanine 133 substitution, CREBM1 cannot be phosphorylated and cannot bind CBP.

Overexpression of CREBM1 inhibited the UVC inducibility of the -711/+45 *fos* CAT promotor construct by more than 50% (28-fold induction in the presence of CREBM1 vs. a 60-fold induction in the absence of CREBM1, **fig. 8B**). This strongly suggests that the participation of the CRE in the transcriptional induction of the c-*fos* gene requires phosphorylation of CREB at serine 133. As expected, the inducibility of the same gene construct by forskolin and IMX was also reduced by more than 50% (5-fold induction in the presence of CREBM1 vs. a 11-fold

induction in the absence of CREBM1). The remaining cAMP inducibility of the promotor construct lacking the -60 CRE (-711/Δ-65/-52/+45 *fos* CAT) was still inhibited by overexpression of CREBM1, suggesting that other promotor elements recognized by CREB mediate at least part of this residual induction (see below; Fisch et al., 1989; Berkowitz et al., 1989; Härtig et al., 1991). However, these other elements addressed by CREB do not seem to contribute to the UV response of the promotor, since the UV inducibility of the -711/Δ-65/-52/+45 *fos* CAT construct is not inhibited by the CREBM1 overexpression (fig. 8B). Parallel transfection experiments, using a -105/-79 HIV TATA CAT promotor construct (Stein et al., 1989a) were performed to prove that CREBM1 specifically interfered only with CREB-dependent but not with CREB-independent transcription. The UVC induction of this promotor construct is mediated by the CREB-unrelated transcription factor NFκB (Stein et al., 1989a). As expected, the -105/-79 HIV TATA CAT promotor construct was induced by UV (4-fold) and the induction was not repressed by overexpression of CREBM1 (fig. 8B).

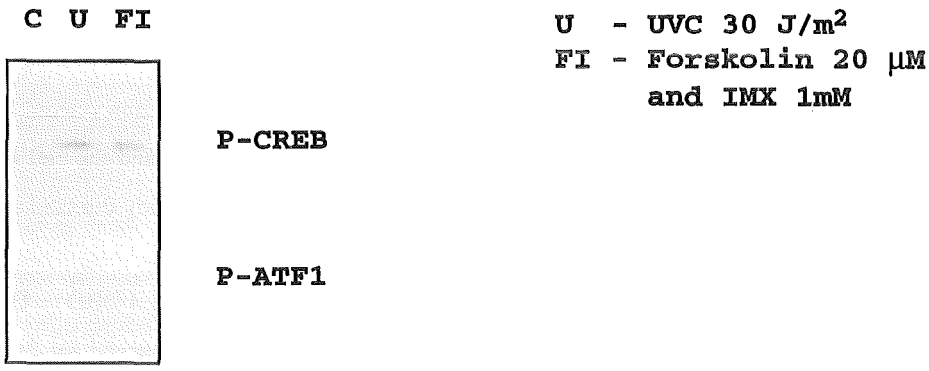
An important outcome of these experiments was that both the cAMP and UVC induction of the -711/+45 *fos* CAT promotor construct were negatively affected by CREBM1, while the cAMP but not the UVC inducibility of the -711/Δ-65/-52/+45 *fos* CAT construct was inhibited by CREBM1. This result shows that the CREB binding sites (CBSs) in the c-*fos* promotor are functionally different in their ability to mediate cAMP or UVC induction of the gene: while more than one CBS contributes to the cAMP inducibility, only the -60 CRE is able to contribute to the UVC response. Berkowitz et al. (1989) have identified two additional CREs in the c-*fos* promotor, which contributed to the cAMP inducibility, though less potently than the -60 CRE: the 5´-CTGCGTCA-3´ motif (positions -295/-288) and the 5´-TCCCGTCA-3´ motif (positions -343/-336). The -295/-288 element binds CREB *in vitro* (Schönthal et al., 1989). Furthermore, microinjection of oligonucleotides representing the c-*fos* -60 CRE abolished the cAMP inducibility of c-*fos* (Berkowitz et al., 1989), suggesting that all three CREs bind the same factor. Thus, the ability of CREBM1 to inhibit the cAMP induction of the -711/Δ-65-52/+45 *fos* CAT promotor construct implies interference of CREBM1 with the binding of the endogenous wild type CREB to the two additional CREs, which take over the cAMP response in the absence of the -60 CRE but are possibly not functional in the presence of the -60 CRE. The observation, however, that CREBM1 did not interfere with the UVC induction of the construct lacking the -60 CRE strongly suggests that the CREs at positions -295/-288 and -343/-336 do not contribute to the UV response of the c-*fos* promotor.

3.2.2. UVC and cAMP, Although They Both Cause CREB Phosphorylation at Serine 133, Differentially Regulate the Transactivation Potential of CREB: the Possible Role of the Co-Activator of CREB, CREB Binding Protein (CBP).

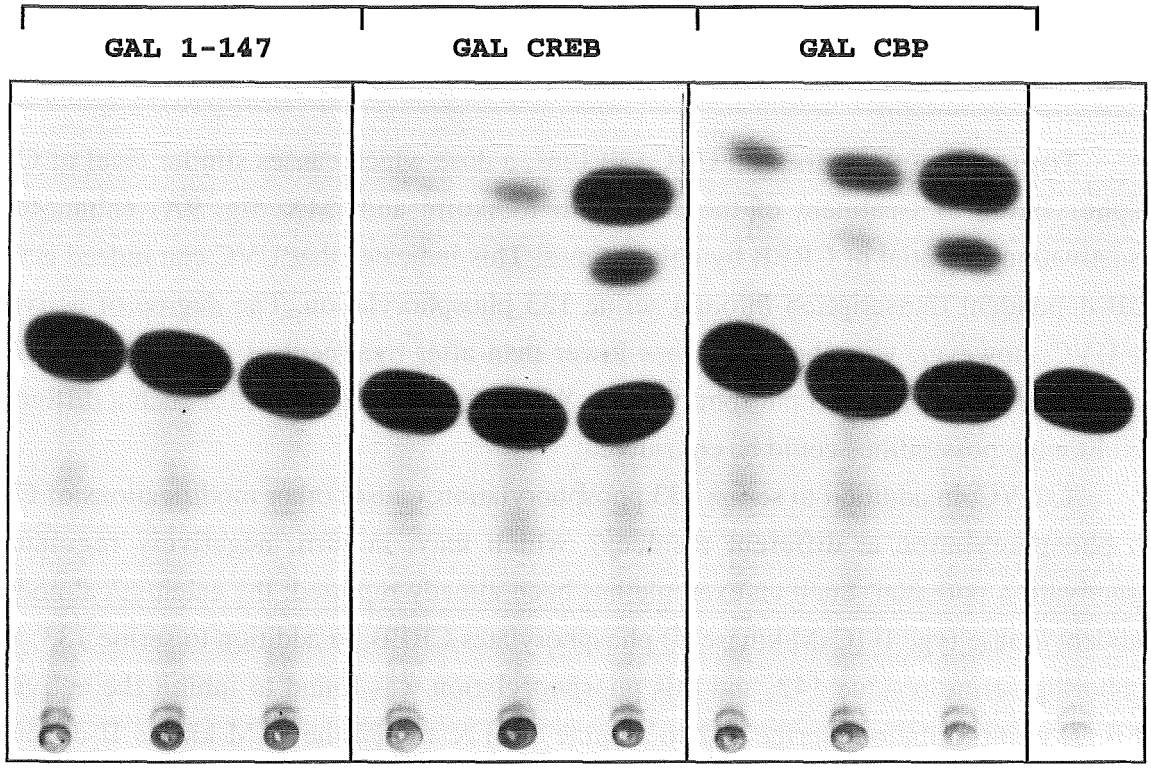
I showed above that CREB participates in the UVC response of a complex promotor such as the c-*fos* promotor. As a logical consequence, the question had to be addressed whether UVC-induced phosphorylation of CREB is sufficient to activate CREB-dependent transcription. This question was provoked by the experiments of Büscher et al. (1988), which suggested that a c-*fos* promotor construct devoid of the SRE and the +18/+38 element, but possessing a functional -60 CRE did not respond to UVC irradiation. To answer this question directly, I investigated the ability of UVC to influence the transactivation potential of CREB under conditions where the transcriptional induction depends only on CREB. Chimeric proteins provide a tool to explore the transactivation capacity of a transcription factor independent of the activity of other interfering factors. In my experiments, they consist of the DNA binding domain (DBD) of the yeast transcriptional activator GAL4 (amino acids 1-147) fused "in frame" with a transcription factor of interest or part of it. GAL4 mediates the induction of different yeast genes by galactose through binding to specific DNA elements (Giniger et al., 1985; Bram et al., 1986). Since mammalian cells are devoid of GAL4 binding activity, the regulatory functions of the fusion protein can be assayed following co-transfection of appropriate cells together with a promotor containing GAL4 binding sites linked to a reporter gene (e.g. CAT). **Figure 9C** shows the structure of the GAL4 DBD and GAL4 ΔCREB proteins used in this study (Hurst et al., 1991, ΔCREB is a naturally occurring splice variant of CREB lacking the α peptide, see Introduction). Since these vectors, transcribed constitutively under the control of the Moloney leukemia virus long terminal repeat (MLV LTR), appear to be poorly expressed in HeLa cells, the experiments were performed in the human chorionic carcinoma cell line JEG-3. **Figure 9A** demonstrates that also in these cells, UVC and cAMP cause phosphorylation of CREB at serine 133 and, to a lower extent, ATF-1 phosphorylation (the low level of ATF-1 phosphorylation may reflect a lower amount of this protein in JEG-3 cells). Importantly, the levels of CREB phosphorylation induced by doses of both stimuli used here and later in the transfection experiments (30 J/m^2 UVC vs 20 μM forskolin and 1mM IMX) were very similar, as detected via an immunoblot assay, suggesting that the right conditions were used to study the transactivation potential of GAL4 CREB after cAMP and UVC stimulation.

When transiently transfected in JEG-3 cells, the GAL4 DBD construct showed no detectable basal or induced transactivation, consistent with the lack of a transactivation domain in the construct (**fig. 9B**). The GAL4 CREB chimeric protein, as expected, manifested a very low level of transactivation in untreated cells which was strongly enhanced upon stimulation of the cells with forskolin and IMX (121 ± 17-fold induction). As phosphorylation at serine 133 is the only known modification of CREB in response to increased cAMP levels, the above result indicates that this phosphorylation event suffices to dramatically induce the transactivation potential of CREB.

A.



B.



C	UV	FI	C	UV	FI	C	UV	FI	UT
-	-	-	1.0	4.8 ±0.5	121 ±17	1.0	2.4 ±0.1	15.3 ±0.1	FOLD INDUCTION

C.

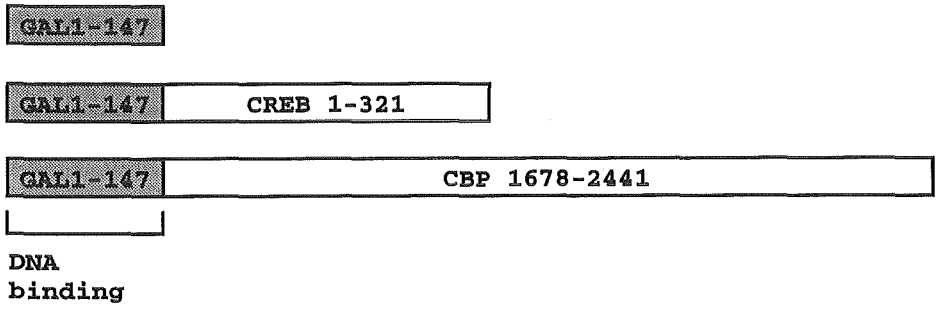


Fig. 9: Although they both cause CREB phosphorylation at serine 133, UVC and cAMP differentially regulate the transactivation potential of CREB.

A. cAMP and UVC cause CREB phosphorylation in JEG-3 human chorionic carcinoma cells. Serum starved JEG-3 cells were treated for 15 min with the indicated doses of forskolin plus IMX or UVC, lysed, and the cell lysates were subjected to an immunoblot assay as described in Materials and Methods, using the antibody directed against CREB phosphorylated at serine 133.

B. JEG-3 cells were transfected with 2 μg GAL4 CAT reporter construct together with 3 μg GAL4 DBD (= GAL 1-147), GAL4 CREB or GAL4 CBP expression vectors as indicated. The cells were serum starved for 24 hours post-transfection and then stimulated with 30 J/m^2 UVC or forskolin and IMX (20 μM and 1 mM) for an additional 36 hours.

C. A schematic representation of the chimeric proteins used in these studies.

Treatment of the cells with UVC (30 J/m^2, a dose which causes similar level of CREB phosphorylation as treatment of the cells with forskolin and IMX, fig. 9A) enhanced the transactivation potential of CREB by only 5 times. This indicates that UVC can indeed activate CREB-dependent transcription through serine 133 phosphorylation. The degree of activation after UVC, however, was about 25 times lower than after treatment of the cells with cAMP despite equal levels of CREB phosphorylation. What might be the reason for such a difference? The following possibilities could be considered:

1. UVC, in addition to serine 133 phosphorylation, causes other modifications of CREB (e.g. phosphorylation at different residues), which may, in turn, negatively regulate the transactivation potential. Such an example has been already reported: the calcium/calmodulin-dependent kinase type II (CaM kinase II) phosphorylates CREB, in addition to serine 133, at the neighboring serine residue 142, and this phosphorylation was found to inhibit the activity of CREB. A point mutation (serine 142 to alanine 142) restricted the CaM kinase II-dependent phosphorylation only to serine 133, and this CREB mutant was strongly activated by CaM kinase II overexpression (Sun et al., 1994).

2. UVC- and cAMP-dependent signal transduction pathways differ in their ability to address the co-activator of CREB, CBP. When fused to the DNA binding domain of GAL4, CBP can activate transcription in a PKA-dependent manner (Chrivia et al., 1993; Kwok et al., 1994). This finding suggests that CREB and its co-activator are *both* regulated in their activity by cAMP, and that this co-regulation is required to achieve maximum transcriptional induction. The PKA-dependence of CBP, however, appears to be indirect, since a point mutation in the only known PKA phosphoacceptor site (serine 1782) failed to inhibit this dependence (Kwok et al., 1994). A GAL4 CREB chimera most likely transactivates through the same mechanism as CREB, namely through binding to CBP. Thus, if UVC, in contrast to cAMP, is not an efficient inducer of CBP activity, the total GAL4 CREB-dependent transcription would be lower.

Although the first possibility was not directly addressed in this study, the results presented below strongly support the second one. Figure 9C shows the structure of a chimeric

protein containing the GAL4 DBD fused to the C-terminal part of CBP (amino acids 1678-2441, Kwok et al., 1994). Transfection experiments in PC12 cells have revealed that when the catalytic subunit of PKA was cotransfected, GAL4 CBP (1678-2441) was strongly activated, indicating that this part of CBP is responsible for the PKA-dependent activation (Kwok et al., 1994). For this reason, the construct was transiently transfected in JEG-3 cells and the ability of cAMP (combined treatment with forskolin and IMX) and UVC to induce a GAL4 CBP-dependent transcription was compared (**fig. 9B**). Forskolin and IMX treatment led to a 15-fold activation of the GAL4 CAT reporter gene. This result clearly shows that the endogenous levels of PKA suffice to increase significantly the transactivation properties of CBP. Again, as in the study with GAL4 CREB, UVC was able to activate GAL4 CBP, but only about 2 times. Thus, the reduced transcriptional activity of GAL4 CREB in response to UVC stimulation is likely to result from the differential capacity of cAMP and UVC to address CBP.

The results described in this chapter clearly show that CREB is an obligatory player in the full UVC response of the c-*fos* promotor. As in the case of the "classical" CREB activator, cAMP, the activation of CREB by UVC is mediated through and depends on the phosphorylation of serine residue 133. It is noteworthy, however, that the transactivation properties of CREB are not equally induced by cAMP and UVC, cAMP being a much stronger inducer. I speculate here that, in JEG-3 cells, this difference is due to the lower potential of UVC to activate CBP, the co-activator of CREB (but see Discussion, 5.2, p. 84).

3.3. SIGNAL TRANSDUCTION PATHWAY(S) LEADING TO UVC-INDUCED CREB PHOSPHORYLATION

3.3.1. Down-Modulation of Signaling Cascades - a Tool to Explore Common Components of Signal Transduction Pathways

Diverse extracellular stimuli (growth factors, peptide hormones, cytokines) involved in changing the genetic program of the cell (e.g. promoting cell proliferation or cell differentiation) exert their action through binding to and activating specific cell-surface receptors with intrinsic protein kinase activity. To date, more than 50 receptor tyrosine kinases (RTKs), which belong to at least 14 receptor families, have been identified (Lemmon & Schlessinger, 1994). Activation of the receptor kinase domain by ligand binding to the extracellular domain leads to downstream activation of multicomponent signal transduction cascades, which in turn leads to the phosphorylation of pre-existing transcription factors and the induced transcription of target genes. Other extracellular stimuli, including many hormones and neurotransmitters, activate signal transduction cascades through receptors without intrinsic kinase activity (e.g. the G-protein-coupled receptors, see Introduction). A common feature of all these signaling cascades is their down-modulation after having been activated by a specific signal, which results in a transient non-responsiveness to subsequent stimulation by the same signal. This down-modulation is caused by exhaustion and/or posttranslational modification (inhibition) of one or more components of the signal transduction chain. During this period of refractoriness, a second

signal that does not use the same components should induce a normal response. If it uses, however, the same component already exhausted by the first stimulation, its ability to "pass" downstream of this component should be affected, as well as the final cellular response (e.g. gene activation). In a more complicated situation, when one or both stimuli use more than one signal transduction pathway and only some of them share common components, prestimulation with the first signal should only partially impair the response to the second signal.

With the idea in mind that UVC radiation, in order to induce gene expression, makes use of signal transduction pathways committed primarily to promoting cellular responses to physiological stimuli, the experimental approach of down-modulation of signaling cascades has been used to determine which cascade is used by UVC. Büscher et al. (1988) showed that down-modulation of protein kinase C (PKC)-dependent signal transduction by pre-treatment of cells with TPA abolishes the responsiveness of the c-*fos* gene to a second TPA treatment, but not to UVC. This led to the conclusion that PKC-dependent signal transduction is not involved in mediating the UVC response (Büscher et al., 1988). Using the same approach, Sachsenmaier et al. (1994) observed that pre-treatment of HeLa cells with a single growth factor (EGF, IL-1α or bFGF) or a mixture of them interferes strongly with UVC-induced activation of p42 MAP kinase and c-*fos* transcription, suggesting that these growth factors and UVC share common signal transduction pathways.

The finding that CREB is a UVC-activated transcription factor raised the problem of which mechanisms and signal transduction cascades are involved in mediating this activation. The following possibilities for UVC-induced signal transduction to CREB could be considered:

1. cAMP-dependent signal transduction: UVC, absorbed by certain cellular (presumably membrane) components, activates adenylyl cyclase, thus increasing the intracellular cAMP levels. cAMP binds to the regulatory subunit (R-subunit) of protein kinase A (PKA) and stimulates the release of the catalytic subunit (C-subunit) which, in the absence of cAMP, is sequestered in the cytoplasm by the regulatory subunit. The released catalytic subunit in turn migrates into the nucleus and phosphorylates CREB at serine residue 133.

2. Signal transduction through routes used by growth factors: UVC makes use of the signaling cascades activated by growth factors (for instance by those which themselves induce CREB phosphorylation). This mechanism has been postulated to explain the UVC-induced p42 MAP kinase activation, the modification of Elk-1 and c-*fos* induction through the serum responsive promotor element (Sachsenmaier et al., 1994).

3. Signal transduction through another "second messenger" (e.g. Ca^{2+}) triggered by UVC.

The experimental approach to down-modulate certain signal transduction chains was chosen to answer the question of which of these possibilities would account for the UVC-induced activation of CREB and CREB-dependent transcription. For this purpose, HeLa cells, which appeared to respond with CREB phosphorylation to a large variety of stimuli, were pre-

treated with forskolin and IMX, individual growth factors (EGF, IL-1α or bFGF), or combinations of them, TPA, or the Ca^{2+}-elevating agents A23187 and thapsigargin, and the ability of UVC to induce CREB serine 133 phosphorylation after such a pre-treatment was studied in immunoblot assays. It was important to compare the responses of CREB after prestimulation and of another UVC-induced endpoint whose behavior toward this prestimulation was already known. The activation of p42 MAP kinase fulfilled this criterion, since it has been used to establish the dependence of the UVC signaling to the c-*fos* promotor on activation of growth factor receptors (Sachsenmaier et al., 1994). The ability of UVC pre-treatment to interfere with CREB phosphorylation in response to a second stimulus different from UVC was also studied.

3.3.2. Down-Modulation of the Adenylyl Cyclase-Dependent Signal Transduction Pathway Does Not Interfere with UVC-Induced CREB Phosphorylation

The adenylyl cyclases are plasma membrane-associated enzymes that convert ATP to a "second messenger" molecule, cyclic AMP, in response to hormones, neurotransmitters and other regulatory molecules (reviewed by Tang & Gilman, 1992; Choi et al., 1993). Activation of adenylyl cyclases is mainly triggered by binding of an extracellular signaling molecule ("first messenger") to specific receptors with seven transmembrane domains, coupled to trimeric guanine nucleotide-binding regulatory proteins (G proteins) (reviewed by Strader et al., 1994; Conklin & Bourne, 1993). Conformational changes in the receptor upon ligand binding are thought to induce dissociation of the G α-subunit from the β/γ subunit complex, which leads to activation of the adenylyl cyclases and production of cAMP. This "second messenger" molecule activates the cAMP-dependent protein kinase (PKA) through binding to its regulatory subunit and allowing the release of the catalytic subunit and its migration into the nucleus (Nigg et al., 1985). The appearance of the catalytic subunit in the nucleus was found to account for cAMP-induced transcription through cAMP responsive enhancer elements (Riabowol et al., 1988). Consistent with this idea, Gonzales & Montminy (1989) identified CREB as a direct target for the catalytic subunit of PKA. It was, therefore, possible to propose a mechanism for the UVC-induced CREB phosphorylation, in which UVC absorbed by certain membrane or peri-membrane components would lead to activation of G protein-coupled receptors and/or adenylyl cyclase. In this case UVC would trigger the same signal transduction pathway as that initiated by physiological stimuli (e.g. hormones and neurotransmitters) elevating the cellular cAMP levels. In order to examine this hypothesis, I pre-treated HeLa cells for 4 hours with forskolin and IMX, and then applied a second treatment with the same agents or with UVC. The phosphorylation of CREB was monitored in an immunoblot assay using the antibody directed against phosphorylated CREB. Forskolin is a direct activator of the mammalian adenylyl cyclases (de Souza et al., 1983). IMX, in turn, inhibits phosphodiesterases that convert cAMP to AMP, thus increasing the cAMP half-life in the cytoplasm (Beavo et al., 1970). If the adenylyl cyclase or a component of the signal transduction pathway downstream of the cyclase is down-

modulated by the first treatment, the endpoint of the signal transduction cascade (CREB) should not respond to a second treatment with forskolin and IMX. This is demonstrated in **fig. 10**.

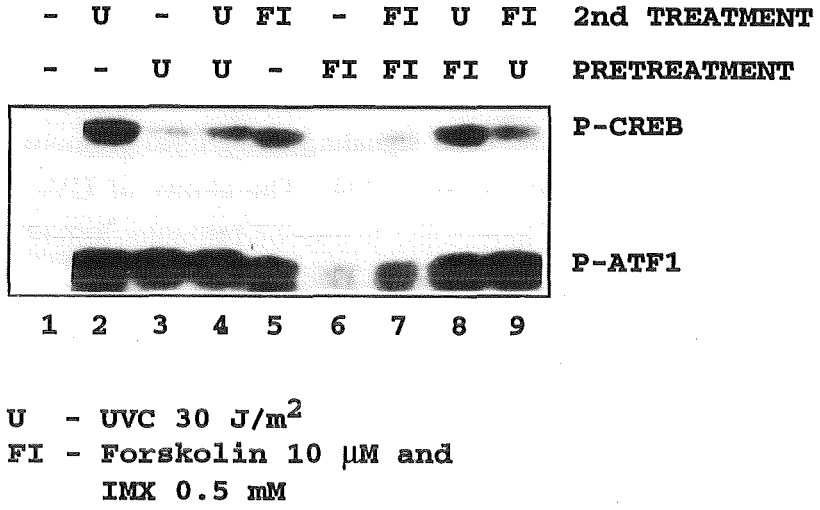


Fig. 10: Down-modulation of the adenylyl cyclase-dependent signal transduction pathway does not interfere with UVC-induced CREB phosphorylation. HeLa tk⁻ were pre-treated with UVC, forskolin and IMX, or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either UVC or forskolin and IMX for 15 min. The cell lysates were analysed using the antibody directed against phosphorylated CREB.

Application of forskolin and IMX for 15 min induced phosphorylation of CREB (lane 5). Four hours after the stimulation, the phosphorylation was already attenuated (lane 6). Two consecutive events have been found to account for the attenuation of CREB phosphorylation (Hagivara et al., 1992). First, following the dissociation of ligand or receptor down-modulation, cAMP levels drop and the catalytic subunit of PKA is inactivated through binding to the regulatory subunit. Second, this drop in PKA activity results in a net increase in phosphatase activity, leading to dephosphorylation of CREB. Most available data indicate that protein phosphatase 1 (PP-1) is the important CREB-phosphatase (Hagiwara et al., 1992). When forskolin and IMX were applied for a second time for 15 min after a 4-hour prestimulation of the cells with the same stimuli, a very weak re-stimulation of CREB phosphorylation was detectable (lane 7). This indicated that, under the experimental conditions used, the adenylyl cyclase-dependent signal transduction pathway was indeed down-modulated by the pre-stimulation with forskolin and IMX.

Similarly, UVC irradiation caused phosphorylation of CREB within 15 min (lane 2), which significantly decreased 4 hours after irradiation (lane 3). Again, as in the case of cAMP stimulation, a second UVC treatment led to much lower levels of CREB phosphorylation (lane 4), suggesting successful down-modulation of the UVC-induced signal transduction to CREB.

Thus, both increased cAMP and UVC showed the predicted down-modulation of their own signal transduction pathways. When, however, UVC irradiation was applied after a 4-hour pre-treatment with forskolin and IMX, the phosphorylation of CREB was equally well manifested as when the cells were subjected to UVC as a single inducer (lane 8). This result strongly suggests that forskolin and IMX, on the one hand, and UVC on the other, do not have common components of the signal transduction cascades downstream of the adenylyl cyclase. There remained a possibility, however, that forskolin pre-treatment down-modulates the cyclase, but not PKA. Then, if UVC would induce dissociation of the PKA catalytic subunit from the regulatory subunit in a cAMP-independent manner, the same result as in fig. 10 would be observed, although both stimuli act through the same kinase, namely PKA. This possibility is, however, not very likely since no evidence exists so far for a cAMP-independent PKA activation. Indeed, further studies showed that a kinase different from PKA is responsible for the UVC-induced CREB phosphorylation (see **3.5**). It is, however, possible that the UVC pre-treatment may indeed down-modulate the adenylyl cyclase, since cells pre-treated with UVC displayed lower levels of CREB phosphorylation in response to forskolin and IMX given as a second stimilus (lane 10). Most likely, this down-modulation reflects some unspecific actions of UVC at the plasma membrane. It will be, for instance, shown, in *3.3.3.(i)* (fig. 12), that UVC causes down-modulation of another class of plasma membrane-associated enzymes, namely growth factor receptor tyrosine kinases, as demonstrated for the EGF receptor.

3.3.3. UVC, Although it Feeds Into Certain Growth Factor-Dependent Signal Transduction Pathways to Activate p42 MAP Kinase, Uses Different Routes to Induce CREB Phosphorylation

The ability of serum and certain individual growth factors to induce transcriptional activation of the c-*fos* gene correlates temporally and functionally with the phosphorylation of a transcription factor, Elk-1 (Zinck et al., 1993), which binds together with the serum response factor (SRF) to the serum response element (SRE) in the c-*fos* promotor (reviewed by Treisman, 1994). Both *in vitro* and *in vivo* evidence exists that MAP kinases (p42/p44) are responsible for the Elk-1 phosphorylation and c-*fos* activation after serum or growth factor stimulation (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993). In HeLa cells, a part of the UVC response which leads to c-*fos* transcriptional activation via the serum responsive element appeared also to require p42 MAP kinase activation, since overexpression of a dominant negative mutant of this kinase hampered the UVC induction of a -711/+45 *fos* CAT promotor construct (Sachsenmaier et al., 1994). Furthermore, activation of p42 MAP kinase by UVC in these cells was found to occur through signal transduction pathways used by several growth factors, among them EGF, IL-1α and bFGF (Sachsenmaier et al., 1994). On the other hand, as shown above (**3.2.1**), CREB and the -60 CRE cooperate with the SRE in the transcriptional induction of the c-*fos* gene by UVC. Thus, the question arose whether UVC uses the same growth factor-dependent signal transduction pathways to induce CREB phosphorylation as for the activation of p42 MAP kinase and Elk-1. This question was addressed by the following four independent approaches:

(i) Specific down-modulation of growth factor-dependent signaling cascades. In this case the ability of a growth factor pre-treatment of HeLa cells to interfere with the UVC signaling to CREB and p42 MAP kinase was examined.

(ii) Inhibition of growth factor receptors by the receptor blocker suramin. The polyanion suramin blocks the binding of growth factors to their membrane receptors and therefore inhibits growth factor-dependent signal transduction (Bestholtz et al., 1986; Coffey et al., 1987; Fantini et al., 1989). The activation of p42 MAP kinase by UVC was found to be suramin-inhibitable, suggesting involvement of growth factor receptors in this process (Sachsenmaier et al., 1994). Here the effects of suramin on both UVC- and growth factor-induced CREB and p42 MAP kinase activation were studied in parallel.

(iii) Inhibition of tyrosine kinase activity by tyrphostin-51. In this case, the ability of an inhibitor of tyrosine kinases which are implicated in mediating the cellular response to growth factors, to interfere with UVC- and EGF-induced CREB phosphorylation was investigated.

(iv) Third stimulus interference. If a certain nuclear endpoint for signal transduction is addressed by three extracellular stimuli (A, B, and C) and if pre-treatment of the cells with stimulus A makes the endpoint non-responsive to a second stimulation with A and B but not to a second stimulation with C, this implies that stimuli B and C use different signal transduction pathways to reach the endpoint. This rationale has been used to show that UVC and phorbol esters, although using the same promotor element to induce c-*fos* transcription (SRE), act via independent mechanisms. In that case a third inducer, increased cytoplasmic Ca^{2+} levels, appeared to interfere with UVC- but not with phorbol ester-induced c-*fos* expression (Büscher et al., 1988). Here, I compared the ability of phorbol ester (TPA) pre-treatment to affect UVC- and growth factor-induced CREB phosphorylation.

All four experimental approaches used led to the conclusion that UVC-induced CREB and p42 MAP kinase activations are separable events and follow different signal transduction routes.

3.3.3.(i). Down-modulation of growth factor-dependent signaling cascades hampers the UVC responsiveness of p42 MAP kinase but not of CREB

Epidermal Growth Factor. First, the ability of a single growth factor (EGF) pre-treatment to interfere with the UVC signaling to CREB and to p42 MAP kinase were compared. As before, CREB activation was monitored in an immunoblot assay, using the antibody directed against phosphorylated CREB. Activation of p42 MAP kinase was detected in an immunoblot assay by the appearance of a slower migrating form of the kinase (mobility shift) (Leevers & Marshall, 1992), indicative of phosphorylation at both threonine and tyrosine residues within the peptide TGFLT*EY*VATR (Payne et al., 1991). This dual phosphorylation is essential for the activation of both p42 and p44 forms of MAP kinases (for review see Marshall, 1994). When serum-starved HeLa cells were subjected to UVC or EGF stimulation for 15 min, both stimuli induced phosphorylation of CREB and activation of p42 MAP kinase (**fig. 11A**, lanes 3 and 6). After application for 4 hours, both UVC- and EGF-induced effects were attenuated (lanes 4 and

7). Both CREB and p42 MAP kinase were non-responsive to a second stimulation with the same signal (lanes 5 and 8). Thus, at the level of the two endpoints (CREB and p42 MAP kinase) UVC and EGF can down-modulate their own signal transduction pathways.

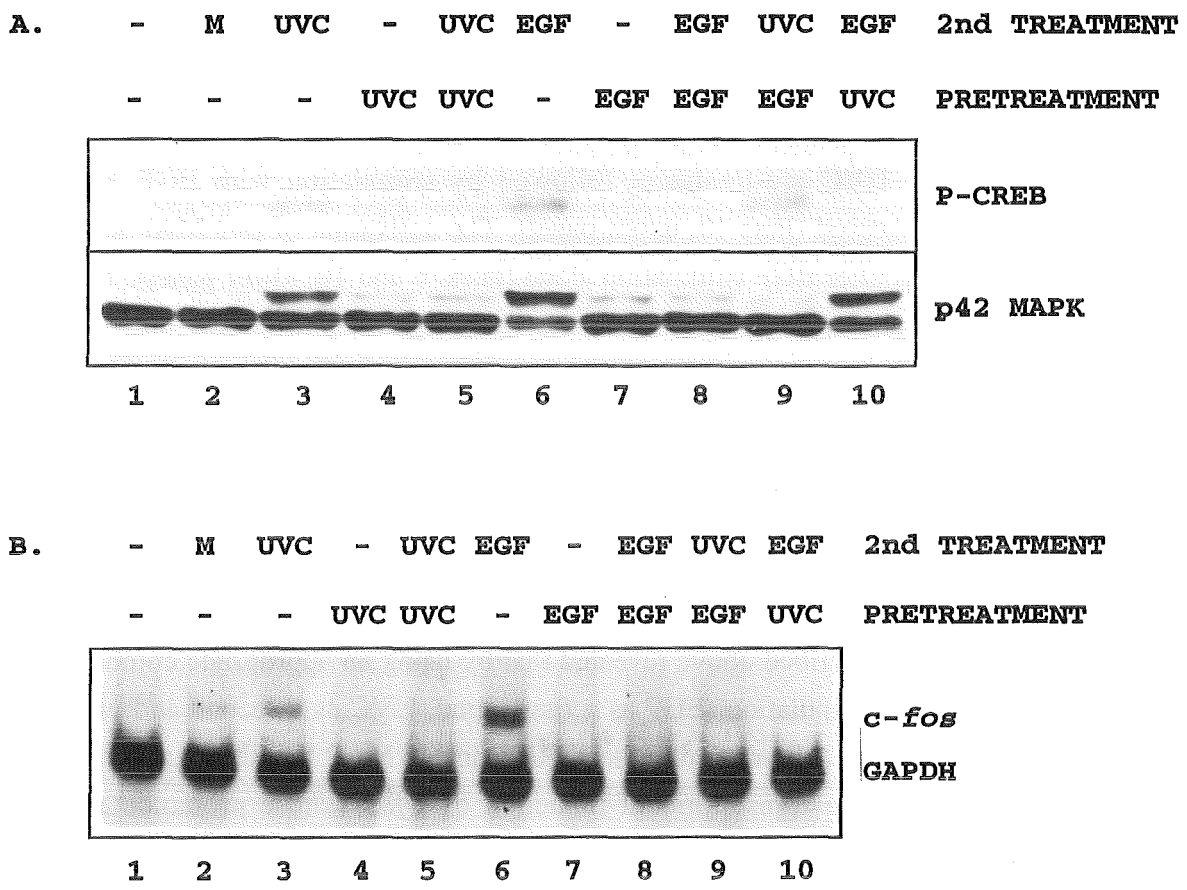


Fig. 11: Down-modulation of the EGF receptor-induced signal transduction pathways hampers the UVC responsiveness of p42 MAP kinase but not of CREB.

A. HeLa tk^- cells were pre-treated with UVC (30 J/m^2), EGF (20 ng/ml), or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either UVC (30 J/m^2) or EGF 20 (ng/ml) for 15 min. The cell lysates were analysed in immunoblot assays using the antibody directed against phosphorylated CREB (upper panel) or using the p42 MAP kinase specific antibody αIcp^{42} (lower panel).

B. Analysis of the c-*fos* mRNA accumulation in a parallel experiment. HeLa tk^- cells were treated as in **A.**, except that the cells were harvested and $polyA^+$ mRNA was prepared 30 min after the second treatment. 5 µg $polyA^+$ mRNA were analysed in Northern blot hybridisation using radioactively labeled c-*fos*- and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a control for the amount of RNA loaded)-specific DNA probes as described in Materials and Methods.

However, in the case of EGF pre-treatment and subsequent UVC stimulation, the same level of CREB phosphorylation was observed as after a single UVC treatment (lanes 3 and 9, upper panel). At the same time, no p42 MAP kinase activation was detected (lanes 3 and 9,

lower panel). This indicates that after pre-treatment with EGF both EGF-induced signal transduction pathways, the pathway leading to CREB phosphorylation and the pathway leading to p42 MAP kinase activation, are down-modulated. In contrast, only the UVC-induced pathway leading to p42 MAP kinase activation is down-modulated in cells pre-treated with EGF, but not the pathway leading to CREB phosphorylation. This suggests that UVC and EGF, in order to induce p42 MAP kinase activation, use a common molecule which is down-modulated by EGF pre-treatment. This is not the case with respect to CREB phosphorylation, indicating that the two pathways are distinct.

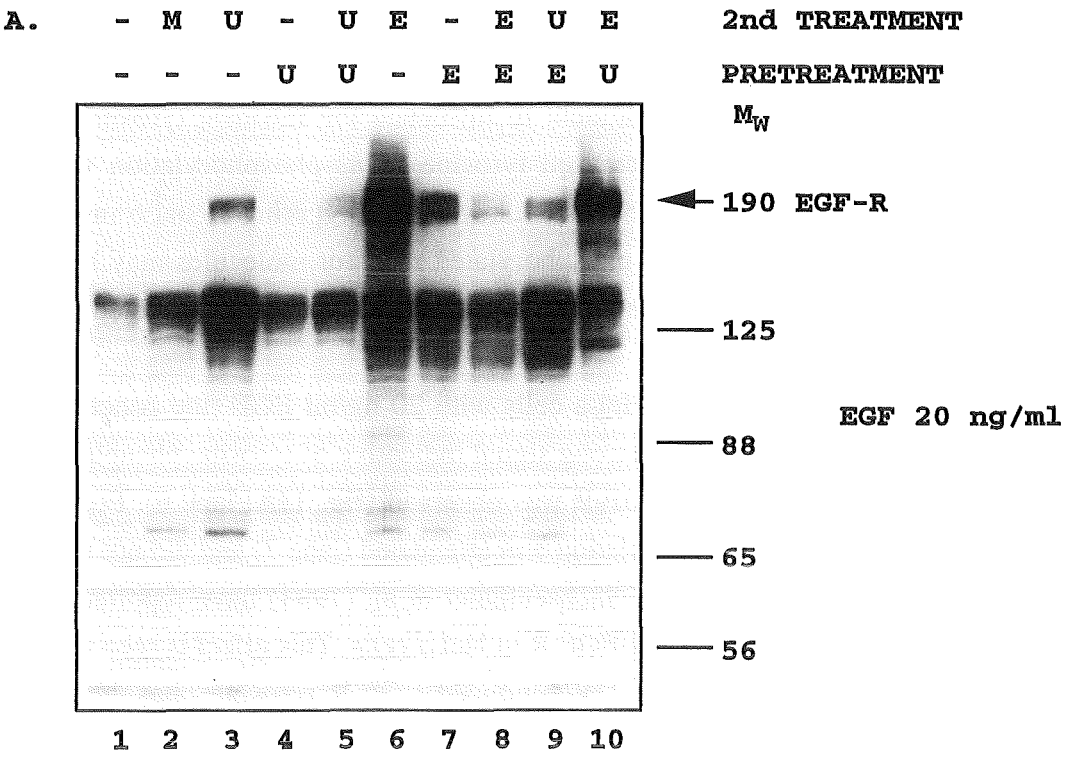
In the case of UVC pre-treatment followed by stimulation with EGF, no CREB phosphorylation was observed (lane 10, upper panel). The activation of p42 MAP kinase was almost as pronounced as after EGF stimulation alone (lanes 6 and 10, lower panel). This result suggests that UVC is able to down-modulate the EGF signaling to CREB but, under the experimental conditions used, not to p42 MAP kinase. Taken together, the results of the study support the model of the UVC-induced p42 MAP kinase activation proposed by Sachsenmaier et al. (1994). According to that model, in HeLa cells, UVC activates p42 MAP kinase and its direct substrate Elk-1 by activating growth factor receptors, especially the EGF receptor. My results, however, strongly argue against the ability of that model to explain the UVC-induced phosphorylation of CREB.

The experiment described above revealed that by using an appropriate combination of pre-treatment and second stimulation (EGF as a first signal, UVC as a second and *vice versa*) preferential and independent activation of either CREB or p42 MAP kinase might be achieved. How does this influence the transcriptional induction of c-*fos*, whose promotor activity is controlled by both endpoints (p42 MAP kinase - Elk-1 and CREB)? To answer this question, the ability of UVC and EGF to induce c-*fos* mRNA accumulation under conditions of pre-treatment with either of those stimuli was investigated in a Northern blot assay. Both UVC and EGF induced c-*fos* transcription when applied for 30 min (**fig. 11B**, lanes 3 and 6), but failed to do so a second time after a 4-hour pre-treatment with the same agent (lanes 4 and 7). When the cells were pre-treated with either EGF or UVC and then subsequently stimulated with the heterologous inducer, the inducibility of the gene was partially restored, though to much lower levels (lanes 9 and 10; in lane 10 the induction can be seen only after a longer film exposure, not shown). This observation supports the conclusion that for the full responsiveness of c-*fos* to EGF and UVC both MAP kinase(s) activation and CREB phosphorylation are required. This notion is also in agreement with the role of CREB and the -60 CRE in the UVC inducibility of the c-*fos* promotor (see **3.2.1**).

How does UVC pre-treatment prevent the EGF-induced CREB phosphorylation? Several signal transduction components found to be rapidly affected by UVC (e.g. growth factor receptors, Sachsenmaier et al., 1994 or p21 Ras, Devary et al., 1992, 1993) are located in the plasma membrane or at its inner surface, assuming that certain membrane constituents may serve as primary targets of UVC absorption. It is therefore reasonable to suggest that some of these components might be down-modulated (in a specific or non-specific way) upon contact

with UVC. In the case of EGF signaling, the EGF receptor (EGF-R) is the most upstream member of the transduction pathway which may be down-modulated by UVC. To prove whether this is the case, the ability of UVC pre-treatment to influence the intrinsic tyrosine kinase activity of the receptor was examined. Increased tyrosine *trans*-phosphorylation within a receptor dimer after ligand binding is thought to be a key event triggering signal transduction (reviewed by Schlessinger, 1993). Tyrosine phosphorylated receptor molecules may be easily detected in an immunoblot assay using whole cell lysates and antibodies directed against phosphotyrosine residues.

Fifteen minutes after treatment of HeLa cells with 20 ng/ml EGF (the concentration used in the experiments shown in fig. 11), a strong signal indicative of tyrosine phosphorylated protein with the characteristic size of the EGF-R was observed (**fig. 12A**, lane 6). Four hours after EGF stimulation, the tyrosine phosphorylation was attenuated (lane 7) and did not reappear upon repeated treatment with EGF (lane 8). Sachsenmaier et al. (1994) have reported on the UVC-induced tyrosine phosphorylation of the EGF-R in HeLa cells. This effect is seen in lane 3 (15 min UVC). It has to be noted that the UVC-induced EGF-R tyrosine phosphorylation requires doses of UVC much higher than those used in this work (Sachsenmaier et al., 1994). Consistently, in most of the repetitions of this experiment, the UVC-induced EGF-R tyrosine phosphorylation was not detectable at 30 J/m^2 (see, for instance, fig. 12B). Pre-treatment of the cells for 4 hours with UVC had, in itself, no detectable effect on the EGF-R phosphorylation (lane 4), but caused slightly reduced responsiveness of the receptor to a second stimulation with EGF (lane 10). Bearing in mind that the concentration of EGF used ($3x10^{-9}$ M) was possibly saturating and therefore did not allow significant manifestation of possible UVC effects, the same experiment was repeated with a lower dose of EGF (2 ng/ml, $3x10^{-10}$ M, **fig. 12B**). As in the previous experiment, 15 min after the stimulation with EGF, its receptor displayed well detectable tyrosine phosphorylation (lane 6), attenuated after 4 hours (lane 7), and was not responsive to second EGF application (lane 8). In this case, however, UVC pre-treatment also significantly reduced the EGF-induced receptor phosphorylation (lane 10). Thus, the EGF-R appeared to be the first point at which UVC interferes with the EGF signaling cascade and the interference of UVC with EGF (and *vice versa*) can be explained by mutual down-modulation of the EGF-R. After EGF-binding, the receptor undergoes ligand-induced down-regulation, a process that involves receptor internalization, endocytotic targeting and lysosomal degradation (Wiley et al., 1991 and references therein). It is not clear whether the UVC-dependent reduction of the EGF-induced tyrosine phosphorylation of the EGF-R reflects similar processes.



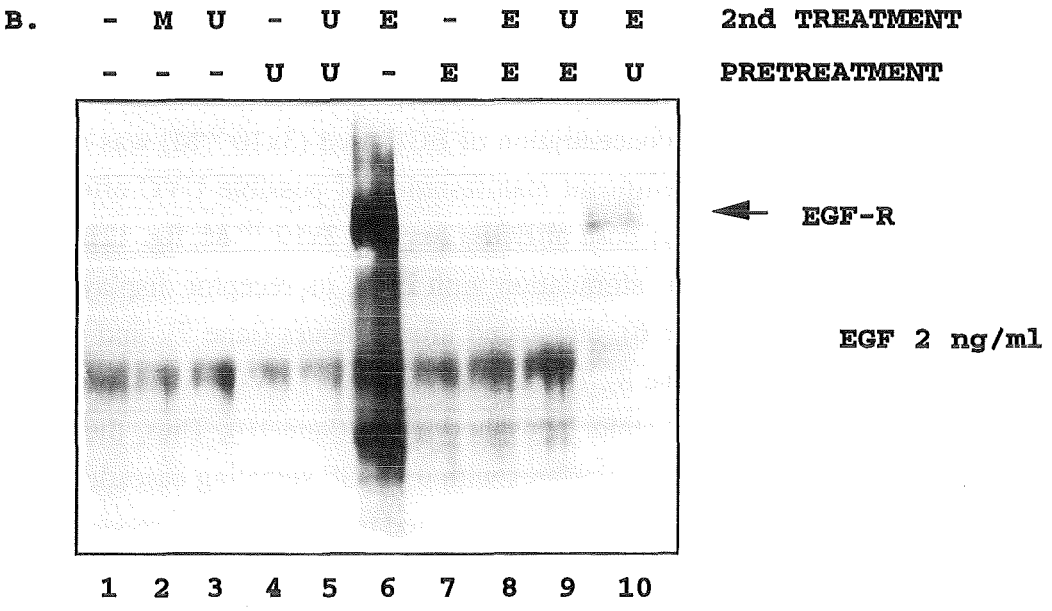


Fig. 12: Pre-treatment of cells with UVC interferes with the EGF-induced tyrosine phosphorylation of the EGF receptor.

A. HeLa tk^- cells were pre-treated with UVC (30 J/m^2), EGF 20 (ng/ml), or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either UVC (30 J/m^2) or EGF (20 ng/ml) for 15 min. Whole cell lysates were analysed in an immunoblot assay using a phosphotyrosine-specific antibody (PY20). The band corresponding to the EGF-R is indicated by an arrow.

B. Same design as in **A.**, except that 2 ng/ml EGF was used.

Combination of growth factors. In order to answer the question whether down-modulation of multiple growth factor-dependent signal transduction pathways interferes with the UVC-induced CREB phosphorylation, I investigated the ability of simultaneous application of three growth factors (EGF, IL-1α, and bFGF) to interfere with the UVC signaling to CREB and to p42 MAP kinase. The cytokine IL-1α induces both CREB serine 133 phosphorylation (fig. 7) and p42 MAP kinase activation (Guesdon et al., 1993), and similar effects have been reported about bFGF in HeLa cells (fig. 7 and Sachsenmaier et al., 1994). Simultaneous prestimulation of HeLa cells with all three growth factors was found to reduce strongly the responsiveness of c-*fos* to UVC (Sachsenmaier et al., 1994). **Figure 13** shows that when HeLa cells were treated for 15 min with UVC or with the combination of EGF, IL-1α, and bFGF (Σ), both stimuli led to CREB phosphorylation and to p42 MAP kinase activation (lanes 3 and 6).

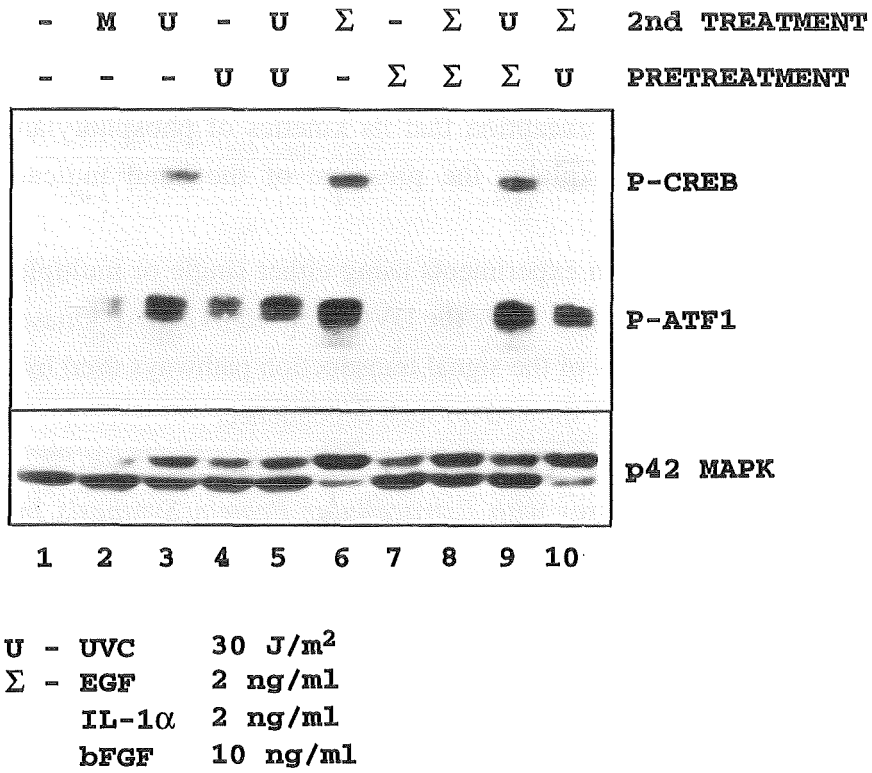


Fig. 13: The UVC-induced phosphorylation of CREB is not affected by pre-treatment of the cells with a combination of growth factors. HeLa tk⁻ cells were pre-treated with UVC, with a combination of three growth factors (EGF, IL-1α, and bFGF, Σ), or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either UVC or Σ for 15 min. The cell lysates were analysed in immunoblot assays using the antibody directed against phosphorylated CREB (upper panel) or using the p42 MAP kinase specific antibody (lower panel).

Pre-treatment with growth factors for 4 hours completely abolished the responsiveness of CREB to the same agents (lane 8, upper panel). The p42 MAP kinase could also be only

weakly restimulated (lane 8, lower panel). This indicated that at least the growth factor-dependent signal transduction pathway to CREB was efficiently down-modulated. Growth factor pre-treatment, however, did not at all affect the ability of UVC to induce CREB phosphorylation (lane 9). Thus, it appeared that even down-modulation of multiple growth factor-dependent signal transduction pathways failed to interfere with the UVC-induced CREB phosphorylation. In this experiment, the analysis of p42 MAP kinase was not possible, because after 4 hours of growth factor pre-treatment the remaining level of activated kinase was still high and comparable to the level of activated kinase seen after 15 min UVC irradiation (lanes 3 and 7). However, as in the case of EGF-pre-treatment, cells pre-treated with a combination of three growth factors showed no further p42 MAP kinase activation in response to UVC (lane 9), again suggesting that UVC-induced CREB phosphorylation and UVC-induced p42 MAP kinase activation follow different pathways. Conversely, when the cells were pre-treated with UVC and then stimulated with EGF, IL-1α, and bFGF, no CREB phosphorylation but full response of the p42 MAP kinase was observed (lane 10), suggesting that UVC pre-treatment down-modulates the growth factor signaling to CREB but not to p42 MAP kinase. The behavior of ATF-1 was similar to that of CREB with the exception that its kinetics of phosphorylation after UVC treatment was prolonged. This explains the presence of the band indicative of ATF-1 phosphorylation in lane 10.

It is worth noting that both experiments using either single or multiple growth factor pre-treatment led to identical outcomes: while the UVC-induced p42 MAP kinase activation was entirely dependent on the intactness of the signal transduction pathways used by certain growth factors, the UVC signaling to CREB showed no such dependence.

3.3.3.(ii). Suramin interferes with Extracellular Protein Synthesis Inducing Factor (EPIF)- and TNF-α-, but not with UVC-induced CREB phosphorylation

Irradiation of human fibroblasts and HeLa cells with UVC leads to the synthesis and secretion of a factor (or a group of factors) which, when applied to nonirradiated cells, cause effects reminiscent of those induced by direct UVC irradiation, namely induction of several UVC-inducible genes (collagenase I gene, HIV-1, metallothionein IIA) (Schorpp et al., 1984; Stein et al., 1989a). This factor was termed Extracellular Protein Synthesis Inducing Factor (EPIF, Schorpp et al., 1984). Krämer et al. (1993) identified two components of EPIF from UVC-irradiated HeLa cells: the cytokine interleukin-1α and basic fibroblast growth factor. Interestingly, these EPIF components appeared to stimulate CREB serine 133 phosphorylation (**fig. 7**). Furthermore, both EPIF- and UVC-induced collagenase I transcription is efficiently inhibited by suramin, indicating that UVC-induced growth factor synthesis and release mediate part of the UVC response (Krämer et al., 1993). Intriguingly, suramin was also found to interfere with some early UVC-induced events (e.g. c-*fos* and c-*jun* transcription, p42 MAP kinase activation, Elk-1 DNA binding activity, and EGF-R tyrosine phosphorylation) which, because of their fast appearance, could hardly result from a UVC-induced synthesis and release of growth factors (Sachsenmaier et al., 1994). Although, in this case, the exact mechanism of the action of

suramin was not clear, appropriate controls indicated that suramin did not affect proteins on the inner side of the plasma membrane. Hence, the conclusion was made that both late and early UVC responses are triggered by activation of suramin-sensitive components which most likely represent growth factor receptors.

In view of this, the ability of suramin to interfere with UVC- and growth factor-induced CREB phosphorylation was tested in an immunoblot assay. As before, CREB phosphorylation was investigated in parallel with the activation of p42 MAP kinase. HeLa cells were pre-treated with suramin for 45 min and then subjected to UVC, TNF-α or EPIF stimulation (EPIF represented culture medium from HeLa cells which were irradiated with UVC and allowed to express and secrete growth factors for 48 hours). **Figure 14** shows that all stimuli induced CREB and ATF-1 phosphorylation (fig. 14A) and p42 MAP kinase activation (fig. 14B).

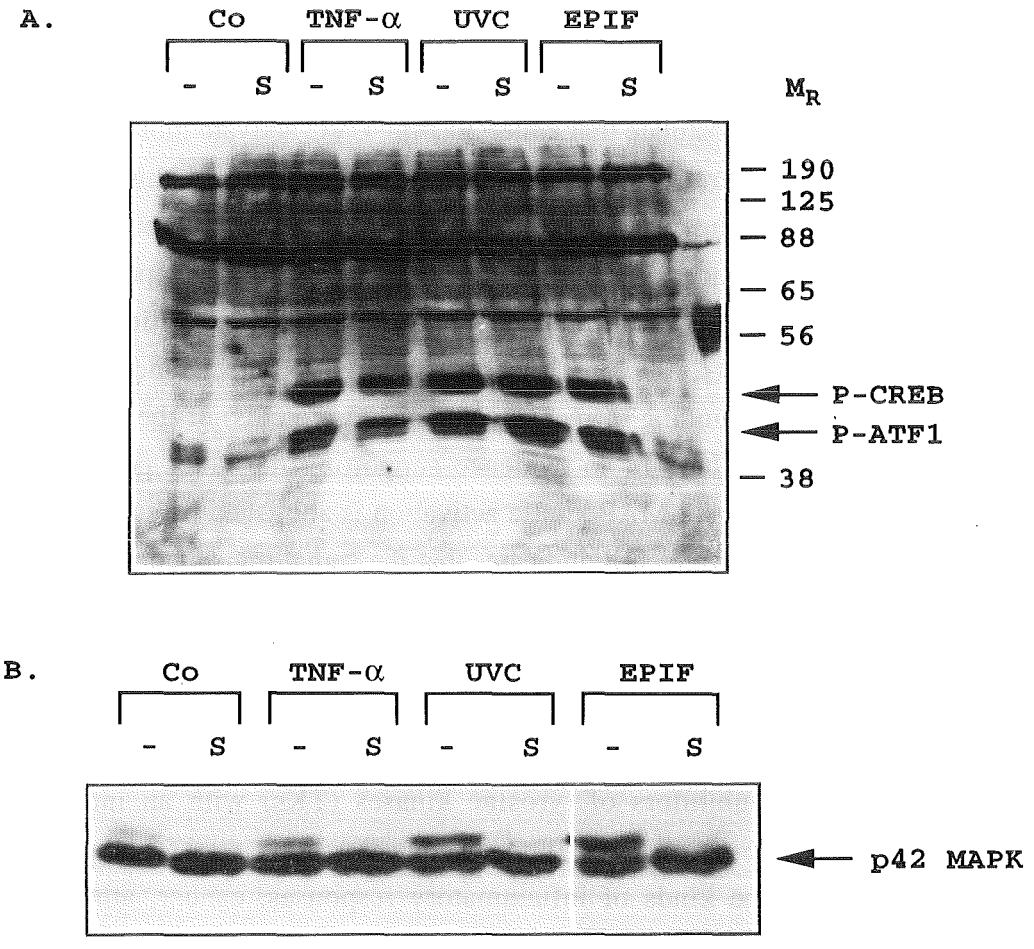


Fig. 14: Suramin interferes with EPIF- and TNF-α-, but not with UVC-induced CREB phosphorylation.

A. HeLa tk^- cells were preincubated where indicated (S) with suramin (0.3 mM) for 45 min and then treated with TNF-α (5 ng/ml), UVC (30 J/m^2), or conditioned medium from HeLa tk^- cells irradiated with UVC (30 J/m^2) and allowed to synthesize and secrete EPIF for 48 hours (EPIF medium). 15 min after the treatment the cells were lysed and the cell lysates were analysed in an immunoblot assay using the antibody directed against phosphorylated CREB.

B. The same cell lysates were used to monitor the effect of suramin on p42 MAP kinase activation.
(The experiment presented here was performed in co-operation with K. Bender, Institut für Genetik, Forschungszentrum Karlsruhe)

As expected, preincubation of the cells with suramin completely blocked the response of p42 MAP kinase to TNF-α and EPIF. The reactions of CREB and ATF-1 were very similar - suramin abolished the EPIF-induced phosphorylation and reduced the responses of these transcription factors to TNF-α. The induction of both endpoints by UVC, however, was differentially affected by suramin: while the activation of p42 MAP kinase was completely blocked, in several experiments no inhibition of CREB and ATF-1 phosphorylation was observed. When interpreting these results the following consideration should be taken into account: *first*, EPIF is a multicomponent mixture of growth factors which exert their action through activation of their receptors, and *second*, the receptor activation upon EPIF stimulation is suramin-inhibitable (fig. 14). Hence, if UVC induces CREB phosphorylation through growth factor receptors, one should postulate that either 30 J/m^2 UVC activate more receptor molecules than EPIF, so that the concentration of suramin is not sufficient to block this process, or that the UVC-induced CREB phosphorylation is mediated through one or more suramin-resistant receptors and these receptors are not activated by EPIF. The former assumption is very unlikely, since very high doses of UVC are needed to obtain functional activation of growth factor receptors. For instance, 1000-2000 J/m^2 UVC are required to detect functional activation (as measured by UVC-induced binding of receptor-associated proteins) of the EGF-R (A. Knebel, personal communication). There is no evidence supporting the latter possibility. The fact, however, that UVC-induced p42 MAP kinase activation is abolished by suramin, while CREB phosphorylation is not affected, clearly indicates that the signal transduction pathways initiated by UVC and leading to CREB phosphorylation and p42 MAP kinase activation are distinct.

3.3.3.(iii). The tyrosine kinase inhibitor tyrphostin-51 inhibits the EGF-induced, but not the UVC-induced CREB phosphorylation

Tyrphostins are inhibitors of tyrosine kinases (TKs) with an increasing medical application in cases where upregulated tyrosine kinase activity was found to correlate with certain diseases (e.g. some kinds of inflammatory responses, cancer, atherosclerosis, and psoriasis, for review see Levitzki & Gazit, 1995 and references therein). Unlike other TK inhibitors (e.g. genistein) which substitute for ATP in a kinase reaction, tyrphostins compete with the natural tyrosine substrates of TKs due to their common pharmacophore unit - 3,4-dihydroxy-*cis*-cinnamonitrile (Yaish et al., 1988). Tyrphostin-51 (2-Amino-1,1,3-tricyano-4-[3´,4´,5´-trixydroxyphenyl]butadiene) is a 3,4-dihydroxy-*cis*-cinnamonitrile derivative and has been used as an EGF receptor tyrosine kinase inhibitor (Levitzki & Gazit, 1995 and references therein). It was, therefore, possible to use this substance to investigate whether UVC and EGF induce CREB phosphorylation via a common signal transduction pathway. The result of this

experiment is shown in **fig.15**. Tyrphostin-51 abolished the response of CREB to EGF, but had no effect on the UVC-induced CREB phosphorylation. This indicates that UVC reaches the endpoint (CREB) independently of certain tyrphostin-sensitive component of the EGF-induced signal transduction pathway. Surprisingly, this tyrphostin-sensitive component does not seem to be the EGF-R, as no effect of the drug on the phosphorylation of the EGF-R at tyrosine residues was detected (**fig. 15**, lower panel). Possibly other tyrosine kinases "downstream" of the receptor are tyrphostin-sensitive.

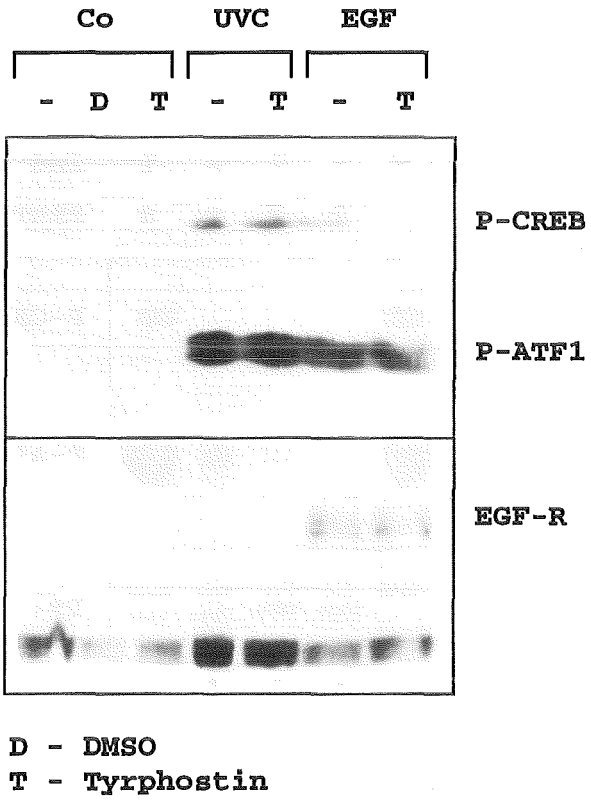


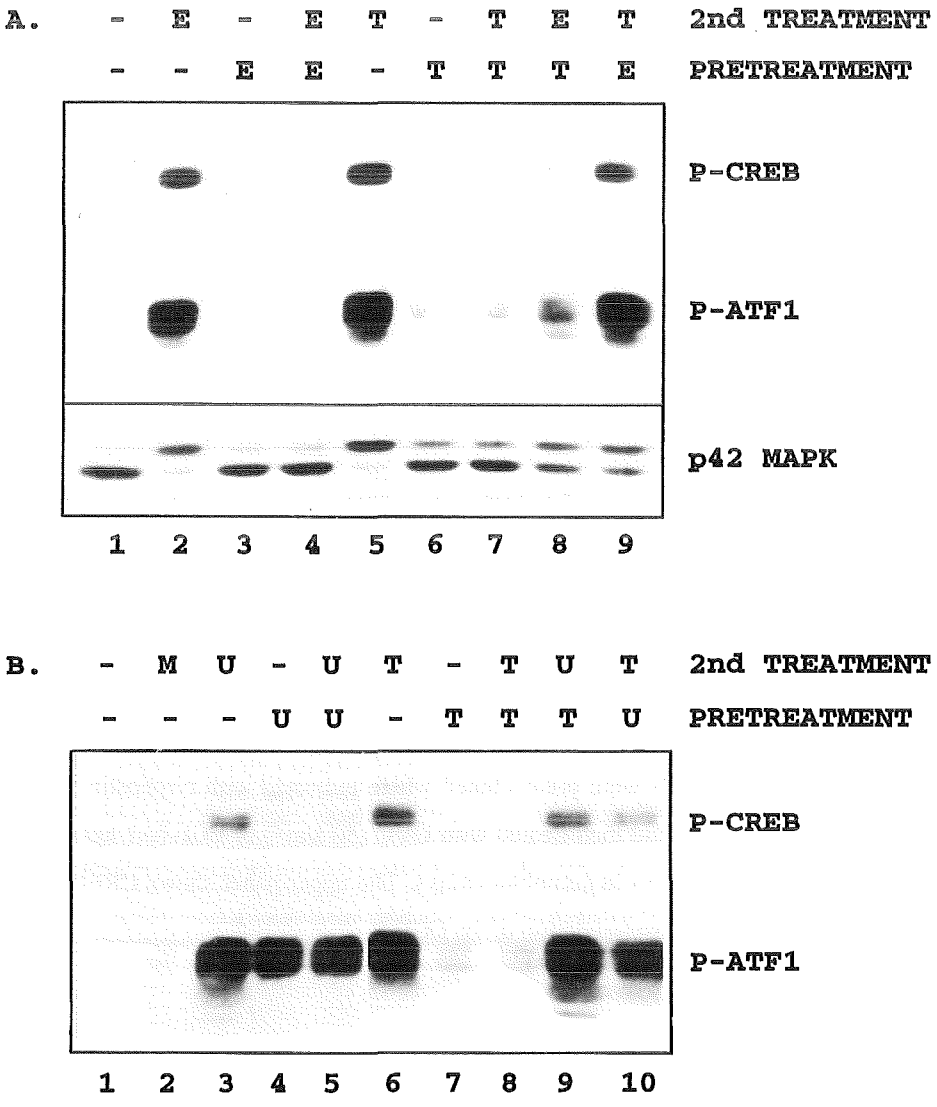
Fig. 15: The tyrosine kinase inhibitor tyrphostin-51 inhibits the EGF-induced, but not the UVC-induced CREB phosphorylation. HeLa tk^- cells were preincubated where indicated with tyrphostin-51 (20 μM), or with DMSO (used as a vehicle) for 10 min and then treated with UVC (30 J/m^2) or EGF (0.1 ng/ml) for additional 15 min. Whole cell lysates were subjected to immunoblot assays. The upper panel shows CREB phosphorylation at serine 133, the lower shows tyrosine phosphorylation of the EGF-R.

3.3.3.(iv). Third stimulus interference: TPA pre-treatment abolishes the responsiveness of CREB to a second stimulation with EGF, but not with UVC

Treatment of human epidermoid carcinoma cells A431 with TPA causes phosphorylation of the EGF receptor at threonine residue 654, an event which was found to result in reduced EGF-stimulated tyrosine kinase activity of the receptor, receptor down-modulation, and its internalization (Cochet et al., 1984; Moon et al., 1984; Lin et al., 1986; King

& Cooper, 1986). In addition, multiple indications exist that growth factor-induced (p21 Ras-dependent) and phorbol ester-induced (PKC-dependent) signal transduction pathways converge to a common point: the p74 Raf kinase (Bruder et al., 1992; Sözery et al., 1992; Kolch et al., 1993). p74 Raf has been found to be an essential element of the linear signal transduction cascade to p42 MAP kinase, being a "MAP kinase kinase kinase" in a broad range of organisms: nematodes, *Drosophila*, and vertebrates (for review see Marshall, 1994).

For these reasons, one might expect that at the level of p42 MAP kinase, EGF and TPA would manifest a cross-refractoriness. Pre-treatment with either of these inducers would decrease the responsiveness of p42 MAP kinase to restimulation with the other signal. This is demonstrated in **fig. 16A**.



U - UVC 30 J/m^2
E - EGF 20 ng/ml
T - TPA 200 ng/ml

Fig. 16: TPA pre-treatment abolishes the responsiveness of CREB to a second stimulation with EGF, but not with UVC.

A. HeLa tk^- cells were pre-treated with TPA, EGF, or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either TPA or EGF for 15 min. The cell lysates were analysed in immunoblot assays using the antibody directed against phosphorylated CREB (upper panel) or using the p42 MAP kinase-specific antibody (lower panel).

B. The same design as in **A.**, except that UVC was used instead of EGF.

HeLa cells pre-treated for 4 hours with TPA responded weaker to a second activation with EGF, compared to the effect of EGF alone, and *vice versa* (fig. 16A, lower panel, lanes 2, 5, 8, and 9). Furthermore, although both TPA and EGF strongly induce phosphorylation of CREB at serine 133, cells pre-treated with TPA hardly respond to EGF with CREB phosphorylation (fig. 16A, upper panel, lanes 2, 5, and 8), indicating either a common and interdependent mechanism of induction, or, more likely, down-modulation of the EGF-R by TPA. TPA pre-treatment, however, did not affect the responsiveness of CREB to UVC (**fig. 16B**, lanes 3 and 9). This once again supports the assumption that UVC induces CREB phosphorylation via mechanism(s) different from those used by EGF. Once again, ATF-1 displayed a behavior similar to CREB under these conditions (figures. 16A and 16B).

3.3.4. The UVC-Induced Signal Transduction to Both CREB and p42 MAP Kinase Is Ca^{2+}-Dependent

The full UVC response of the c-*fos* gene requires three major promotor elements: the serum responsive element (SRE), the +18/+38 element (Büscher et al., 1988), and the proximal cAMP responsive element (-60 CRE, this work). It is now known that two of these elements (SRE and -60 CRE) mediate the transcriptional induction of c-*fos* via UVC-induced modifications of the transcription factors Elk-1/p62 TCF and CREB which operate through them (Sachsenmaier et al., 1994; and this work). Intriguingly, Büscher et al. (1988) demonstrated that UVC-induced c-*fos* transcription may be completely obliterated by pre-treatment of NIH 3T3 cells for 4 hours with the calcium ionophore A23187. This effect appeared to be UVC-specific, since calcium ionophore pre-treatment had no effect on the induction of c-*fos* by TPA (Büscher et al., 1988). The question, however, through which mechanisms the intracellular calcium levels influence the ability of UVC to induce gene expression has not been addressed. An apparent possibility is that Ca^{2+} plays a role in the UVC-induced signal transduction to CREB and/or to Elk-1. The following hypotheses should be considered:

1. Ca^{2+} is required for the enzyme activity of certain element(s) of the UVC-induced signaling cascade (e.g. as a co-factor).

2. Ca^{2+} is a "second messenger" in the UVC-induced signal transduction: UVC irradiation leads to elevation of the cytoplasmic calcium levels and to activation of calcium-dependent signaling cascades.

The former hypothesis does not require UVC-induced elevation of the cytoplasmic calcium levels, while the latter would. In order to find out whether and through which mechanisms the UVC-induced signal transduction is Ca^{2+}-dependent, the ability of increased cytoplasmic Ca^{2+} to induce CREB phosphorylation and p42 MAP kinase activation, and to interfere with UVC-induced CREB phosphorylation and p42 MAP kinase activation, was investigated. Further, in order to prove or to reject the "second messenger" hypothesis, I examined the Ca^{2+} levels in the cytoplasm upon UVC irradiation.

3.3.4.(i). Elevation of the cytosolic Ca^{2+} levels in HeLa cells causes both phosphorylation of CREB at serine 133 and p42 MAP kinase activation

In PC12 pheochromocytoma cells, increased calcium influx through voltage-sensitive calcium channels after membrane depolarization causes phosphorylation of CREB at serine 133 and CREB-dependent transcriptional activation of c-*fos* via the "-60CRE" (Sheng et al., 1990). Both *in vitro* and *in vivo* data suggest that calcium/calmodulin kinases types II and IV may be responsible for this phosphorylation (Sheng et al., 1991; Dash et al., 1991; Matthews et al., 1994; Sun et al., 1994). Moreover, in numerous cell types, elevated cytoplasmic calcium levels lead to activation of MAP kinases. Several independent mechanisms, all converging at the point of increased cytoplasmic Ca^{2+}, account for this phenomenon: activation of voltage-sensitive calcium channels in PC12 cells (Rosen et al., 1994), activation of ionotropic glutamate receptors in hippocampal and cortical neurons (Bading & Greenberg, 1991; Fiore et al., 1993) and mobilization of intracellular calcium stores in fibroblasts (Chao et al., 1992).

Two drugs causing elevation of cytoplasmic Ca^{2+} levels via two different mechanisms have been widely used to analyze the role of this ion in the regulation of gene expression: the Ca^{2+} ionophore A23187 and the sesquiterpene lactone thapsigargin (Schönthal et al., 1991, and references therein). A23187 is a membrane-soluble calcium-to-proton exchanger: at low pH and low Ca^{2+} concentration (conditions close to those inside the cell) the carboxyl group of the molecule is protonated. The protonated A23187-COOH may pass through the plasma membrane into the extracellular space characterized by higher pH and higher Ca^{2+} concentration. There the carboxyl group looses its proton, two deprotonated carboxyl groups from two A23187 molecules (A23187-COO^-) bind one calcium ion, thus forming a Ca-(OOC-A23187)$_2$ complex. This complex may then re-enter the cell through the plasma membrane and release the calcium ion, thereby allowing the carboxyl groups to be protonated again. Thus, treatment of cells with A23187 leads to a rapid increase in the intracellular Ca^{2+} concentration, due to enhanced transport of the Ca-(OOC-A23187)$_2$ complexes through the plasma membrane (Ca^{2+}-influx) and through the membranes of the intracellular Ca^{2+} stores - endoplasmic reticulum and mitochondria (Pressman, 1976). Thapsigargin exerts its action via highly specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1987). It induces acute responses in a large variety of cell types, but in all cases its effects are initiated by a single event: a rapid increase in cytosolic free Ca^{2+}, avoiding some of the pleiotropic effects of A23187, e.g.

stimulation of the adenylyl cyclase through release of C_{20} fatty acids and their conversion to prostaglandins (Schönthal et al., 1991, and references therein).

In order to study the effects of increased cytosolic Ca^{2+} levels on CREB phosphorylation and p42 MAP kinase activation in HeLa cells, I treated the cells with A23187 or thapsigargin and then analyzed both endpoints using immunoblot assays, as before. The results are presented in **fig. 17**.

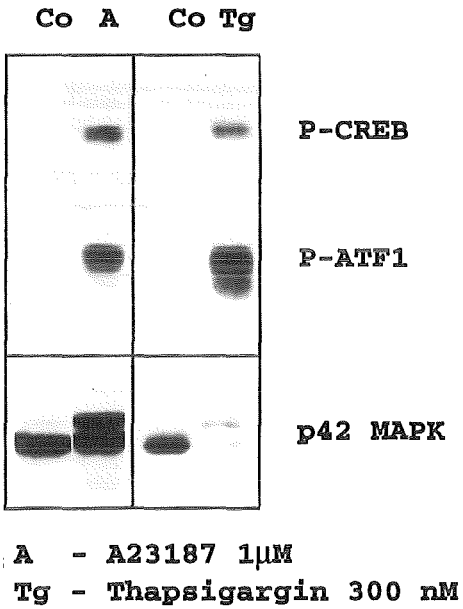
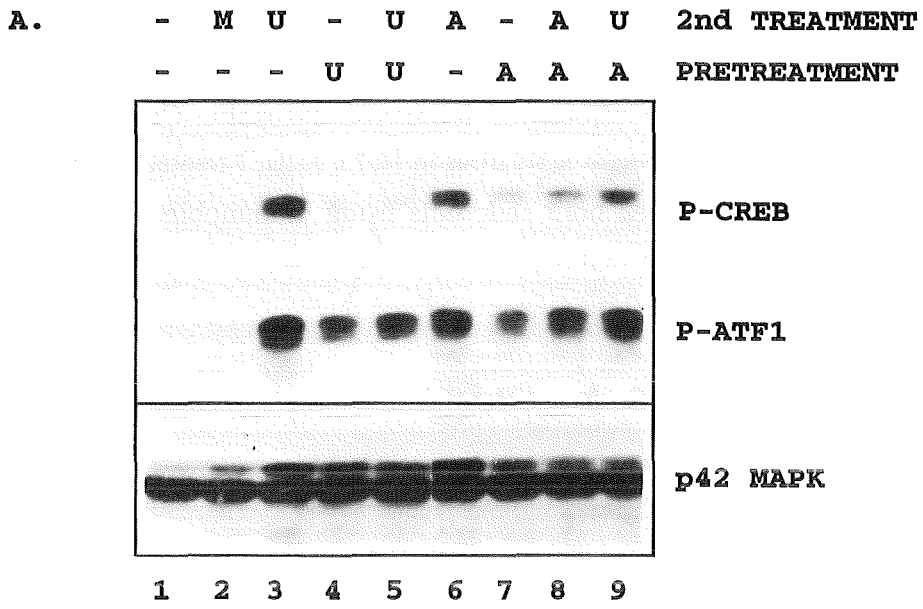


Fig. 17: Elevation of cytosolic Ca^{2+} levels causes both phosphorylation of CREB at serine 133 and p42 MAP kinase activation. Confluent HeLa tk^- cells were serum starved for 24 hours and then stimulated with thapsigargin or calcium ionophore A23187 for 15 min. The cell lysates were analysed in immunoblot assays using the antibody directed against phosphorylated CREB (upper panel) or using the p42 MAP kinase-specific antibody (lower panel).

Fifteen minutes after application of A23187 or thapsigargin a dramatic increase in phosphorylation of CREB and of ATF-1, and activation of p42 MAP kinase was observed. Thus, elevated intracellular calcium appeared to be a strong inducer of signal transductions both to CREB and ATF-1, and to p42 MAP kinase in HeLa cells.

3.3.4.(ii). Depletion of the intracellular Ca^{2+} stores prevents UVC signaling to CREB and to p42 MAP kinase

Next the ability of calcium signaling to interfere with the UVC-induced CREB phosphorylation and p42 MAP kinase activation was investigated, using the signal transduction down-modulation approach.



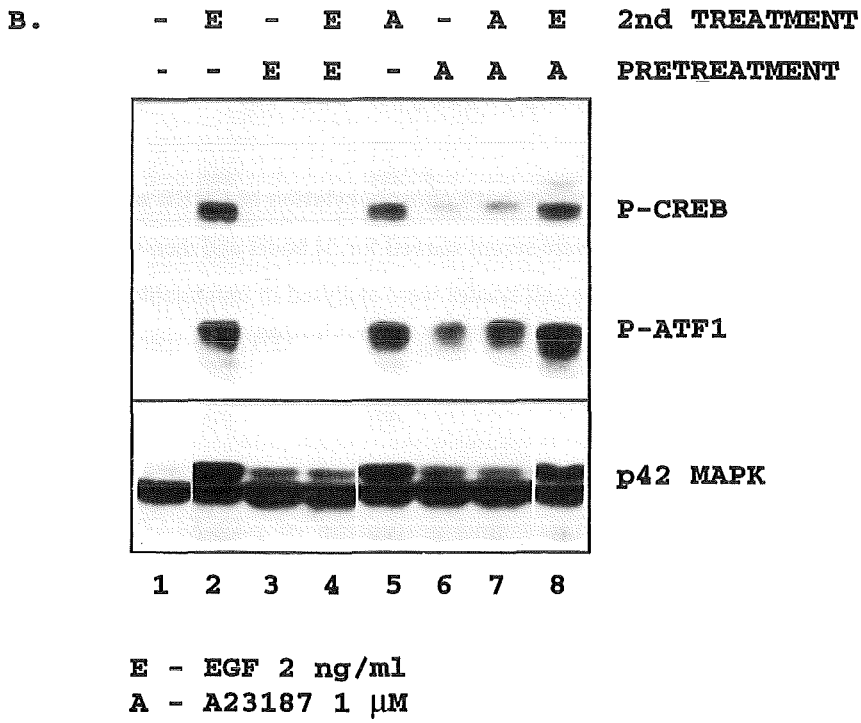


Fig. 18. Pre-treatment of the cells with the calcium ionophore A23187 interferes with the UVC-induced, but not with the EGF-induced CREB phosphorylation and p42 MAP kinase activation.

A. HeLa tk^- cells were pre-treated with UVC (30 J/m^2), A23187 (1μM), or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either UVC (30 J/m^2) or A23187 (1μM) for 15 min. The cell lysates

were analysed in immunoblot assays using the antibody directed against phosphorylated CREB (upper panel) or using the p42 MAP kinase specific antibody (lower panel).

B. The same design as in **A.**, except that EGF (2 ng/ml) was used instead of UVC.

A23187. When serum-starved HeLa cells were subjected to either UVC irradiation or to A23187 (**fig. 18A**) for 15 min, both CREB and p42 MAP kinase responded as expected (lanes 3 and 6). It is noteworthy that the Ca^{2+} signaling appeared to display the same signal transduction pathway down-modulation as was shown above for UVC, growth factors or TPA. Thus, 4 hours after A23187 application, both CREB and p42 MAP kinase effects were attenuated (lane 7) and were non-responsive to a second ionophore stimulation (lane 8). When the cells were subjected to UVC irradiation as a second stimulus following A23187 pre-treatment, total non-responsiveness of the p42 MAP kinase and reduced responsiveness of CREB were observed (lane 9). In parallel, the cross-refractoriness between A23187 and EGF was studied (**fig. 18B**) in order to analyze whether the effects observed reflected a specific interference between Ca^{2+}- and UVC-dependent signal transduction pathways. No significant change in the CREB or p42 MAP kinase response to EGF stimulation under conditions of ionophore pre-treatment was detected (lane 8). Thus, the interference between A23187 pre-treatment and UVC-dependent signal transduction pathways seems to be specific and indicates that the UVC response (as measured here at the level of CREB and p42 MAP kinase, and by Büscher et al. (1988) at the level of c-*fos* transcription) depends on the cytosolic Ca^{2+} concentration.

Simultaneous application of A23187 and thapsigargin in the absence of extracellular calcium: depletion of the intracellular Ca^{2+} stores. To answer to the question of whether Ca^{2+} is an essential component of the UVC-induced signal transduction chain, conditions were required under which the cellular Ca^{2+} stores are maximally depleted and no free calcium influx is possible. To achieve such a state, HeLa cells were pre-treated simultaneously with A23187 and thapsigargin for 4 hours. Although maximum release of calcium from the intracellular stores occurs in HeLa cells within minutes after such a treatment (see *3.3.4.(iii)*, fig. 20), this long period of preincubation was necessary for the cell to attenuate the phosphorylation of CREB and p42 MAP kinase induced by the drugs themselves (i.e. to allow the second signal to reach these same endpoints). Fifteen minutes before the second stimulation with A23187 plus thapsigargin, UVC or EGF, the extracellular Ca^{2+} (in both untreated and pre-treated cells) was complexed by adding a 5-fold molar excess of the chelating agent ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) to the cell culture medium. **Figure 19** presents the effect of the Ca^{2+} depletion on the UVC- and EGF-induced signaling to CREB and to p42 MAP kinase. The cells which received only EGTA treatment 15 min before induction displayed both CREB phosphorylation and p42 MAP kinase activation after stimulation with UVC or EGF (lanes 3 and 4). Depletion of the intracellular calcium stores and complexation of the extracellular calcium, however,

obliterated the UVC signaling to both endpoints (lane 8). The effect on EGF signaling was in part different: while the ability of EGF to induce CREB phosphorylation was challenged, more than 50% of the p42 MAP kinase molecules remained responsive to EGF (lane 9). The responsiveness of the p42 MAP kinase to EGF also argues against the assumption that the cells did not respond to UVC because of a possible cytotoxic effect of the calcium depletion. Thus, it appeared that Ca^{2+} is indeed an essential component of the UVC-induced signal transduction to CREB and p42 MAP kinase.

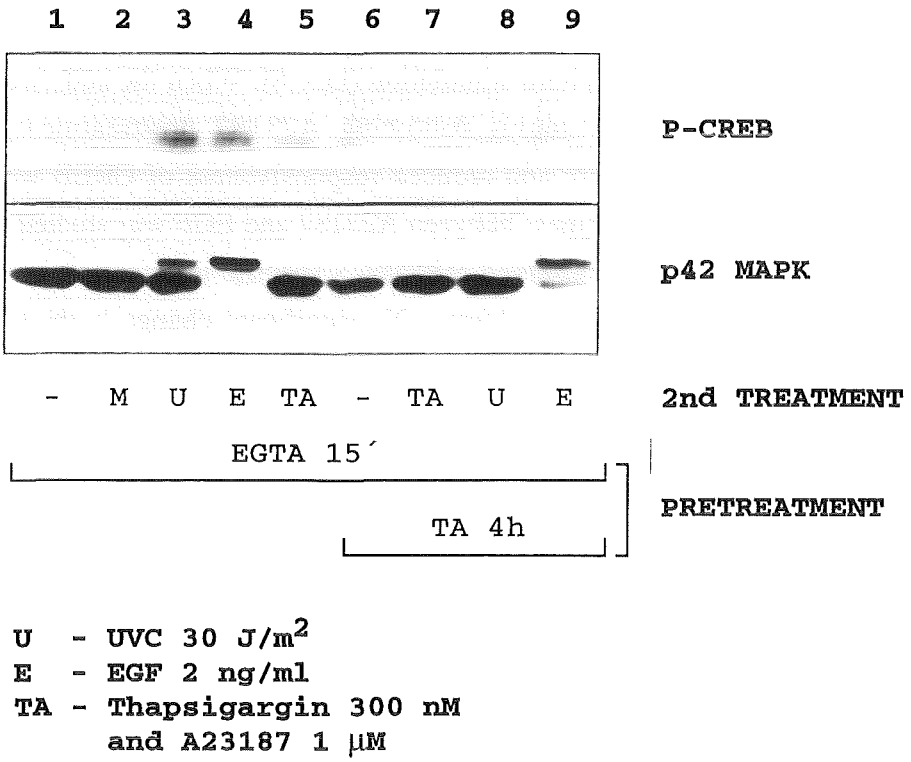


Fig.19: Depletion of the intracellular Ca^{2+} stores prevents UVC signaling to CREB and to p42 MAP kinase. HeLa tk^- cells were pre-treated simultaneously with A23187 (1µM) and thapsigargin (300 nM) for 4 hours or not pre-treated. Fifteen minutes before the second stimulation the extracellular Ca^{2+} (in *both* untreated and pre-treated cells) was complexed by adding a 5-fold molar excess of EGTA (f.c. 9 mM) to the culture medium. For induction, the cells received A23187 (1µM) plus thapsigargin (300 nM), UVC (30 J/m^2), or EGF (2 ng/ml) for 15 min. The cell lysates were analysed in immunoblot assays using the antibody directed against phosphorylated CREB (upper panel) or using the p42 MAP kinase specific antibody (lower panel). M - mock treated cells.

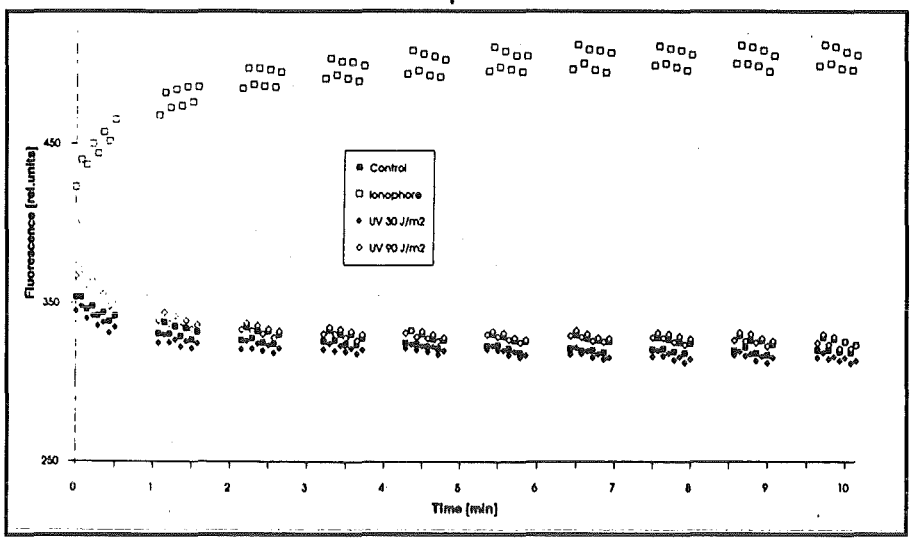
3.3.4.(iii). UVC does not induce elevation of the free cytosolic calcium: an argument against the "second messenger" hypothesis

How is Ca^{2+} involved in the UVC response? An attractive possibility is that upon absorption, UVC causes elevation of the cytosolic Ca^{2+} levels (via Ca^{2+} influx and/or enhanced release of Ca^{2+} from the intracellular stores), thus triggering calcium-dependent signal transduction. Increased cytosolic calcium mediates as a "second messenger" the effects of many hormones, neurotransmitters and growth factors on numerous cellular functions, including cell motility and contractility, carbohydrate metabolism, cell cycle, gene expression and neuronal plasticity (for reviews see Kenedy, 1989, Hanson & Schulman, 1992, Schulman, 1993). These intracellular effects of calcium are transmitted by many effector systems: protein kinases and phosphatases, ATPases, ion channels, proteases and phospholipases (Schulman, 1993). If UVC were to induce gene expression via elevated Ca^{2+}, this elevation should be measurable. I therefore determined the levels of cytosolic free calcium after UVC stimulation. For this purpose the fluorescent dye *fluo-3* was used, which can be accumulated in the cell and can form stable complexes with Ca^{2+} there. These complexes (but not the Ca^{2+}-free dye) emit fluorescence at 530 nm upon excitation with monochromatic light at 485 nm, and this fluorescence may be used as a marker for the concentration of cytosolic Ca^{2+} (Rijkers et al., 1990; Merritt et al., 1990). Two methods, spectroscopic and microscopic, both based on *fluo-3*-emitted fluorescence, were used for the study.

Spectroscopic analysis. This method allows quantitative measurement (as relative fluorescence units) of the Ca^{2+} alterations in a sufficiently representative number of cells. Serum-starved HeLa cells were treated with the calcium ionophore A23187 and thapsigargin (positive control), with EGF or with different doses of UVC: 30, 90, 500, and 2000 J/m^2. The results of the study are presented in **fig. 20**. A23187 treatment led to a rapid increase of the cytosolic calcium (fig. 20A). The elevation reached its maximum 4 min after the stimulation and remained stable during the whole period of measurement (15 min). The effect of thapsigargin was less pronounced (fig. 20C), though above the basal level in the untreated cells. However, neither a saturating dose of EGF (200 ng/ml, which corresponds to $3x10^{-8}$ M, fig. 20C), nor even the highest UVC dose applied (2000 J/m^2, fig. 20B), were able to cause any detectable increase in the intracellular Ca^{2+} concentration. Neither of the stimuli applied, UVC, EGF or thapsigargin, could affect the ability of the cells to respond with an elevation of the free cytosolic calcium to a second stimulation with A23187 (figures 20B and 20C, the time-point of the A23187 application is indicated by an arrow). Thus, although all four stimuli tested - A23187, thapsigargin, EGF, and UVC - induce changes in CREB and p42 MAP kinase of a similar intensity (see figures 17, 18A and 18B), they manifest significant differences in their ability to elevate the cytosolic free calcium. This strongly argues against the hypothesis that UVC (or EGF) uses Ca^{2+} as a "second messenger" and the calcium-dependent signaling pathways for inducing CREB and/or p42 MAP kinase.

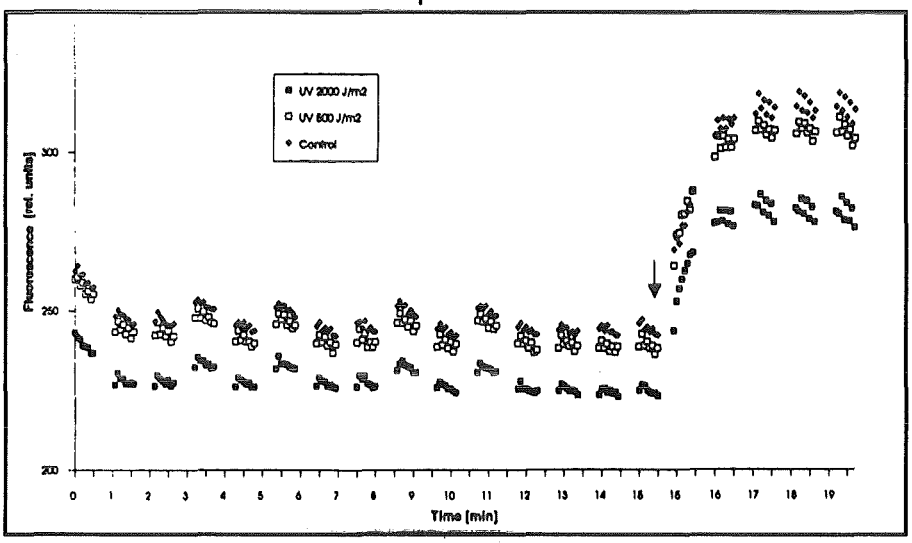
UVC 30 and 90 J/m²
A23187 1 µM

A.



UVC 500 and 2000 J/m²
A23187 1 µM

B.



Thapsigargin 300 nM
EGF 200 ng/ml
A23187 1 µM

C.

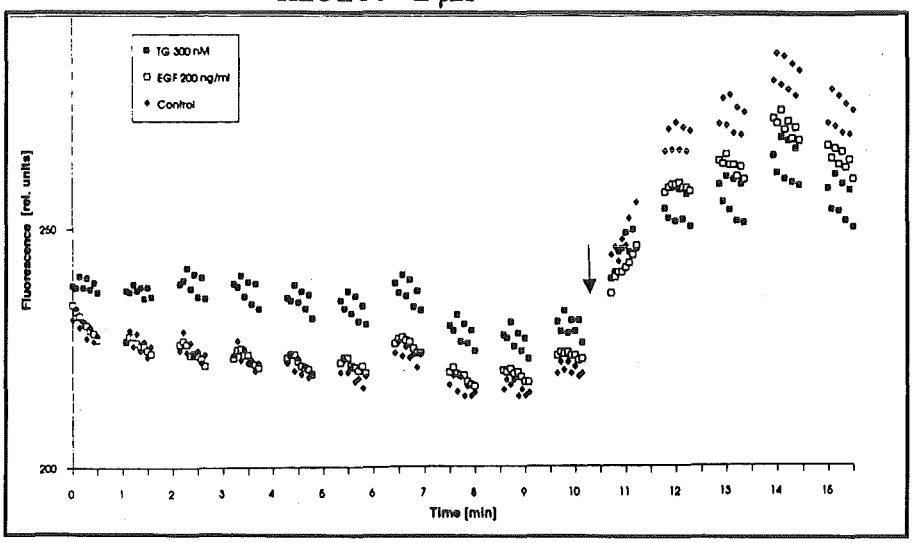


Fig. 20: UVC does not induce elevation of the free cytosolic calcium *(spectroscopic analysis)*: HeLa tk^- cells were pre-treated with the fluorescent dye *fluo-3* as described in Materials and Methods and then treated with: (**A**) A23187 (1μM) or UVC (30 and 90 J/m^2); (**B**) UVC (500 and 2000 J/m^2); (**C**) thapsigargin (300 nM) or EGF (200 ng/ml). The levels of free cytosolic calcium were measured as described in Materials and Methods.

It is worth noting that the inability of even saturating doses of EGF (in the HeLa cells used, increasing of the EGF concentration above 200 ng/ml does not lead to a further increase in the EGF-R tyrosine phosphorylation, not shown) to cause calcium elevation in the cytosol was somehow unexpected. In many cell types, the EGF-R tyrosine autophosphorylation was found to cause elevation of cytosolic calcium through binding of the receptor to phospholipase Cγ (PLCγ) and its activation (Fantl et al., 1993; Kazlauskas, 1994 and references therein). PLCγ is a key enzyme in phosphatidylinositol(4,5)diphosphate turnover, which produces two potent second messengers: inositol trisphosphate(I-3P) and diacylglycerol (DAG). I-3P binds to its receptor and initiates a rise of intracellular calcium (reviewed by Berridge, 1993). The role of the growth factor-induced PLCγ activation is still not clear. The majority of the studies aimed at answering this question led to the conclusion that PLCγ is not essential for the growth factor-dependent mitogenic response and that its activation possibly serves only as a "back up" trigger of cell proliferation (Kazlauskas, 1994 and references therein). In HeLa cells, at least a part of the UVC-induced signal transduction shares components with that induced by EGF (Devary et al., 1992,1993; Radler-Pohl et al., 1993; Sachsenmaier et al., 1994). Since another part of the UVC response, namely CREB activation, seems to follow different signal transduction routes (3.3.3), it was interesting to know whether the influence of both stimuli on the Ca^{2+} levels belongs to the common or to the inducer-specific effects. The results of these studies, however, suggest that, in HeLa cells, neither UVC nor EGF are able to significantly influence the intracellular levels of Ca^{2+}.

Microscopic analysis. An important disadvantage of the spectroscopic method used above is that it cannot detect differences in the Ca^{2+} levels which occur during UVC irradiation. More than two minutes are required in order to irradiate the cells with a dose of 2000 J/m^2 with the UVC source used. Thus, a significant but transient elevation of the free cytoplasmic calcium in response to UVC may remain undetected due to a rapid attenuation of the calcium increase after the treatment. These problems are overcome using microscopic analysis, since with this method measurement of the Ca^{2+} levels during treatment is possible. **Figure 21** demonstrates the result of the experiment. HeLa cells were irradiated with 2000 J/m^2 UVC (2 min 13 sec) and the levels of cytoplasmic calcium were monitored within this period and up to 5 min after the beginning of the irradiation. Only few cells displayed a slight increase in the calcium level, while the majority of them were unaffected. In contrast, the same cells reacted very well to the calcium ionophore A23187 within 1 min, indicating that the cells were able to mobilize their intracellular Ca^{2+} stores and to increase the uptake of extracellular calcium.

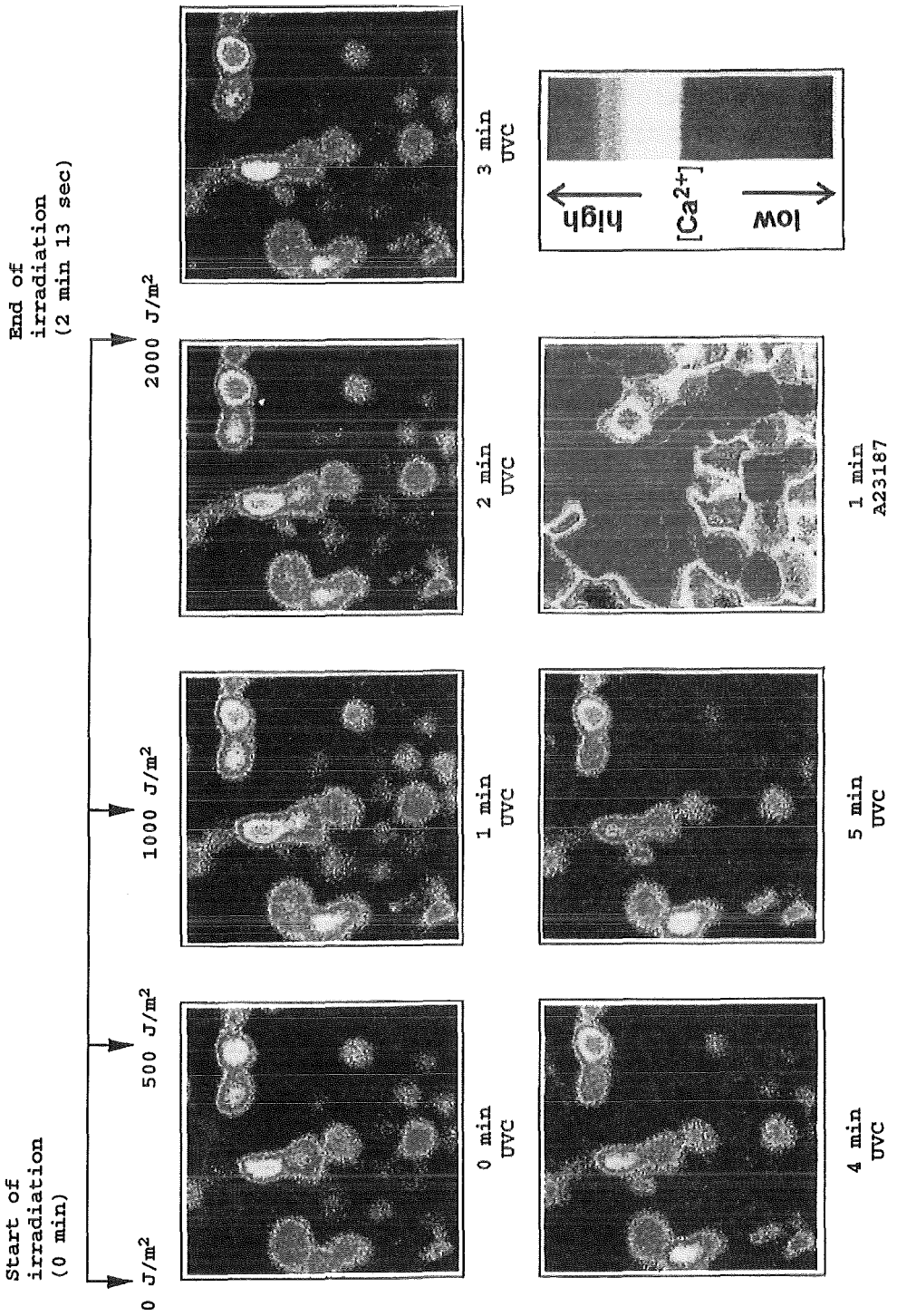


Fig. 21: UVC does not induce elevation of the free cytosolic calcium *(microscopic analysis)*: HeLa tk$^-$ cells were pre-treated with the fluorescent dye *fluo-3* as described in Materials and Methods and then treated with UVC (2000 J/m^2). The levels of free cytosolic calcium were monitored *during* (1st and 2nd min) and *after* (3rd, 4th, and 5th min) the UVC irradiation as described in Materials and Methods. (The experiments presented in figures 20 and 21 were performed in co-operation with T. Ade, Institut für Toxikologie, Forschungszentrum Karlsruhe)

Although both methods used have some limitations in their sensitivity, it is still safe to say that, in HeLa cells, UVC, even when applied at doses about two orders of magnitude higher than those sufficient to induce the UVC response, cannot cause significant changes in the cytoplasmic calcium levels. The microscopic analysis revealed that *some* but not *all* of the cells increase slightly the cytosolic Ca^{2+} concentration in response to UVC irradiation (fig. 21). However, the UVC-induced CREB phosphorylation (as measured by immunofluorescence in NIH 3T3 cells, fig. 5) or other UVC-induced endpoints such as cFos accumulation in HeLa cells (A. Kolbus, personal communication) are seen in almost every irradiated cell. It is, therefore, unlikely that Ca^{2+} is the "second messenger" which mediates the signal transduction from the primary target of UVC absorption to the nucleus.

3.3.5. Identification of the UVC-Inducible CREB Kinase

Several serine/threonine kinases have been found to regulate CREB activity *in vivo* and *in vitro* through phosphorylation of serine residue 133. These protein kinases include the cAMP-dependent protein kinase A (PKA, Gonzales & Montminy, 1989), the Ca^{2+}/calmodulin-dependent kinases (CaMKs) type II and IV (Sheng et al., 1991; Dash et al., 1991; Matthews et al., 1994; Sun et al., 1994) and a novel p105 CREB kinase which was identified to phosphorylate CREB in response to nerve growth factor (NGF) stimulation of the pheochromocytoma cell line PC12 (Ginty et al., 1994).

Which kinase is responsible for the UVC-induced CREB serine 133 phosphorylation? To answer this question a modification of a technique was used originally described as "*in situ* (in gel) kinase assay" (Geahlen et al., 1986, quoted from Hutchcroft et al., 1991). **Figure 22** shows a schematic presentation of the method. As a specific substrate for co-polymerization in the gel a peptide was used representing the amino acid sequence 123-136 of CREB (CREBtide, Ginty et al., 1994, KRREILSRRPS*YRK). The control gels were either poured without a co-polymerized substrate (fig. 23) or with the co-polymerized irrelevant peptide DGDSSMDPRGGFDTV (fig. 24). For these studies serum starved HeLa cells were treated with UVC, EGF, and forskolin and IMX for 15 min, and crude nuclear extracts were prepared. The extracts were resolved electrophoretically via 7.5% SDS PAGE and processed further for "in gel kinase assay" as outlined in fig. 22. **Figure 23** (left panel) shows that nuclear extracts from the UVC treated and from the EGF treated cells, but not from the control or cAMP stimulated cells, contain a CREB-specific protein kinase activity with an apparent molecular weight of 108 kD (indicated by an arrow). Another kinase activity, with a molecular weight of 96 kD, is also induced by UVC and EGF. It is, however, unlikely that this 96 kD kinase is the one responsible for the phosphorylation of CREB *in vivo*, since this activity was also reproducibly detected in the nuclear extracts from untreated cells, while the same extracts showed no CREB phosphorylation as measured in immunoblot assays (not shown). Thus, a UVC-induced signal transduction cascade appeared to feed into the same kinase (the 108 kD kinase) which phosphorylates CREB in response to EGF.

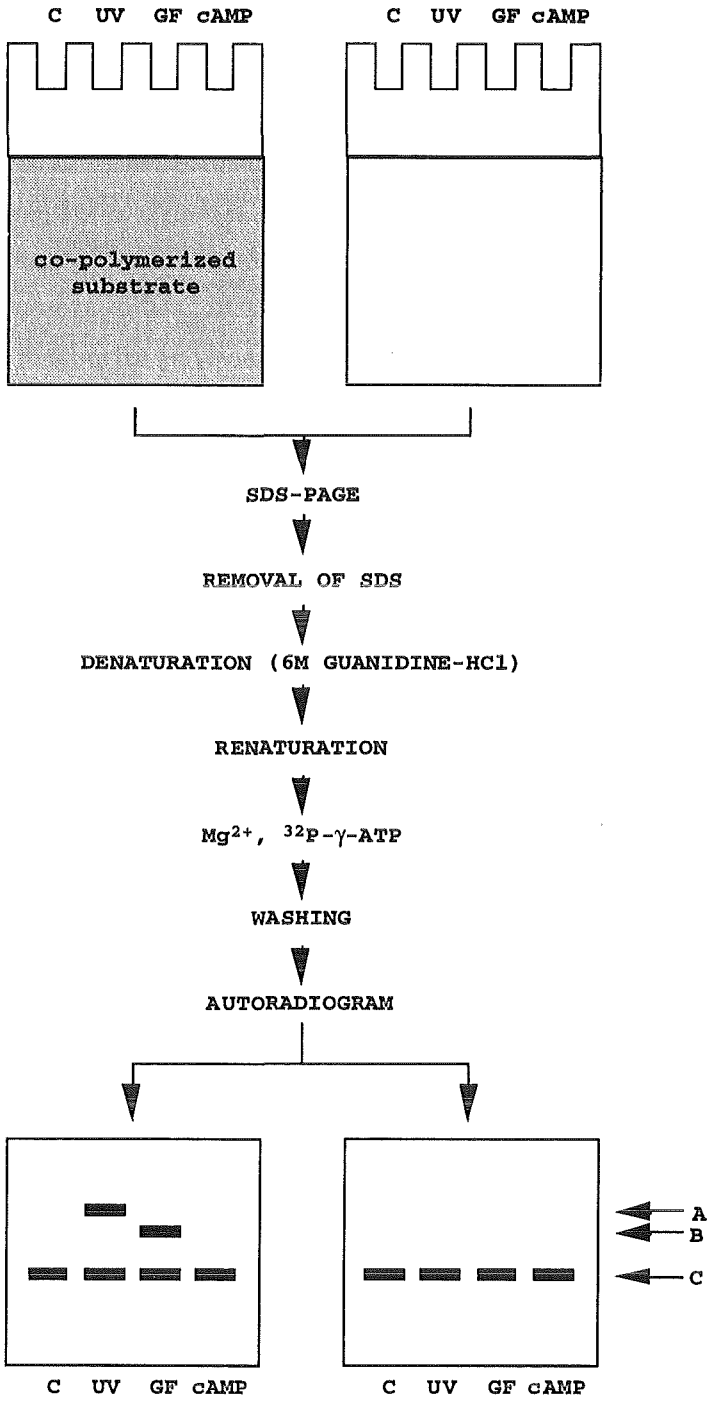


Fig. 22: Scematic representation of the "in gel kinase assay" (modified from Hutchcroft et al., 1991, see also Materials and Methods). Nuclear extracts from untreated (control) and treated (e.g. with UVC, growth factors, or cAMP-elevating agents) cells are resolved in SDS-PAGE, using 7.5% gels with and without a co-polymerized specific kinase substrate. A recombinant protein or a synthetic peptide representing the phosphoacceptor site of interest may serve as a substrate. Since the substrate has been physically "trapped" during the polymerization process, it is electrophoretically inerted and remains throughout the gel during the electrophoresis. Upon removal of the SDS, the proteins in the gels are shock-denatured with 6 M guanidine-HCl and then allowed to renature slowly for 15 hours. The *in gel* kinase reaction is initiated by providing ^{32}P-γ-ATP and Mg^{2+}. During the kinase reaction a specific kinase, induced by a particular stimulation, would phosphorylate the substrate in such a position in the gel

which corresponds to its molecular weight . Thus, the kinase manifests itself as a band of characteristic molecular size in the X-ray film (lower part of the scheme, left panel, bands A and B). The bands present in *both* gels (with or without substrate) represent autophosphorylating kinases (left and right panels, band C).

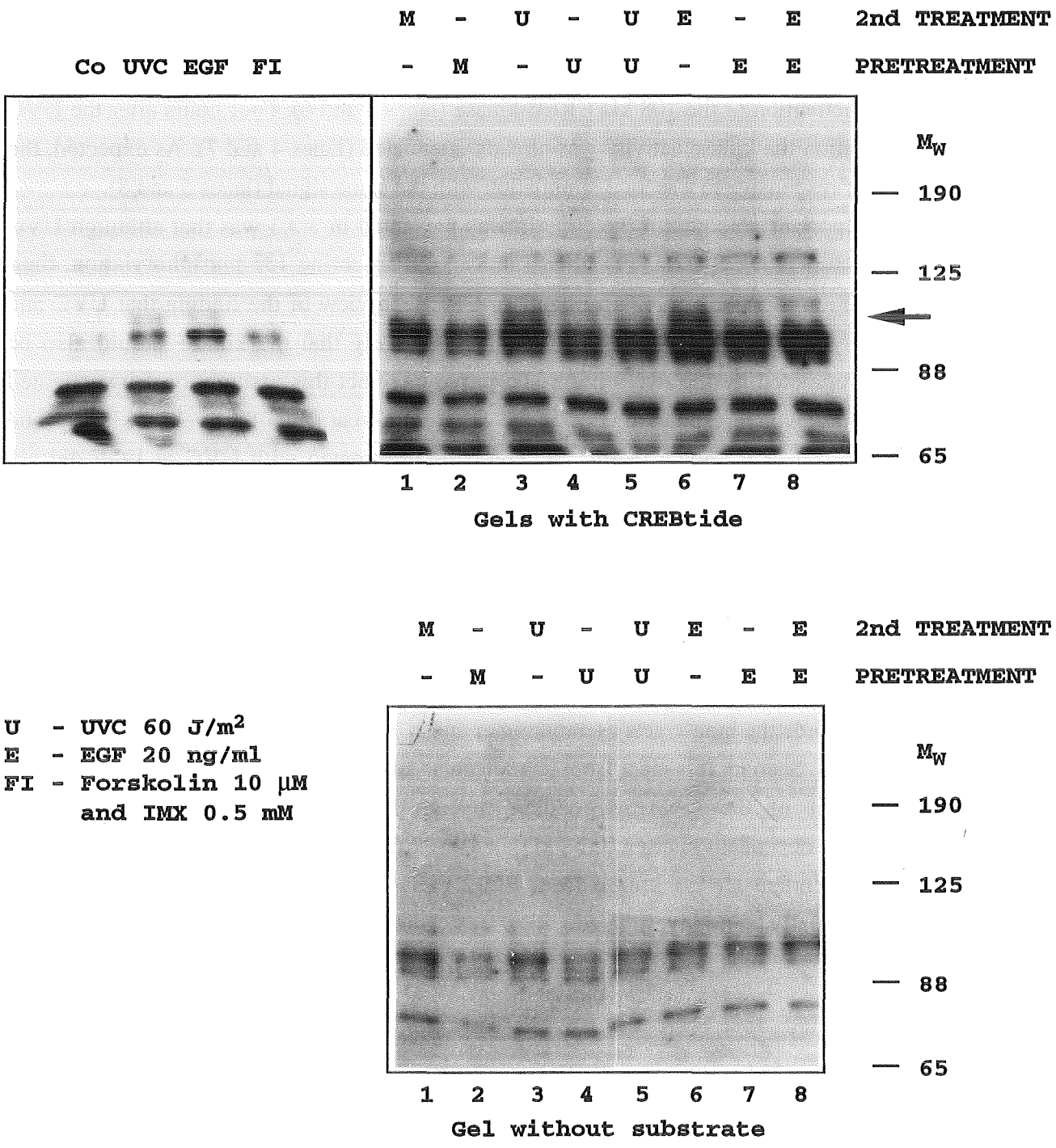


Fig. 23: UVC and EGF, but not cAMP induce a 108 kD CREB kinase in HeLa cells. Serum-starved HeLa tk$^-$ cells were treated with the indicated doses of UVC, EGF, or forskolin plus IMX for 20 min. The nuclear extracts were prepared and the "in gel kinase assays" were performed as described in Materials and Methods and as outlined in fig. 22. The UVC- and EGF-induced 108 kD CREB kinase is indicated by an arrow.

In 3.3.3 (figures 10 and 11) it was shown that CREB did not respond with serine 133 phosphorylation neither to UVC nor to EGF stimulation if the cells had been pre-treated with the same stimulus (signaling cascade down-modulation). It was, however, still reactive to another stimulus (e.g. cAMP), indicating that not CREB itself, but rather an "upstream" component of the signaling cascade was down-modulated. It is, therefore, to be expected that the 108 kD CREB kinase should behave the same way as CREB. That this is indeed the case is demonstrated in **fig. 23** (right panel). Treatment of the cells with UVC or EGF for 15 min resulted in an activation of the 108 kD CREB kinase (lanes 3 and 6). Four hours after the UVC or EGF application the kinase activity was already attenuated (lanes 4 and 7). As expected, the kinase was non-responsive to a second stimulation with the same signal (lanes 5 and 8).

An important outcome of the experiments presented in 3.3.3 was that although UVC and growth factors (EGF, IL-1α, and bFGF) induce CREB serine 133 phosphorylation, their signal transduction routes diverge "upstream" of CREB. In view of the finding that UVC and EGF phosphorylate CREB via the same kinase, it is likely that the kinase should also be differentially regulated by the two stimuli. To prove or reject this assumption I investigated whether the kinase would respond to UVC in the case of pre-treatment of the cells with EGF and *vice versa*. The result is shown in **fig. 24**. The cells pre-treated with UVC or EGF did not reactivate the kinase when the same signal was applied (lanes 4 and 7). The cells prestimulated with EGF, however, were still responsive to UVC and *vice versa* (lanes 8 and 9). Thus, the observation that UVC and EGF use different mechanisms to induce phosphorylation of CREB was confirmed also at the level of the kinase responsible for this phosphorylation.

What properties of the 108 kD kinase may be deduced from the experiments presented above? First, the substrate recognition and the kinase activity are within a single polypeptide chain, in other words the kinase acts as a monomer and it does not need other subunits for either of these functions. Second, it is clear from its molecular size that the kinase is different from the catalytic subunit of the cAMP-dependent protein kinase A (41 kD) and from the Ca^{2+}/calmodulin-dependent kinases II (54 kD) or IV (52.6 kD). It is very likely that it is similar to the NGF-dependent CREB kinase from PC12 cells (105 kD, Ginty et al., 1994). This possibility is currently being investigated.

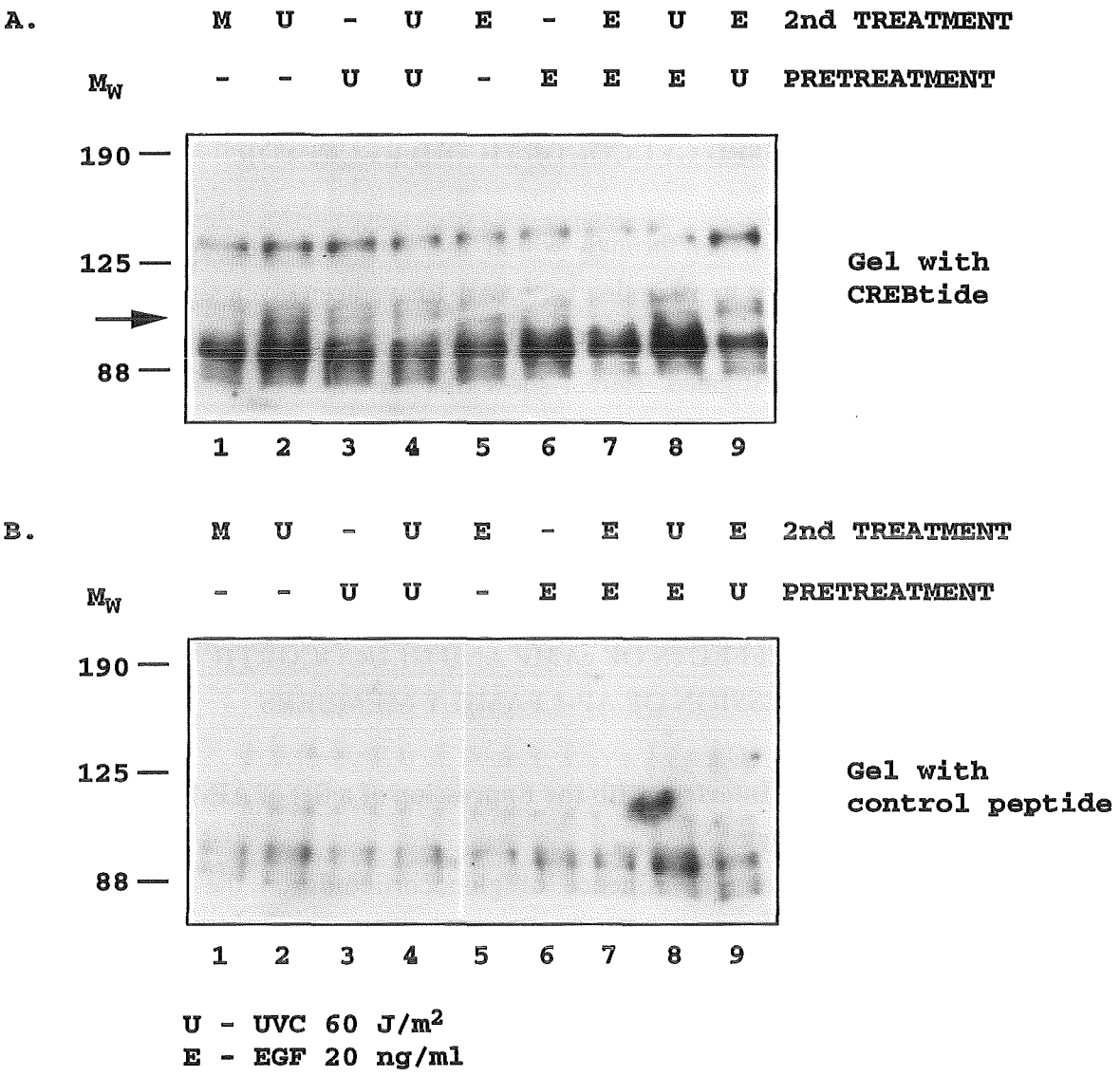


Fig. 24: The UVC- and EGF-induced CREB kinase is differentially and independently regulated by the two stimuli. HeLa tk^- cells were pre-treated with UVC (60 J/m^2), EGF (20 ng/ml), or not pre-treated (-), followed by a second treatment 4 hours after the pre-treatment with either UVC (60 J/m^2) or EGF (20 ng/ml) for 20 min. The CREB kinase is indicated by an arrow.

4. RESULTS (PART TWO)

CREB AND THE PROXIMAL c-*fos* CRE AS TARGETS FOR REPRESSION BY THE ACTIVATED GLUCOCORTICOID RECEPTOR

Although the mechanisms through which the activated glucocorticoid receptor interferes with the *activity* of the transcription factor AP-1 have been given significant attention in the last years, the question of whether and how glucocorticoids affect the *expression* of AP-1 has not been intensively investigated. Here I show that the AP-1 expression *is* subject to glucocorticoid-induced regulation, namely that glucocorticoids antagonize both the positive and the negative effects of increased cytosolic cAMP levels on the expression of three cAMP-regulated AP-1 family members, c-*fos*, c-*jun*, and *jun*B. I further investigate the mechanisms through which the interference of GR and CREB regulate the activity of the c-*fos* promotor.

4.1. ANTAGONISTIC EFFECTS OF cAMP AND GLUCOCORTICOIDS ON THE EXPRESSION OF AP-1 FAMILY MEMBERS

4.1.1. Glucocorticoids Interfere with the Expression of a Set of AP-1 Family Members Regulated by cAMP

In order to investigate the influence of glucocorticoids on the cAMP-regulated expression of various members of the AP-1 family, NIH 3T3 quiescent fibroblasts were pre-treated for 15 min with 10^{-7} M dexamethasone or not pre-treated, and then treated with forskolin. The expression of c-*fos*, c-*jun* and *jun*B was analyzed via Northern blot (**Fig. 25**). As described before (Büscher et al., 1988; Chiu et al.; 1989, Mechta et al., 1989), forskolin transiently induced the transcription of both c-*fos* and *jun*B with maximum mRNA accumulation 60 minutes after stimulation, but repressed by 15 fold the basal level of c-*jun* mRNA after 2 hours of forskolin treatment (lanes 1-5). Compared to stimulation with forskolin only, cells pre-treated with dexamethasone showed reduced forskolin-induced c-*fos* and *jun*B mRNA accumulation (lanes 6-10). Interestingly, dexamethasone also alleviated the negative action of cAMP on c-*jun*, which was no longer repressed in forskolin-treated cells (lanes 6-10). Thus, glucocorticoids appear to counteract simultaneously at least two different endpoints caused by elevated intracellular levels of cAMP, namely the induction of c-*fos* and *jun*B and the repression of c-*jun* expression.

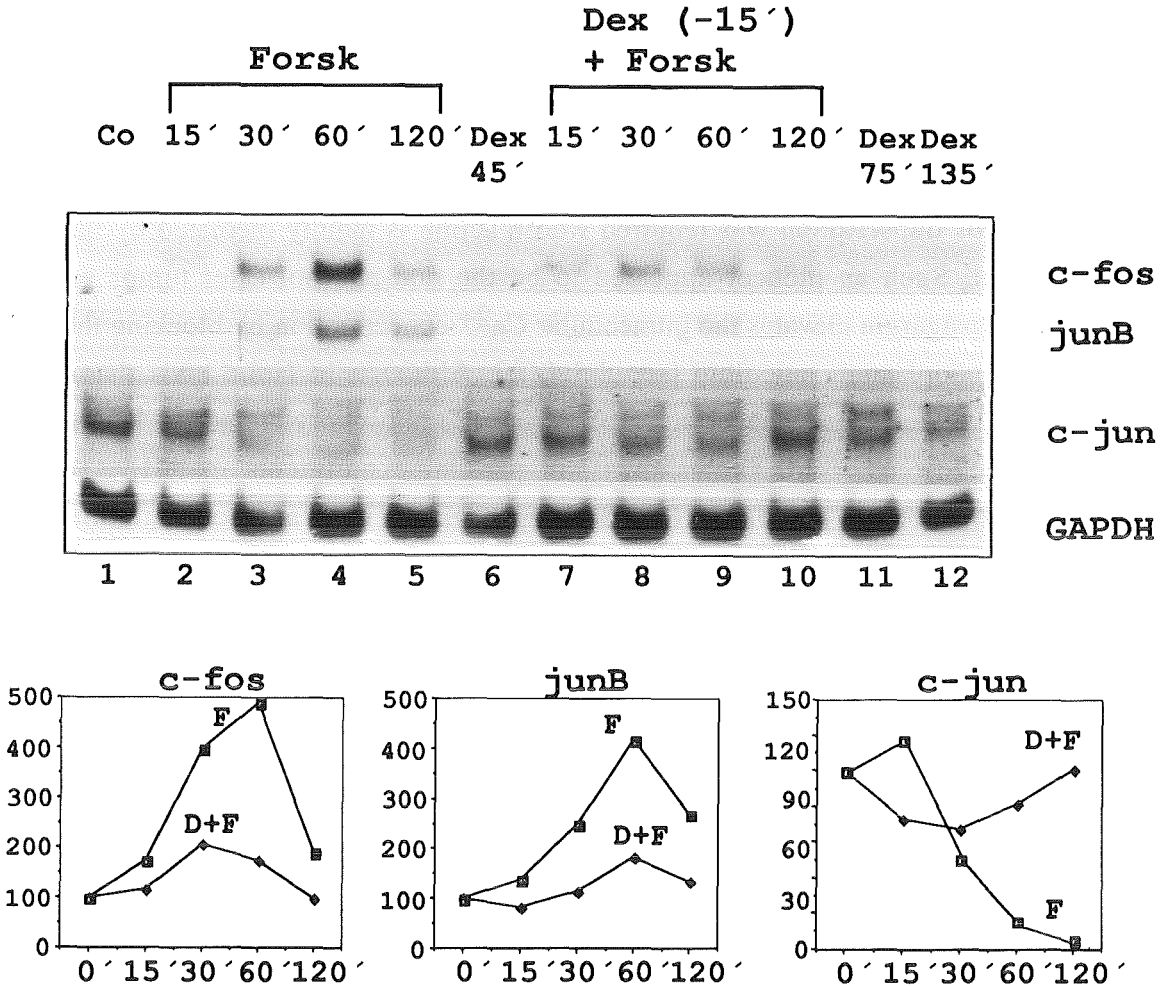


Fig. 25: In NIH 3T3 cells, glucocorticoids interfere with the expression of a set of AP-1 family members regulated by cAMP. Quiescent NIH 3T3 cells were pre-treated with 10^{-7} M dexamethasone (Dex) for 15 min or not pre-treated and then treated with 10 µM forskolin for the indicated time. PolyA$^+$ mRNA was prepared as described in Materials and Methods. 5 µg polyA$^+$ mRNA were analysed in Northern blot hybridisation using radioactively labeled c-*fos*-, c-*jun*-, *jun*B-, and GAPDH-specific DNA probes as described in Materials and Methods (upper panel). Lower panels - graphic representation of the data shown in the upper panel, normalized to GAPDH mRNA amounts. D+F - dexamethasone- and forskolin-treated cells. F - cells treated only with forskolin.

4.1.2. Glucocorticoids Specifically Repress c-*fos* Transcription Induced by cAMP

As the activation of c-*fos* gene transcription by cAMP has been intensively investigated, I further concentrated on the effect of glucocorticoids on the cAMP-induced c-*fos* transcription. First, the question of whether glucocorticoids interfere specifically with the cAMP induced c-*fos* expression (i.e. with the transcriptional activation mainly mediated by CREB and the CREs of the c-*fos* promotor) or whether induction of c-*fos* transcription by other agents is also affected, was addressed. With most inducers examined (such as phorbol esters and ultraviolet radiation),

some inhibition by a glucocorticoid pre-treatment of the cells was detected. For instance, the inhibition of the UVC-induced c-*fos* transcription by dexamethasone was about 30 to 40% (not shown). The transcriptional induction of c-*fos* by UVC is mediated through the serum response element (SRE) located between positions -320 and -300 (Büscher et al., 1988) but also other promotor elements contribute significantly to this induction: the -60 CRE (see Results, Part One) and the +18/+38 element (Büscher et al., 1988). For this reason, to answer the initial question, an inducer of c-*fos* transcription was required whose action is mainly mediated by the serum response element. Such an inducer appeared to be the non-phorbol ester tumor promotor thapsigargin. Thapsigargin elevates the intracellular Ca^{2+} concentration by blocking the Ca^{2+}-ATPase in the endoplasmic reticulum [see *3.3.4.(i)*]. Although in HeLa cells thapsigargin appeared to be a very efficient inducer of CREB serine 133 phosphorylation (fig. 17), even at a concentration as low as 3 nM (not shown), in parallel experiments in NIH 3T3 cells, thapsigargin application did not lead to CREB phosphorylation even at a concentration of 300 nM (not shown), suggesting that the effects of increased Ca^{2+} levels induced by this drug on certain processes may be cell type-specific. Furthermore, in NIH 3T3 cells, thapsigargin treatment could not induce transcription of transfected reporter gene constructs containing multimerized c-*fos* CREs (Schönthal et al., 1991) or a single c-*fos* CRE (not shown), while efficiently inducing a SRE-directed construct (Schönthal et al., 1991) and transcription of endogenous c-*fos* (Schönthal et al., 1991 and fig. 26). Thus, the data available suggest that, in NIH 3T3 cells, thapsigargin addresses the c-*fos* promotor exclusively through the SRE. Consistent with this, in contrast to c-*fos* transcription induced by elevated cAMP (either by treatment with forskolin or with dibutyryl cAMP), thapsigargin-induced c-*fos* transcription was not affected by glucocorticoids (**Fig. 26**). I conclude from these experiments that glucocorticoids specifically repress cAMP-induced c-*fos* transcription while not (or only slightly) interfering with inducers working through the SRE.

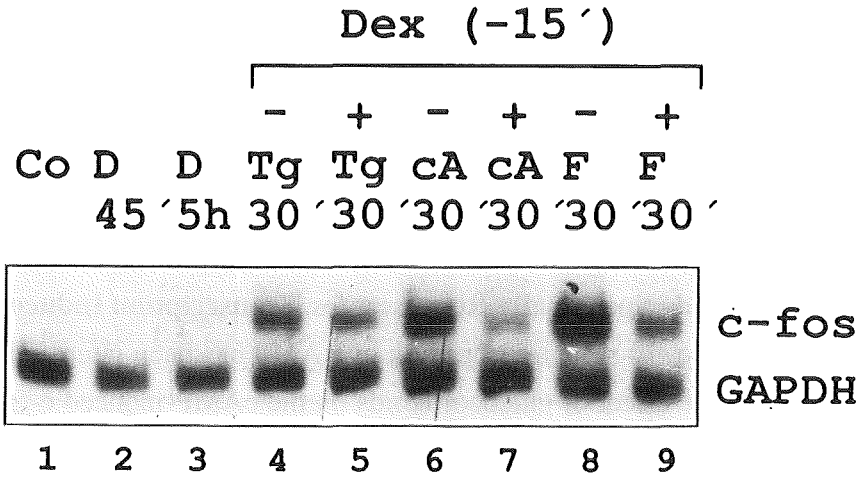


Fig. 26: Glucocorticoids repress specifically c-*fos* transcription induced by cAMP. Quiescent NIH 3T3 cells were pre-treated with 10^{-7} M dexamethasone (Dex) for 15 min or not pre-treated and then treated with 300 nM thapsigargin (Tg), 1 mM dibutyryl cAMP (cA), or 10 µM forskolin (F) for 30 min. PolyA$^+$ mRNA was prepared as described in Materials and Methods. 5 µg polyA$^+$ mRNA were analysed in Northern blot hybridisation using radioactively labeled c-*fos*- and GAPDH-specific DNA probes as described in Materials and Methods.

4.2. MECHANISM OF THE GLUCOCORTICOID-INDUCED REPRESSION OF THE cAMP-INDUCED c-*fos* TRANSCRIPTIONAL ACTIVATION

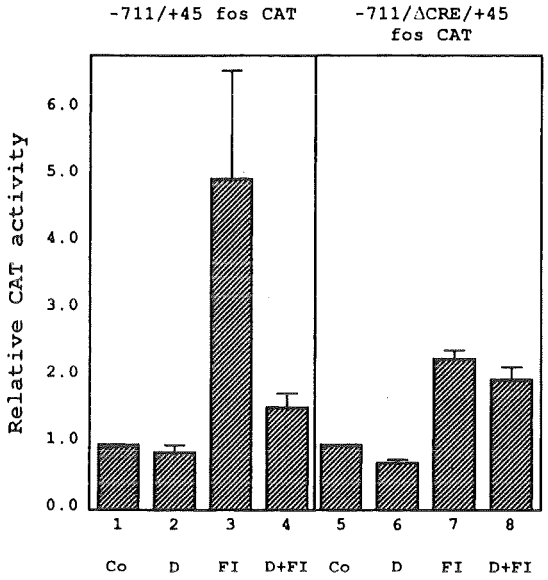
4.2.1. The Repression of cAMP-Induced c-*fos* Expression by Glucocorticoids Is Mediated Through the Major CRE in the c-*fos* Promotor

One major element mediating cAMP induced c-*fos* transcription is the proximal cAMP responsive element (-60 CRE) which binds CREB (Sassone-Corsi et al., 1988; Härtig et al., 1991). Upon destruction of this element by either 5' deletion or by internal mutation, cAMP induction of the c-*fos* promotor is reduced, but not lost completely (Büscher et al., 1988; Fisch et al., 1989; Berkowitz et al., 1989; Härtig et al., 1991; and see also fig. 8). When cloned as single elements in front of heterologous, non-responsive promotors, several other sections of the c-*fos* promotor confer cAMP inducibility. These include the cAMP-inducible element located in the transcribed, but not translated region of the c-*fos* gene (+18/+38, Härtig et al., 1991), the 5´-CTGCGTCA-3´ motif (positions -295/-288) and the 5´-TCCCGTCA-3´ motif (positions -343/-336), both found by Berkowitz et al. (1989). The contributions to the cAMP response of these diverse portions of the promotor in their natural context are only poorly understood.

In order to investigate which element in the c-*fos* promotor mediates glucocorticoid-induced inhibition, the inducibility by cAMP and the inhibition by glucocorticoids of a wild type *fos* promotor gene construct (-711/+45 *fos* CAT) and a gene construct with a deleted CREB binding site at position -60 (-711/ΔCRE/+45 *fos* CAT) were compared (**fig. 27**). The wild type promotor was induced around 5-fold by cAMP (lane 3). This induction was strongly inhibited by glucocorticoids (lane 5), suggesting that the promotor-reporter gene construct contains all of the elements required for the response. Upon destruction of the CRE at position -60, the response of the promotor to cAMP was strongly diminished, but not abolished (compare lanes 3 and 7). This residual induction was not affected by glucocorticoids (lanes 7 and 8). These data suggest that the repression of cAMP-induced c-*fos* transcription by glucocorticoids is mediated through the CRE at position -60 and that cAMP induction through the residual element(s) of the c-*fos* promotor is not influenced by glucocorticoids.

Fig. 27: The repression of cAMP-induced c-*fos* expression by glucocorticoids is mediated through the major CRE in the c-*fos* promotor. NIH 3T3 cells were transfected with 10 µg -711/+45 *fos* CAT or -711/Δ-65/-52/+45 *fos* CAT promotor constructs together with 1 µg RSV GR expression vector as described in Materials and Methods. The cells were serum starved for 10 hours post-transfection and then stimulated with forskolin and IMX (10 µM

and 0.5 mM), with (D+FI) or without (FI) 10^{-7} M dexamethasone (D) given 30 min before the cAMP induction, for an additional 36 hours. Relative CAT activities (induction factors) were determined as described in Materials and Methods. Results of seven (-711/+45 *fos* CAT) or five (-711/Δ-65/-52/+45 *fos* CAT) independent repetitions (±SEM) are presented.



4.2.2. The Repression of cAMP-Induced c-*fos* Transcription by Glucocorticoids is Mediated Through the Interference of the Activated Glucocorticoid Receptor and CREB

The glucocorticoid-induced repression of the activity of leucine zipper proteins belonging to the AP-1 family is mediated through the activated glucocorticoid receptor (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990; for reviews see Cato et al., 1992; Herrlich & Ponta, 1994). In view of this, I investigated whether the activated glucocorticoid receptor is also involved in the repression of the c-*fos* gene. In order to answer this question, several glucocorticoid receptor-negative cell lines (murine F9 teratocarcinoma cells, CV-1 cells, COS-7 cells) were transfected with the -711/+45 c-*fos* promotor-CAT construct, with and without an expression vector coding for the human glucocorticoid receptor. However, the cell lines used proved to be non-responsive to cAMP in transient transfection assays with the -711/+45 c-*fos* promotor-CAT construct (not shown). Turning back to NIH 3T3 cells, the observation was made that upon transfection of high amounts (10 μg) of the reporter, cAMP inducibility was only weakly affected by glucocorticoids (in contrast to the transfection of 1 μg), suggesting that the endogenous factor required for repression is limiting. In order to check whether the glucocorticoid receptor is this limiting factor, increasing amounts of an expression plasmid encoding the glucocorticoid receptor were co-transfected with 10 μg of the reporter and tested for their ability to restore the glucocorticoid-induced repression (**fig. 28**). Indeed, upon co-transfection of NIH 3T3 cells with increasing amounts of the glucocorticoid receptor, cAMP

induced expression of the reporter was efficiently inhibited suggesting that the activated GR is a necessary intermediate component of repression. While cotransfection of 1 µg of GR did not influence cAMP-induced expression of a promotor with a deleted CRE, cotransfection of 5 µg of GR-encoding plasmid did, suggesting that probably a high amount of the activated GR also interferes with the transcription factors working at the residual CRE(s).

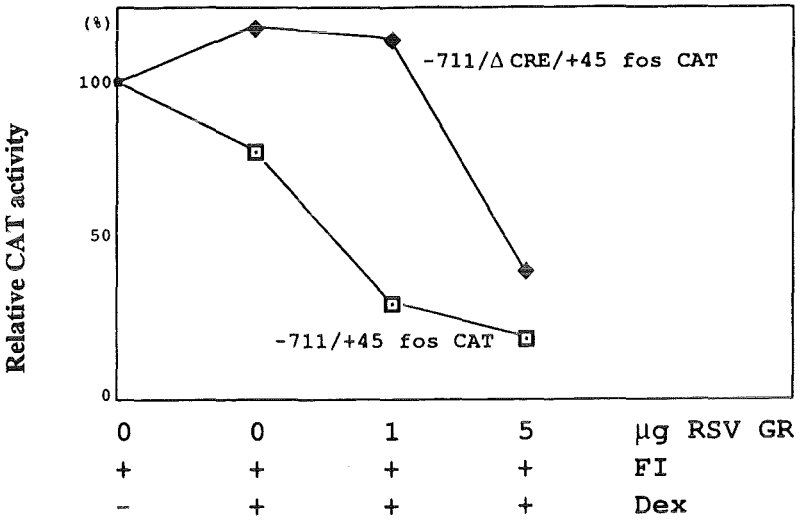


Fig. 28: The repression of cAMP-induced c-*fos* expression by glucocorticoids depends on the amount of activated GR. NIH 3T3 cells were transfected with 10 µg -711/+45 *fos* CAT or -711/Δ-65/-52/+45 *fos* CAT promotor constructs together with three different amounts of RSV GR expression vector (0, 1, and 5 µg) and treated as described in fig. 27. The cAMP-induced (in the absence of dexamethasone) promotor activity is considered 100%. FI - forskolin and IMX treatment. Dex - dexamethasone treatment.

Thus, the results presented in figures 27 and 28 indicate that the repression of cAMP-induced c-*fos* gene transcription by glucocorticoids is mainly mediated through the CRE element at position -60 and that it requires the activated GR. The -60 CRE of the c-*fos* promotor is constantly occupied by protein(s) even in non-stimulated cells (Runkel et al., 1991 and fig. 31) If, then, the repression occurs through interference between the c-*fos* CRE-bound CREB and the activated GR, it is possible that overexpression of CREB (i.e. by providing an excess of CREB that is not bound to DNA) may reduce this repression. That this is the case is seen in **fig. 29**.

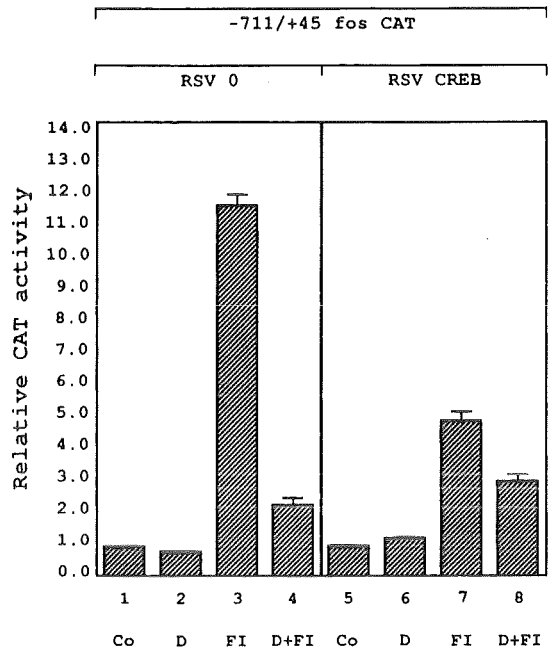


Fig. 29: Overexpression of CREB decreases the ability of dexamethasone to repress the cAMP-induced c-*fos* promotor activity. NIH 3T3 cells were transfected with 10 µg -711/+45 *fos* CAT promotor construct together with 1 µg RSV GR expression vector and 5 µg RSV 0 or RSV CREB expression vectors as described in Materials and Methods. The cells were serum starved for 10 hours post-transfection and then stimulated with forskolin and IMX (10 µM and 0.5 mM), with (D+FI) or without (FI) 10^{-7} M dexamethasone (D) given 30 min before the cAMP induction, for an additional 36 hours. Relative CAT activities (induction factors) were determined as described in Materials and Methods. Results of three independent repetitions (±SEM) are presented.

When overexpressed in NIH 3T3 cells, CREB reduced rather than increased the inducibility of the reporter by cAMP (compare lanes 3 and 7), possibly because of squelching of either a cAMP-dependent CREB kinase (PKA) or a coactivator. Most importantly, however, overexpression of CREB decreased the ability of dexamethasone to repress the cAMP-induced c-*fos* promotor activity (compare lanes 3 and 4 with lanes 7 and 8), suggesting that CREB which is not bound to DNA may interact with GR and prevent it from negatively interacting with CREB bound to the c-*fos* promotor.

4.2.3. The Activated GR Represses the c-*fos* Promotor without Binding to DNA

The finding that overexpression of CREB reduces glucocorticoid receptor-mediated repression of the c-*fos* promotor suggests that CREB and GR may interact without binding of the GR to the c-*fos* promotor. With regard to the repression of AP-1-dependent genes by glucocorticoids, two different mechanisms have been proposed for different genes. First, GR can

confer negative regulation upon binding to promotor sequences bearing overlapping GR and AP-1 binding sites, an example being the rat α-fetoprotein gene (Zhang et al., 1991). In this case, in the presence of both transcription factors, there is a competition for the same binding site by the two factors, which leads to mutual inhibition of each other's response. Second, the GR may interact directly or through a third partner with DNA-bound AP-1, thereby inhibiting its activity. This mechanism has been proposed for the repression of the collagenase gene (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990). Although by computer-aided search no binding sites for GR in the c-*fos* promotor could be found, I tried to address experimentally the question of whether DNA binding of GR was a prerequisite for the repression. The anti-glucocorticoid RU486 provides a suitable tool to distinguish between GR-mediated activities which require or do not require DNA binding. This drug abolishes the transactivation potential of the glucocorticoid receptor by forming an abortive ligand-receptor complex with a very low affinity to DNA, but still allowing the release of the receptor from the heat-shock protein/GR complex and translocation of GR into the nucleus (Heck et al., 1994). Thus, the ability of RU486 to cause changes in cAMP-induced c-*fos* expression, similar to those induced by dexamethasone, was investigated via Northern blot. **Figure 30** shows that RU486 repressed cAMP-induced c-*fos* transcription as efficiently as glucocorticoids (lanes 4-7). This suggests that the repression occurs without DNA-binding of GR, and that it most likely involves interference between the activated GR and CREB, the transcription factor mediating cAMP-inducibility of c-*fos*.

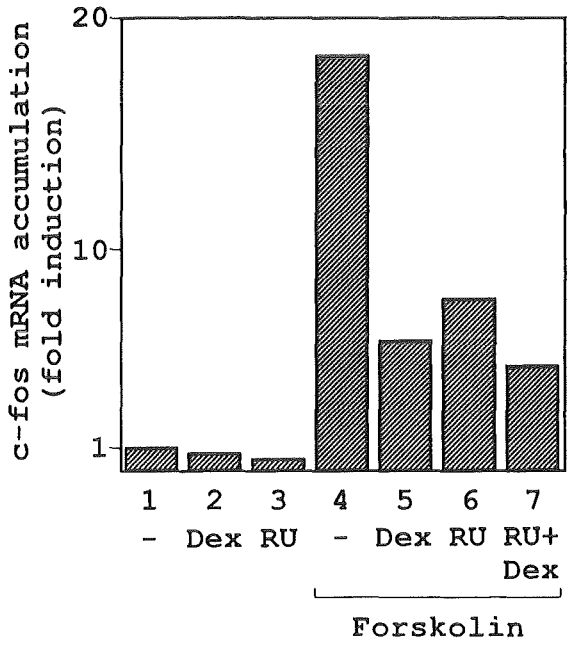


Fig. 30: The antiglucocorticoid RU486 represses cAMP-induced c-*fos* transcription as efficiently as glucocorticoids, suggesting that the repression occurs without DNA-binding of GR. Graphic representation

(normalized to the amount of GAPDH mRNA) of a Northern blot analysis. Quiescent NIH 3T3 cells were pre-treated with 10^{-7} M dexamethasone (Dex), 10^{-7} M RU486 (RU), or both (RU+Dex, each 10^{-7} M) for 15 min or not pre-treated and then treated with 10 μM forskolin for 30 min. PolyA$^+$ mRNA was prepared as described in Materials and Methods. 5 μg polyA$^+$ mRNA were analysed in Northern blot hybridisation using radioactively labeled c-*fos*- and GAPDH-specific DNA probes as described in Materials and Methods.

I further examined the mechanism(s) by which the activated GR and CREB mediate the glucocorticoid-induced repression of c-*fos*. The following possibilities could be considered:

1. The activated GR interferes with the binding of CREB to the c-*fos* CRE.
2. The activated GR interferes with cAMP-induced phosphorylation of CREB at serine 133.
3. The repression of the cAMP-induced c-*fos* transcription by glucocorticoids results from interference between the activated GR and CBP, the co-activator of CREB. In such a case, CBP but not CREB would be the direct target of repression. Although the results from the experiments in which CREB was overexpressed (fig. 29) do not support such a hypothesis, it was worthwhile to address this question experimentally.
4. The activated GR interferes with the transactivation properties of CREB without affecting the DNA binding or the phosphorylation of CREB. The most likely event in such a scenario would be GR-induced conformational changes in the CREB molecule which, in turn, would prevent the formation of a proper CREB-CBP-basal transcriptional machinery complex.

The results presented below strongly argue against the first and the second hypotheses. Furthermore, with the approach chosen to address the third possibility, no evidence was found in its support. I, therefore, conclude that the experimental data available tend to favor the forth hypothesis.

4.2.4. Glucocorticoids Do Not Alter the Pattern of Occupation of the c-*fos* CRE *in vivo*.

In order to investigate whether the activated GR interferes with the DNA-binding properties of CREB, genomic dimethyl sulfate (DMS) footprinting analyses of the c-*fos* CRE in NIH 3T3 cells were performed. This method allows *in vivo* monitoring of the binding of sequence-specific DNA-binding proteins (such as transcription factors) to their recognition sites in the promotors of the genes of interest. Runkel et al. (1991) have shown that, in A431 cells, the human c-*fos* CRE is constitutively occupied by protein(s) and that this occupation is not altered under conditions of cAMP stimulation. In the experiment presented below this observation was extended to the mouse c-*fos* CRE in NIH 3T3 cells (**fig. 31**). Neither at 15 min nor at 60 min after treatment of the cells with forskolin there was a change in the occupation of the c-*fos* CRE as compared to non-treated cells (compare lane 2 with lanes 3 and 4 and lane8 with lanes 9 and 10). More importantly, dexamethasone pre-treatment of the cells did not alter the pattern of *in*

vivo occupation of the CRE (compare lanes 3 and 6 and lanes 9 and 12). This indicates that the activated glucocorticoid receptor is not able to prevent the binding of CREB to its recognition sequence in the c-*fos* promotor. It cannot be ruled out, however, that upon activation of GR a rapid exchange of factor(s) bound to the CRE takes place. Also, when using nuclear extracts from forskolin- or forskolin plus dexamethasone-treated cells (with the c-*fos* -60 CRE as a probe in gel retardation analyses), no decreased c-*fos* CRE-binding activity in glucocorticoid treated cells could be detected (not shown).

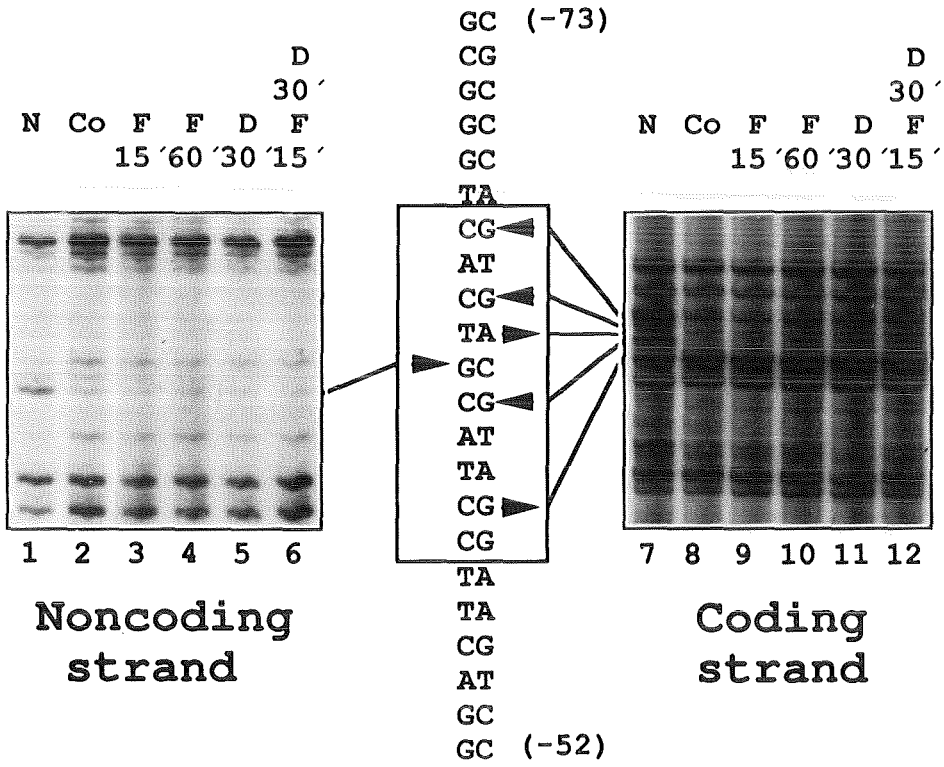


Fig. 31: Genomic (DMS) footprint analysis shows that, in NIH 3T3 cells, the *in vivo* occupation of the c-*fos* CRE is not altered under conditions of forskolin and/or dexamethasone treatment. The sequence of the mouse c-*fos* CRE is shown in the middle. The left-pointing arrows indicate the protected *in vivo* G bases, the right-pointing ones - the hypermethylated *in vivo* G or A bases. N - "naked", *in vitro* methylated DNA. Co - *in vivo* methylation pattern in untreated cells. F - *in vivo* methylation pattern after forskolin (10 µM, 15 or 60 min) treatment. D - *in vivo* methylation pattern after application of dexamethasone, either alone (30 min) or 15 min prior to the forskolin treatment.

4.2.5. Glucocorticoids Do Not Prevent PKA-Mediated Phosphorylation of CREB at Serine 133.

Upon cAMP stimulation of cells, the inactive form of CREB is activated via phosphorylation of the protein by the cAMP-dependent Protein Kinase A at the essential serine residue 133 (Gonzales & Montminy, 1989). To examine whether glucocorticoid treatment

interfere with this process, immunofluorescent staining and immunoblot analysis with the antibody exclusively recognizing the phosphorylated form of CREB (and of ATF-1, see Results, Part One, 3.1.1) were performed. The immunofluorescent staining of NIH 3T3 cells using this polyclonal serum directed against the serine 133-phosphorylated form of CREB showed a low level of CREB serine 133 phosphorylation in the nuclei of control and dexamethasone-treated cells (**fig. 32**, dark nuclei) and a transition to the phosphorylated state of CREB in the nuclei of the cells treated either with forskolin alone or with forskolin after dexamethasone pre-treatment (positively stained nuclei).

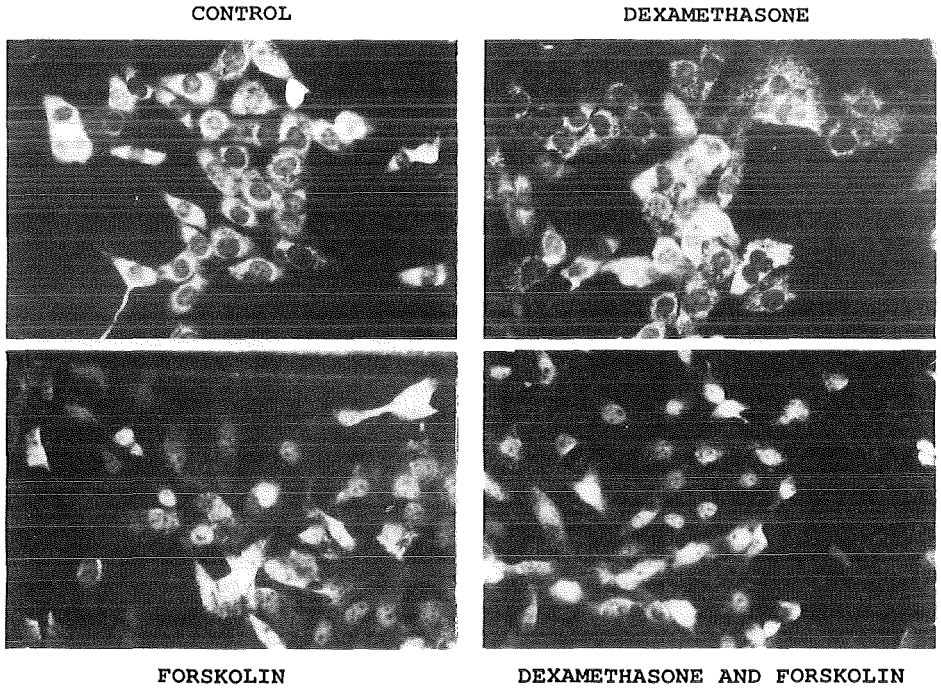


Fig. 32: Glucocorticoids do not prevent PKA-mediated phosphorylation of CREB at serine 133 (immunofluorescent analysis). NIH 3T3 cells were serum starved for 24 hours (DMEM, 0.5% FCS), pre-treated with dexamethasone (10^{-7} M) for 15 min or not pretreated, and then stimulated with forskolin (10 μM). 15 min later the cells were fixed in 4% *p*-formaldehyde and processed for immunofluorescent analysis as described in Materials and Methods, using the antibody against phosphorylated at serine 133 CREB. The dark stained nuclei are indicative of the non-phosphorylated state of CREB. The serine 133 phosphorylation is manifested by a positive nuclear staining.

An immunoblot analysis (**fig. 33**) with the same antibody and the same treatment conditions proved that the increased nuclear signal after cAMP and dexamethasone plus cAMP stimulation is, by and large, due to phosphorylation of CREB and only partially due to phosphorylation of the CREB-related transcription factor ATF-1 recognized by the same antibody (W. Schmid, personal communication, see also Results, Part One, 3.1.1). These results

clearly indicated that glucocorticoids repress the cAMP-induced c-*fos* transcription without interfering with the cAMP-induced CREB phosphorylation.

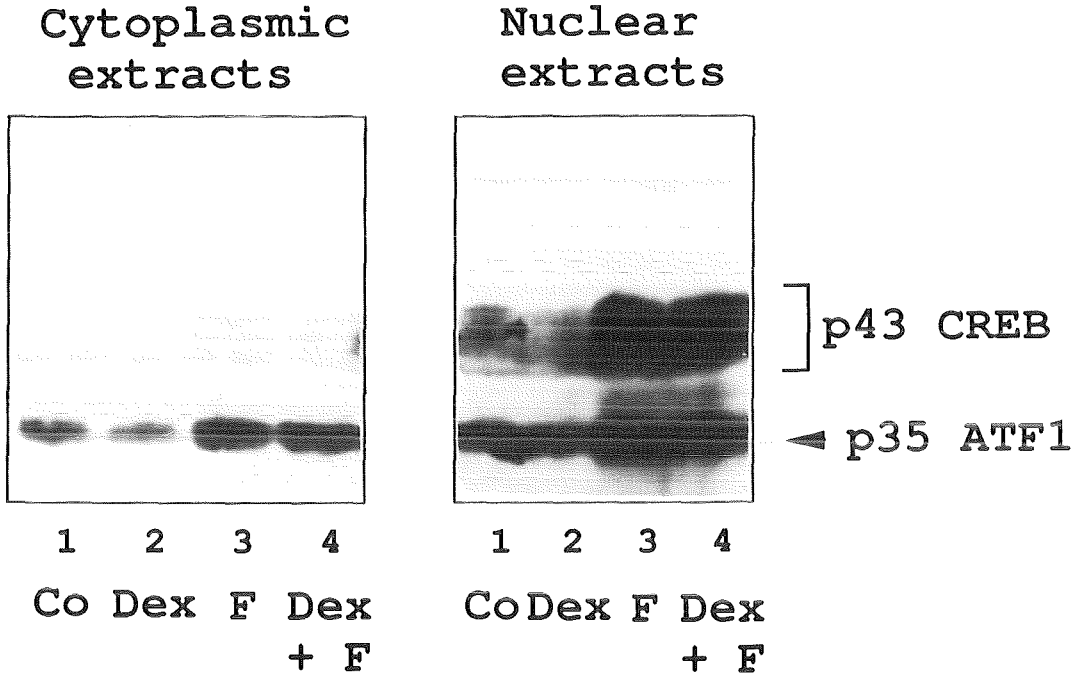


Fig. 33: Glucocorticoids do not prevent PKA-mediated phosphorylation of CREB at serine 133 (immunoblot analysis). NIH 3T3 cells were treated as in fig. 32 except that, 15 min after the forskolin treatment, the cells were harvested and nuclear and cytoplasmic extracts were prepared as described in Materials and Methods. The PAGE and the immunoblot analysis were performed as described in Materials and Methods, using the same antibody as in fig. 32. The bands corresponding to phosphorylated CREB and ATF-1 are indicated.

4.2.6. Glucocorticoids Do Not Inhibit the Transactivation Potential of a GAL4-CBP Chimeric Protein

Several groups have tried to demonstrate direct protein-protein interactions between CREB and GR. Imai et al. (1993) could show co-immunoprecipitation of CREB with an anti-GR antibody using *in vitro* transcribed and translated proteins. Stauber et al. (1992), however, have made several attempts aimed at detecting direct protein-protein interactions between CREB and GR *in vitro*, but failed to do so. It is, therefore, possible to imagine a mechanism of interaction involving a third partner protein. Such a protein may be present in the reticulocyte lysates used for the production of *in vitro* transcribed and translated CREB and GR, thus allowing detection of an indirect interaction (CREB-X-GR). This may also imply a mechanism of repression in which the activated GR represses CREB-mediated transcription through interference with the function of such a third factor that is required for the CREB-dependent transactivation. Could CBP, the only known co-activator of CREB, be this limiting third partner? In order to experimentally address this question I used the GAL4-CBP chimeric protein which can enhance the transcription of a Gal4-CAT reporter construct independently of CREB (see Results, Part

One, 3.2.2) and investigated whether its transactivation potential is inhibited by the activated GR in NIH 3T3 cells. As shown in **fig. 34**, neither the basal, nor the induced (by co-transfecting the catalytic subunit of PKA) activities of GAL4-CBP were negatively affected by dexamethasone treatment, although the levels of GR in the cells were increased by co-transfection of a GR expression vector (lanes 9-12).

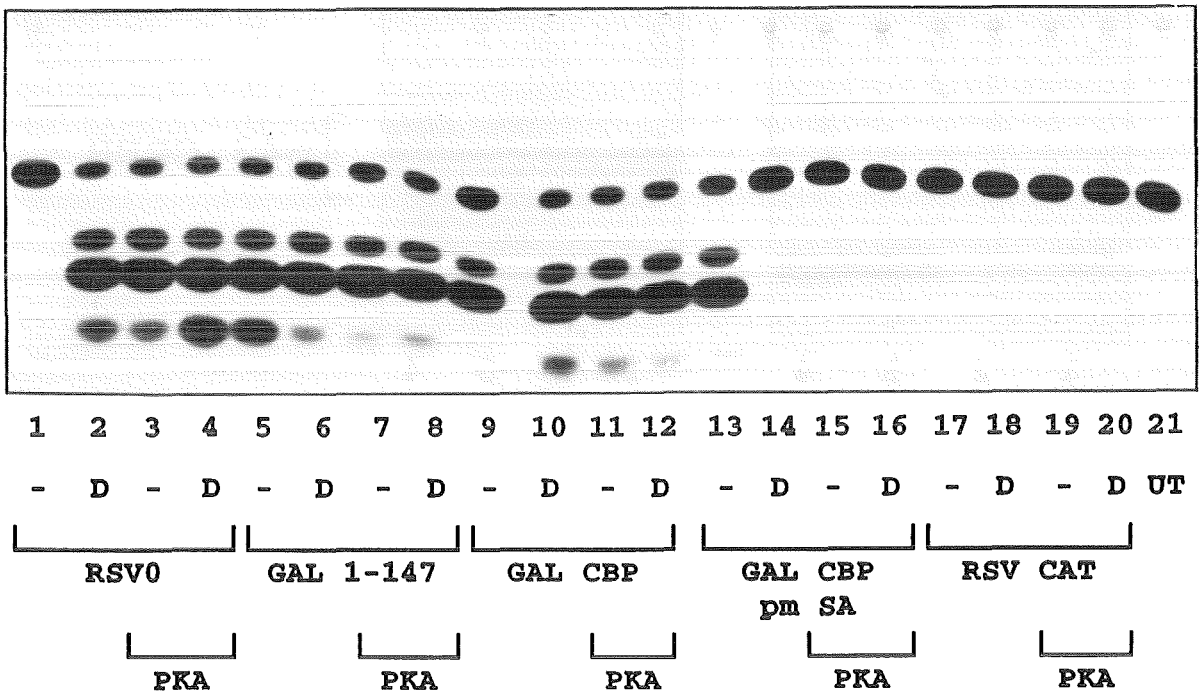


Fig. 34: Glucocorticoids do not inhibit the transactivation potential of a GAL4-CBP chimeric protein. NIH 3T3 cells were transfected with 2 μg GAL4 CAT reporter construct, together with 1 μg RSV GR expression vector and 3 μg of the indicated expression vectors (GAL4 1-147, GAL4 CBP or GAL4 CBP pm SA) as described in Materials and Methods. The cAMP induction was mimicked by co-transfection, where indicated, of an expression vector for the catalytic subunit of PKA. 10 hours post-transfection the cells were treated with 10^{-7} M dexamethasone (D) or not treated for 24 hours.

Interestingly, the activity of the chimeric protein was even increased by GR activation, indicating that CBP may indeed be a target for GR-mediated interference, but in a direction opposite to the effects of GR on c-*fos* transcription. It has been shown previously that, in PC12 cells, the induction of CBP by cAMP does not require direct phosphorylation of the only PKA consensus phosphoacceptor site in CBP, namely serine 1782 (Kwok et al., 1994). Here I observed an identical result in NIH 3T3 cells by using a non-phosphorylatable GAL4-CBP chimeric protein with a serine 1782-to-alanine 1782 substitution (lanes 13 and 15). Furthermore, the positive action of GR on the GAL4-CBP-dependent transcription was also not dependent on

this serine (lanes13-16). The actual induction of both the "wild type" and the mutant GAL4-CBP chimeras by PKA overexpression and the positive effect of GR on the PKA-induced GAL4-CBP transactivation may have been higher than observed, as PKA co-transfection appeared to slightly repress the RSV promotor directing the expression of both GR and CBP expression vectors (as shown by using a RSVCAT control transfection, lanes 17-20).

These results suggest that the C-terminal portion of CBP (amino acids 1678-2441), which bears the transactivation domain of the protein (Kwok et al., 1994), may not be a target for a GR-mediated repression. The positive effect of GR on the CBP-directed transcription deserves further investigation, as this is the first observation that a steroid receptor may interfere with the function of a co-activator of another transcription factor.

5. DISCUSSION

PART ONE

THE *cAMP RESPONSIVE ELEMENT BINDING PROTEIN* (CREB) AS A PARTICIPANT IN THE MAMMALIAN UVC RESPONSE

5.1. Phosphorylation of CREB at Serine 133 Is a Convergence Point for a Large Variety of Extracellular Signal Transduction Pathways

For a long time, the phosphorylation of CREB at serine 133 was regarded as an exclusive property of signal transduction pathways highly specialized in mediating the biological effects of two common "second messengers", namely cAMP and Ca^{2+} (Gonzales & Montminy, 1989; Sheng et al., 1990 and 1991; for review see Lee and Masson, 1993). Consistently, CREB was found to bind to and operate through a specific promotor sequence termed CRE (for cAMP Responsive Element, Montminy et al., 1986; Montminy &Bilezikjian, 1987) or CaRE (for Ca^{2+} Responsive Element, Sheng et al., 1988). With very few exceptions (e.g. the somatotroph cells of the anterior lobe of the pituitary), cAMP is not a mediator of mitogenic, growth-, or tumor-promoting responses in many cell types. Neither CREB nor any of its family members possess oncogenic, cell-transforming or cell-immortalizing properties, as is the case for many transcriptional activators addressed by growth factors, tumor promotors and adverse environmental agents (Herrlich & Ponta, 1989; Herrlich & Rahmsdorf, 1994), the best studied example being the AP-1 complex (Fos/Jun, Angel and Karin, 1991). For these reasons it seemed unlikely that CREB and CREB family members might be addressed by extracellular stimuli promoting, for instance, proliferative responses. Only in the last year it has become obvious that this view of CREB and its relatives deserves significant re-consideration. Ginty et al. (1994) showed that, in PC12 pheochromocytoma cells, a *ras*-dependent signal transduction pathway initiated by nerve growth factor (NGF) stimulation causes phosphorylation of CREB at serine 133, and that this phosphorylation contributes to the NGF-induced c-*fos* transcriptional activation. Furthermore, Böhm et al. (1995) described growth factor-induced CREB serine 133 phosphorylation in primary human melanocytes and de Groot et al. (1994) reported increased phosphorylation of serine 117 of CREMτ (which functionally corresponds to serine 133 of CREB) in response to serum stimulation of quiescent cells.

In this work I have described a novel and very potent inducer of CREB serine 133 phosphorylation, namely short-wavelength ultraviolet radiation (UVC). Furthermore, I have reported that this serine residue is a point of convergence for many extracellular signal transduction pathways initiated by numerous other stimuli, such as certain growth factors, pro-inflammatory cytokines, and phorbol esters. **Table III** summarizes the knowledge to date on extracellular signals and "second messengers" inducing CREB serine 133 phosphorylation.

Table III

Extracellular Stimuli and "Second Messengers" Causing Phosphorylation of CREB at Serine 133	**Reference**
cAMP	Gonzales & Montminy (1989)
Free cytosolic Ca^{2+}	Sheng et al. (1990; 1991) and this work
Nerve Growth Factor	Ginty et al. (1994)
Epidermal Growth Factor	Ginty et al. (1994) and this work
Basic Fibroblast Growth Factor	Ginty et al. (1994) and this work
Brain-Derived Neurotrophic Factor	Ginty et al. (1994)
Interleukin-1α	This work
Tumor Necrosis Factor-α	This work
Extracellular Protein Synthesis Inducing Factor (EPIF)	This work
TPA	This work
UVC	This work
UVB*)	A. Knebel (personal communication)
Cycloheximide	B. Magun (personal communication)
Cellular Stress (e.g. serum deprivation of F9 teratocarcinoma stem cells)	M. Iordanov (unpublished)

*)The UVB dose sufficient to induce, in HeLa cells, a well manifested CREB serine 133 phosphorylation (500 J/m^2), corresponds approximately to the amount of solar UV radiation a person would be exposed to during a 10 min walk on a sunny summer day at the latitude of New York. This dose does not suffice to induce a detectable p42 MAP kinase activation in these cells, but is able to induce c-*fos* transcription (A. Knebel, personal communication).

5.2. The Extracellular Stimuli Which Cause CREB Serine 133 Phosphorylation Differ in Their Ability to Promote CREB-Dependent Transcription

The problem of whether certain post-translational modifications of a transcription factor play a role in the transactivation mediated by this factor is complex and may be roughly divided into two questions:

(i) does the post-translationally modified transcription factor *participate* in the overall transcriptional induction of a natural promotor in response to a certain stimulus? (This implies the situation in which the post-translationally modified transcription factor *cannot* alone promote transcription, but can do so in a co-operation with other factors whose binding sites are present in the promotor, and which are also addressed by the stimulus.)

(ii) does the postranslational modification studied *suffice* to induce the transactivation potential of the transcription factor? (In such a situation the post-translationally modified transcription factor *can* alone promote transcription independent of other co-operating factors.)

To answer the first question, studies with promotor mutants of the gene of interest are usually performed. The role of the transcription factor in mediating the transcriptional induction of this gene is elucidated by mutating the binding site(s) of *this* factor or by overexpressing interfering mutants of the factor (examples are the experiments shown in fig. 8, and see below table IV, panel I).

To answer the second question, the function of the transcription factor is studied on a single or multiple binding sites of the factor, cloned in front of a minimal promotor (e.g. a TATA box), by mutating *other* responsive elements important for the induction by the same stimulus in the promotor of interest (table IV, panel II), or by using chimeric proteins, conferring a different DNA-binding specificity to the factor (e.g. GAL4 DBD-transcription factor chimeras, as shown in fig. 9, and table IV, panel III).

All these approaches have been used in this and other works (references in table IV) with the task of investigating the role of the CREB serine 133 phosphorylation in response to different stimuli. The results of these studies are summarized in **Table IV**. As shown in this table, the inducers of CREB serine 133 phosphorylation may be classified, according to the criteria outlined in (*i*) and (*ii*), in the following three groups:

Group I: cAMP and Ca^{2+}. These inducers promote CREB-dependent transcription even in the absence of a natural promotor context.

Group II: UVC. CREB participates in the activation of the full lenght c-*fos* promotor by UVC (fig. 8). However, the CRE is not sufficient to confer UVC inducibility to a c-*fos* promotor lacking the SRE and the region between +18/+38 (Büscher et al., 1988). UVC also cannot induce transcription from a minimal CRE construct, but is able to stimulate a GAL4 CREB chimeric transcription factor (in JEG-3 cells, fig. 9, and also in HeLa cells, M. Iordanov, unpublished), suggesting that, under certain conditions (like, perhaps, a beneficial conformation in the case of the GAL4 chimera), the serine 133 phosphorylation is sufficient to promote transactivation.

Group III: Growth factors such as EGF and, probably, NGF. These inducers promote CREB-dependent transcription only in the context of an intact c-*fos* promotor and require, most likely, co-operation with other responsive elements and transcription factors to induce transcription.

Table IV

Function of CREB	**Stimulus Causing Serine 133 Phosphorylation**		**Reference**
I. CREB- and CRE-dependent transcriptional induction in the context of the intact c-*fos* promotor (i.e. containing all other responsive elements)	cAMP	+	Sassone-Corsi et al. (1988); Fisch et al. (1989); Berkowitz et al. (1989); Härtig et al. (1991)
	Ca^{2+}	+	Sheng et al. (1990, 1991)
	NGF	+	Ginty et al. (1994)
	EGF	+	M. Iordanov (unpublished)
	TPA	n.d.	-
	UVC	+	This work (fig. 8)
II. Ability to transactivate: 1) minimal CRE constructs (e.g. CRE TATA CAT, CRE tk CAT, -71 somatostatin CAT) 2) a c-*fos* promotor construct with mutated SRE and +18/+38 elements	cAMP[1)2)]	+	Gonzales & Montminy (1989) Härtig et al. (1991) Büscher et al. (1988)
	calcium[1)]	+	Sheng et al. (1991)
	NGF[1)]	-	Ginty et al. (1994)
	EGF	n.d.	-
	TPA	n.d.	-
	UVC[1)2)]	-	Büscher et al. 1988; H. J. Rahmsdorf (unpublished); M. Iordanov (unpublished)
III. Ability to transactivate a GAL4 reporter gene by a GAL4 CREB chimeric protein	cAMP	+	Hurst et al. (1991)
	Ca^{2+}	+	Sun et al. (1994)
	NGF	n.d.	-
	EGF	-	M. Iordanov (unpublished)
	TPA	-	M. Iordanov (unpublished)
	UVC	+	This work and M. Iordanov (unpublished)

+ = function induced, - = function not induced, n.d. = not determined

What makes the extracellular signals, all causing similar levels of serine 133 phosphorylation, so different with respect to their ability to induce the transactivation potential of CREB? As already mentioned in 3.2.2, there are two most likely possibilities:

1. "Weak" inducers of CREB-dependent transcription may cause, in addition to serine 133 phosphorylation, other modifications of CREB (e.g. phosphorylation at different residues), which may, in turn, negatively regulate its transactivating potential. Examples of repressing

phosphorylation events have been already reported: the calcium/calmodulin-dependent kinase type II (CaM kinase II) phosphorylates CREB, in addition to serine 133, at the neighboring serine residue 142, and this phosphorylation was found to inhibit the activity of CREB (Sun et al., 1994). Another example is provided by cJun. Phosphorylation of serine and threonine residues, mapped close to the DNA-binding domain of the protein, decrease its DNA-binding activity and, thereby, cJun transactivation properties (Boyle et al., 1991).

2. "Weak" and "strong" inducers of CREB-dependent transcription may differ in their ability to address (activate) the co-activator of CREB, CBP. When fused to the DNA binding domain of GAL4, CBP can activate transcription in a PKA-dependent manner (Chrivia et al., 1993; Kwok et al., 1994). This finding suggests that CREB and its co-activator are *both* regulated in their activity by cAMP, and that this co-regulation is required to achieve maximum transcriptional induction.

While in JEG-3 cells results supporting the second possibility, were obtained (fig. 9), some recent experiments in HeLa cells argue against it. When transfected in these cells, a RSV-driven GAL4 CREB chimera was induced to transactivate a GAL4 CAT reporter construct by cAMP (forskolin and IMX) treatment or by UVC, but not by EGF or TPA treatments. A GAL4 CBP construct, however, was induced to transactivate by all the agents, with EGF being the most potent inducer (M. Iordanov, unpublished).

With regard to the UVC inducibility of c-*fos*, the data available to date suggest that although CREB and the -60 CRE contribute about 50% to the overall induction of the c-*fos* promotor by UVC (since deletion of this CRE or overexpression of the dominant negative CREB mutant decrease the inducibility by about 50%, fig. 8), they can do so only in concert with other factors binding to the c-*fos* promotor (since a reporter gene construct whose expression is directed by a single CRE is not inducible by UVC, H. J. Rahmsdorf, unpublished, M. Iordanov, unpublished, and since a c-*fos* promotor construct lacking the SRE and the +18/+38 element but containing the -60 CRE is also not UVC responsive, Büscher et al., 1988). The possible biological meaning of these complicated control mechanisms of the c-*fos* transcriptional induction by UVC will be discussed in **5.6**.

5.3. Is the UVC Induced Signal Transduction to CREB Initiated by Activation of Growth Factor Receptors?

Two major hypotheses, mainly based upon the nature of the hypothetical "primary target of UVC absorption", have been proposed to explain the initial events of the UVC-induced signal transduction to the nucleus:

1. DNA damage-dependent signal transduction (reviewed by Herrlich et al., 1992; Herrlich & Rahmsdorf, 1994; Blattner et al., 1994). According to this hypothesis, UVC-induced DNA lesions (in the nucleus or, as sometimes speculated, in mitochondria) serve as a

recognition signal (or at least as an intermediate step) which triggers the UVC response. An attractive and challenging assumption of this hypothesis is that, in such a case, the UVC response would probably require a *"reverse"*, nucleus-to-membrane signaling, as membrane components (e.g. p21 Ras, Devary et al., 1992, 1993) and cytosolic enzymes (e.g. Raf-1, Radler-Pohl et al., 1993; and Src kinases, Devary et al., 1992) seem to be obligatory mediators of the UVC response. Apart from the obvious overlap between the action spectra of UVR and the ability of UVR to cause DNA damage (see Introduction), there is substantial experimental evidence in support of this hypothesis, mainly based on investigations of the UV response in cells with defined deficiencies in repairing certain types of DNA lesions (Stein et al., 1989b; Yarosh et al., 1993; H. J. Rahmsdorf, unpublished). However, while the involvement of DNA damage in the late UVC response (as measured by induction of the collagenase I gene and of transiently transfected reporter gene-CAT constructs) seems to be sufficiently proven, its role in mediating the early UVC effects (e.g. induction of plasma membrane-associated and cytosolic kinases) is still a controversial subject.

2. Plasma membrane-dependent signal transduction. According to this hypothesis, the primary targets of UVC absorption are located in or at the plasma membrane and may be, for instance, lipid components of the membrane, transmembrane proteins or membrane-associated proteins. The UVC response in such a case would be independent of DNA damage and would not require a "reverse" signal transduction. The main arguments in support of this hypothesis are:

-the very fast (within 1-5 min post-irradiation) response to UVC of certain membrane components (as shown, for instance, for the tyrosine phosphorylation of the EGF receptor, Sachsenmaier et al., 1994);

-the ability of enucleated cells to display a "cytoplasmic UVC response" (e.g. UVC-induced *in vitro* DNA-binding activity of NF-κB, Devary et al., 1993).

Although the hypothetical membrane primary targets of UV radiation have not yet been defined, there is already some evidence about what the first functional step in the initiation of the UVC response might be. Systematically following the UVC-induced signal transduction pathways in the "upstream" direction (induced genes - activated transcription factors - nuclear and cytoplasmic kinases - membrane components such as p21 Ras), striking similarities between the UVC- and growth factor-initiated signal transduction pathways have been observed (references in 1.2.3 and 3.3.3). Thus, the idea has emerged that the most "upstream" components of the UVC signal transduction chains may be also common for UVC and growth factors. Such common components were identified to be, for instance, growth factor receptors (Sachsenmaier et al., 1994).

Where and how is, in view of the above mentioned hypotheses, the UVC-induced signal transduction to CREB initiated? The possible role of the DNA damage in this process was only partially addressed. Cells deficient in the repair of DNA double strand breaks appeared to

induce CREB phosphorylation upon UVC irradiation as well as the respective wild type cells (not shown). As this kind of DNA damage is atypical for UVC, the results of these experiments cannot be used to rule out the possibility that UVR-damaged DNA may initiate a signal transduction chain leading to CREB serine 133 phosphorylation.

However, the ability of growth factor receptors to serve as major mediators of the response of CREB to UVC was thoroughly investigated in this thesis [Results (Part One), 3.3.3]. Several receptors, previously implicated in the UVC response (Sachsenmaier et al., 1994), and whose activations themselves induce CREB phosphorylation (fig. 7), were investigated in this study. Down-modulation of an individual growth factor receptor (EGF-R, fig. 11A) or of a set of them (the ones for EGF, bFGF and IL-1α, fig. 13) rendered CREB non-responsive to stimulation with the respective growth factors, while allowing full responsiveness to UVC. Blocking of receptors by the receptor inhibitor suramin completely abolished the ability of EPIF (conditioned medium from UVC-irradiated cells, whose activity is mainly ascribed to secreted growth factors) to induce CREB phosphorylation, but had no effect on the same phosphorylation induced by UVC (fig. 14). The tyrosine kinase inhibitor tyrphostin-51 inhibited the EGF-induced CREB phosphorylation but again had no effect on the respective UVC response (fig. 15). Furthermore, phorbol ester pre-treatment appeared to interfere strongly with the EGF-induced, but not with the UVC-induced phosphorylation of CREB (fig. 16). It is noteworthy that, in almost all of these experiments, an endpoint for UVC signal transduction, namely the p42 MAP kinase, whose activation by UVC has been shown to depend on growth factor receptor induction (Sachsenmaier et al., 1994), behaved indeed in a growth factor receptor-dependent manner. These results strongly argue against the involvement of the growth factor receptors studied in mediating the UVC-response of CREB. On the other hand, EGF and UVC induce, in HeLa cells, the same 108 kD kinase that is responsible for the phosphorylation of CREB at serine 133 (fig. 23).

For these reasons, I conclude that the UVC-induced signal transduction pathway leading to CREB serine 133 phosphorylation only partially shares common components with the signal transduction routes used by certain growth factors (in particular EGF). The point of convergence of these routes is *downstream* of the growth factor receptors but it is *upstream* of CREB itself and, probably, of the 108 kD CREB kinase (for a schematic representation see **5.6, fig. 36**). Current studies aim at detecting this convergence point more precisely (e.g. by using interfering mutants of some known members of the UVC- and GF-induced signalings, such as p21 Ras). There are additional considerations that suggest this conclusion should be made with caution. Obviously, no experimental approaches are available to date which can block the function of *all* types of receptor molecules at the cell surface. Furthermore, only some (and, probably, a minor part) of the UVC-addressed growth factor receptors are known. On the other hand, it is unlikely that all of the modifications of cell surface proteins induced by an adverse physical agent such as UVC will prove to cause functional activation of these proteins.

5.4. How is the UVC-induced CREB phosphorylation attenuated - UVC-regulated phosphatase activity?

Every extracellular signaling has to be, at a certain time point, attenuated. This allows the cell to return to the non-stimulated state and to be re-stimulated, if required, by the same or another stimulus. In the case of transcription factors regulated by phosphorylation, the attenuation is usually achieved by dephosphorylation via specific phosphatases (reviewed by Hunter & Karin, 1992; Karin, 1994; Hunter, 1995).

The attenuation of CREB serine 133 phosphorylation has been intensively studied only with respect to the cAMP-induced, PKA-mediated phosphorylation event. Two reasons have been found to account for the dephosphorylation of CREB (Hagivara et al., 1992). First, following the dissociation of ligand or receptor down-modulation, cAMP levels drop and the catalytic subunit of PKA is inactivated through binding to the regulatory subunit. Second, this drop in PKA activity results in a net increase in phosphatase activity, leading to dephosphorylation of CREB. Most available data indicate that protein phosphatase 1 (PP-1) is the relevant CREB-phosphatase (Hagiwara et al., 1992). Virtually nothing is known about CREB dephosphorylation after Ca^{2+}, growth factor, or UVC stimulation. The finding, however, that EGF and UVC phosphorylate CREB through the same kinase and that the kinetics of induction of this kinase is similar for both stimuli (3.3.5, fig. 23), may help us to shed some light upon the involvement of phosphatases in regulating the UVC-induced signal transduction to CREB. **Figure 35** shows the time-dependence of serine 133 phosphorylation of CREB after UVC (upper panel) and EGF (lower panel) stimulation of HeLa cells. It is clearly seen that the EGF-induced phosphorylation is rapidly attenuated and that 45 min after induction almost no phosphorylated CREB is detectable. The UVC-induced phosphorylation, however, lasts much longer and decays to levels corresponding to 45 min EGF treatment at a time point 6 hours after UVC irradiation. Taking into consideration that, 4 hours after either EGF or UVC treatment the UVC- and EGF-inducible kinase shows no detectable activity (fig. 23), these results strongly suggest that the differences in the EGF- and UVC-induced levels of CREB phosphorylation at later time points are due to differences in a phosphatase activity. It may be speculated that either EGF *induces* a CREB-specific phosphatase or UVC *inhibits* such a phosphatase. Turning back to the questions discussed in 5.2, one can speculate that the longer persistance of CREB phosphorylation after UVC treatment might be yet another reason why UVC but not EGF activated the transactivation potential of GAL4 CREB.

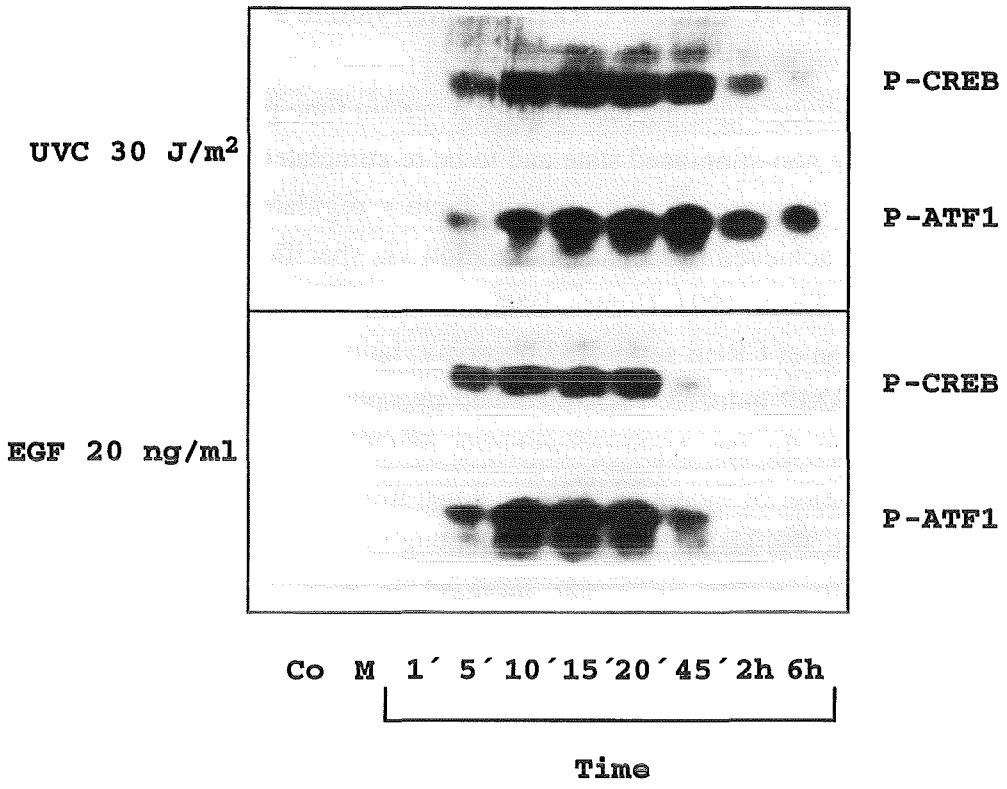


Fig. 35: The phosphorylation of CREB (and ATF-1), induced by UVC, persist longer than the EGF-induced phosphorylation. Serum starved HeLa tk^- cells were stimulated with UVC (30 J/m^2, upper panel) or EGF (20 ng/ml, lower panel) and cell extracts were prepared at 1 min, 5 min, 10 min, 20 min, 45 min, 2 h, and 6 h post-stimulation. Both stimuli induced a detectable CREB phosphorylation 5 min after treatment, which reached maximum values between 10 and 20 minutes. The EGF-induced phosphorylation was rapidly attenuated and 45 min after stimulation almost no phosphorylated CREB was detectable. Similar levels of attenuation could be seen 6 hours after the UVC irradiation. M - mock (15´) treated cells (only for the UVC treatment, the same lane in the lower panel represents untreated cells).

5.5. The UVC- and EGF-Induced CREB Kinase Most Likely Belongs to a Class of Serine/Threonine Kinases Different from the MAPK/JNK/SAPK Superfamily of "Proline-Directed" Protein Kinases

With the exception of NF-κB, the protein kinases involved in the regulation of the transcription factors so far known to mediate the UVC response [AP-1 (Fos/Jun), Jun/ATF-2, and TCF/Elk-1] are relatively well investigated. Consistent with the current ideas about the UVC-induced signal transduction routes as overlapping, at least partially, with the pathways used by phorbol esters, certain growth factors, pro-inflammatory cytokines and oncogenes (Herrlich et al., 1992; Herrlich & Rahmsdorf, 1994), no *exclusively UVC-specific* kinases have been found. Rather, UVC appears to induce protein kinases committed primarily to phosphorylate transcription factors in response to "physiological" stimuli. These include members of the Mitogen-Activated Protein Kinases (MAPKs), and of the Jun N-terminal

Kinases (JNKs) (the latter are also referred to as Stress-Activated Protein Kinases, SAPKs) (references in Table V). Some of the current knowledge about the involvement of these kinases in the regulation of the group of UVC-activated transcription factors is summarized in **Table V**. The substrate specificity, the fact that they are activated through dual phosphorylation at adjacent threonine and tyrosine residues separated by one amino acid, and some distant homology in their primary structure indicate that the MAPKs and JNKs/SAPKs belong to a superfamily of serine/threonine protein kinases. A common feature of each of them is that they phosphorylate serine or threonine residues within a consensus motif **(P)-X-S*/T*-P-X**, where X is any amino acid (Davis, 1993; Karin, 1994). This motif is characterized by the presence of an invariant proline residue immediately succeeding the phosphoacceptor serine or threonine. For this reason these kinases are also referred to as "Proline-Directed Kinases" (Davis, 1993).

Could the UVC- and EGF-inducible CREB kinase (3.3.5, fig. 23) be a member of the MAPK/JNK/SAPK superfamily of kinases? Two facts indicate that this possibility is unlikely. First, the molecular weight of this kinase (108 kD) makes it impossible to be one of the known MAPK/JNK/SAPK members (although it might be a new member). Second, the phosphoacceptor site of CREB (and the corresponding sites of its closest relatives ATF-1 and CREM, see Introduction) is quite different from the consensus sequence phosphorylated by proline-directed kinases:

CREB: 130-**RRPS*YRK**-136
ATF-1: 60-**RRPS*YRK**-66
CREM: 114-**RRPS*YRK**-120

These phosphoacceptor sites lack the invariant proline and resemble those recognized by the cAMP-dependent protein kinase A (PKA), by the Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) and by the α, β, and γ isoforms of protein kinase C (PKC) (Kemp & Pearson, 1990 and references therein):

PKA: **xRRxS*x**
CaM kinase II: **xRxxS*x**
PKC: **xRxxS*xRx**

In the "evolutionary tree" of the protein kinases, based upon their primary structure, *PKA*, *CaM kinase II* and *PKC* appear to be placed on a separate branch of this tree together with the *cyclic GMP-dependent kinase* and the *myosin light chain kinase*, indicating that these kinases are relatives (Alberts et al., 1994). It is, therefore, possible that the 108 kD CREB kinase is another distant relative of this group of kinases, which, however, has acquired during evolution the properties enabling it to mediate the response of CREB to UV radiation and to growth factor signaling.

Table V

Transcription Factor (and inducers)	**Protein Kinase** (mol. weight)	**Phosphorylated Motif**	**Reference**
Elk-1 (phorbol esters, growth factors, H-*ras*, UVC)	MAP kinase-1 (42 kD) MAP kinase-2 (44 kD)	**TLT^{363}PV** **LLT^{368}PS** **TLS^{383}PI** **TST^{417}P** **VLS^{422}P**	Marais et al. (1993); Zinck et al. (1993) Radler-Pohl et al. (1993); Sachsenmaier et al. (1994)
cJun (phorbol esters, growth factors, H-*ras*, DNA-damaging agents, UVC)	JNK1 (46 kD) JNK2 (55 kD) (SAPKs) MAPKs (?)	**LTS^{63}PD** **LAS^{73}PE**	Hibi et al. (1993); Derijard et al. (1994); Kyriakis et al. (1994); Pulverer et al. (1991); van Dam et al. (1995)
cFos (EGF, H-*ras*)	FRK (Fos-regulating kinase, 88 kD)	**AST^{232}PE**	Deng & Karin (1994)
ATF-2 (pro-inflammatory cytokines, DNA-damaging agents, UVC)	JNK1/JNK2 or close JNK/SAPK family members	**NQT^{69}PT** **TPT^{71}PT**	Gupta et al. (1995); van Dam et al. (1995)

S^{X} or **T^{X}** represent the phosphoacceptor serine or threonine with its position in the primary structure of the protein.

5.6. CREB and the -60 CRE in the Regulation of the UVC-induced c-fos Transcription: Redundancy or Necessity?

Why should UVC target the CRE of the c-*fos* promotor (3.2.1, fig. 8) if this promotor already possesses a *bona fide* UVC responsive element, namely the SRE (Büscher et al., 1988) which alone can confer UVC-inducibility to a heterologous UVC-irresponsive promotor (Sachsenmaier et al., 1994)? Furthermore, why do extracellular signals often address more than one element in the c-*fos* promotor (e.g. PDGF which works through the SIS element and through the SRE, Wagner et al., 1990; Büsher et al., 1988; cAMP which works through several CREB-binding sites and through the +18/+38 element, Fisch et al., 1989; Berkowitz et al., 1989; Büscher et al., 1988; Härtig et al., 1991)? One explanation might be that multiple promotor

elements are required in order to ensure that even weak stimulants (or stimulants applied at low doses) will activate the promotor. In such a case, multiple promotor elements would provide "back up" mechanisms resulting in overall *hypersensitivity* of the c-*fos* promotor. Such hypersensitivity, at least in cell culture, is a well documented phenomenon. An elegant *in vivo* confirmation of this hypothesis appeared recently, when Robertson and co-workers (1995) created transgenic mice carrying c-*fos* promotor-*lacZ* fusion genes with clustered point mutations in each of several distinct regulatory sequences: the SIS element, the SRE, the c-*fos* AP-1-like site, and the -60 CRE (a schematic representation of the c-*fos* promotor region is shown in fig. 8). Analysis of Fos-lacZ expression in the central nervous system (CNS) and in cultured cells demonstrated that all of the regulatory elements tested were required in concert for the tissue- and stimulus-specific regulation of the c-*fos* promotor. The authors postulated that the regulation of c-*fos* expression requires the concerted action of multiple control elements that direct the assembly of an interdependent transcription complex.

In view of these recent findings, it seems likely that also the full UVC-inducibility of the c-*fos* promotor depends on the co-operative action of multiple responsive elements and of the respective transcription factors binding to these elements. Why is it so important for the cell to maintain the c-*fos* expression after UV irradiation under such a tight, "backed up" control? A possible answer comes again from experiments with transgenic mice. Embryonic fibroblasts from c-*fos* knock-out mice (Wang et al., 1992) show increased sensitivity to UV radiation, suggesting that c-*fos* expression may accelerate the ability of the cells to resist genotoxic stress (Haas & Kaina, 1995; B. Baumann & P. Angel, personal communication).

Finally, the multiple UVC-initiated signal transduction pathways leading to transcriptional activation of c-*fos* described here and in other works (Büscher et al., 1988; Radler-Pohl et al., 1993; Sachsenmaier et al., 1994) and their relations to the growth factor-induced signaling cascades may be summarized in the following scheme (**fig. 36**). UVC initiates signal transduction cascades leading to MAPKs and Elk-1 activation and to c-*fos* induction through the SRE via a direct or indirect activation of certain growth factor receptors. The full responsiveness of the promotor requires the proximal c-*fos* CRE and the phosphorylation of CREB at serine 133 by the 108 kD CREB kinase as well. In this case UVC uses signal transduction routes which are independent of the set of growth factor receptors involved in the transmission of the signal to Elk-1 and to the SRE and which only partially share common components with the growth factor-initiated signaling cascades. It is currently being investigated whether these components (collectively indicated in fig. 36 as Y) do or do not include p21 Ras and/or p74 Raf-1 kinase.

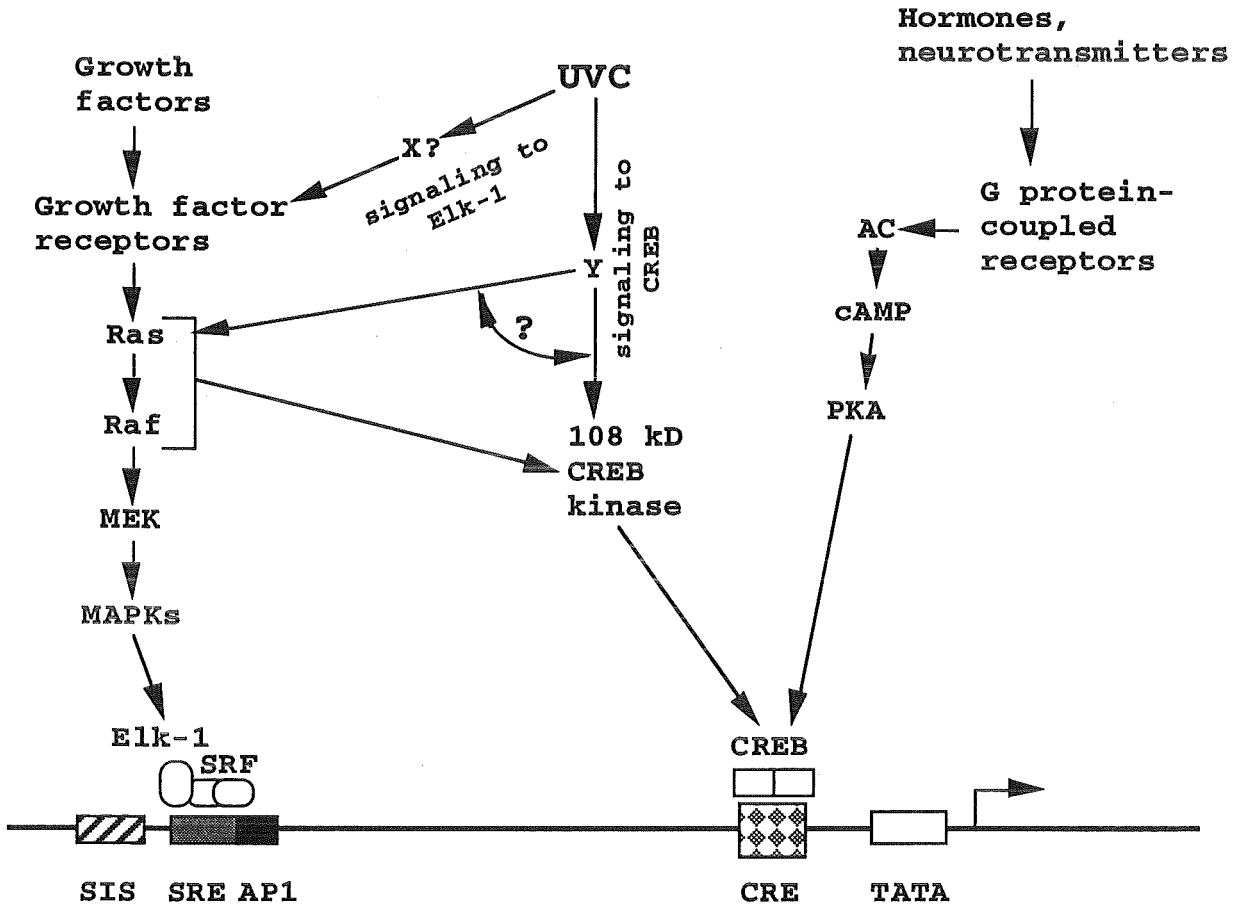


Fig. 36: UVC-induced signal transduction pathways leading to transcriptional activation of the c-*fos* promotor. See explanations in the text.

PART TWO

CREB AND THE PROXIMAL CRE IN THE c-*fos* PROMOTOR AS TARGETS FOR REPRESSION BY THE ACTIVATED GLUCOCORTICOID RECEPTOR

5.7. What Biological Role May the Regulation of AP-1 Expression by Glucocorticoids Play in the Cell Cycle and in AP-1-Dependent Gene Regulation?

In this thesis, I show that cAMP and glucocorticoids antagonistically regulate the expression of three genes of the AP-1 family in quiescent NIH 3T3 mouse fibroblasts: while cAMP induces strongly the transcription of c-*fos* and *jun*B, repressing at the same time the expression of c-*jun* mRNA, glucocorticoids act in the opposite direction, repressing the cAMP-dependent c-*fos* and *jun*B induction and alleviating the repression of c-*jun* (fig. 25). At least for c-*fos*, these effects were also detected at the protein level: dexamethasone-pre-treated cells displayed lower levels of cFos accumulation in response to forskolin treatment than the cells treated only with forskolin (not shown). What consequences for the cells may these effects have?

In quiescent BALB/c-3T3 mouse fibroblasts, elevated intracellular concentrations of cAMP inhibit the serum-induced mitogenic stimulation of the cells by arresting them in the mid-G_1 phase of the cell cycle (Leof et al., 1982). With the NIH 3T3 cells used in this thesis, similar results were obtained: quiescent cells, pre-treated with forskolin, showed significantly lower numbers of cells entering the S phase in response to a 20% serum stimulation than the non-pre-treated cells (M. Iordanov, unpublished). One proposed explanation of this phenomenon is that cAMP antagonizes p21 Ras-dependent signal transduction pathways (Burgering et al., 1993; Cook & McCormick, 1993) via PKA-mediated inhibitory phosphorylation of the Raf-1 kinase (Hafner et al., 1994). Indeed, forskolin pre-treatment of NIH 3T3 cells blocked the serum-induced p42 MAP kinase activation in these cells (M. Iordanov, unpublished). There might be, however, other explanations, one of them being the effect of cAMP itself on the expression of AP-1 and its action on the mitogen-induced AP-1 expression. In numerous cell types, including NIH 3T3, it has been reported that activation of adenylyl cyclase strongly represses the basal levels of c-*jun* mRNA and abolishes c-*jun* induction by serum (Chiu et al., 1989; Mechta et al., 1989). This latter effect, however, may be a consequence of the repression of the basal level: when using high amounts of polyA$^+$ mRNA from NIH 3T3 cells in Northern blot analysis, I could still detect normal serum-induction of c-*jun* in forskolin pre-treated cells (not shown). This induction may have been missed before because of the strongly repressed basal levels of c-*jun* mRNA at the beginning of the induction. cJun is thought to play an important (however, probably not decisive) role in proliferation and cell cycle progression in certain cell types. Fibroblasts derived from homozygous c-*jun* knock-out embryos (a lethal phenotype) show a reduced growth rate in culture and subnormal mitogenic responses to serum and individual growth factors (Johnson et al., 1993). Furthermore, sequestering of cJun in the cytoplasm via

microinjection of anti-cJun-specific antibodies in Swiss 3T3 cells blocks the entrance to the S phase of serum-stimulated or asynchronously growing cells (Kovary & Bravo, 1991a). Thus, it is possible that the cAMP-induced repression of cJun levels may be involved in the cAMP-dependent G_1 arrest. This possibility raised the question of whether glucocorticoids, by antagonizing the cAMP actions on AP-1 expression, may also revert the cAMP-induced G_1 arrest. Preliminary experiments (not shown) suggest that this may be the case.

There is another aspect of the glucocorticoid-regulated AP-1 expression which is worth mentioning. cFos cannot form homodimers or dimerize with any of the Fos proteins (FosB, Fra-1 or Fra-2) but efficiently heterodimerizes with all of the Jun proteins (cJun, JunB and JunD) (Angel & Karin, 1991 and references therein). Upon cAMP stimulation, under conditions of low c-*jun* expression and a rapid decay of the level of pre-existing cJun (cJun has a half-life of approx. 1.5 hours, Oehler et al., 1993 and P. Angel, personal communication), it is unlikely that the newly synthesized cFos would form cFos/cJun heterodimers and it would probably heterodimerize with the product of the other cAMP-induced gene, namely JunB (and/or JunD). Consistent with this assumption, elevated cAMP levels do not induce collagenase I gene expression in Hela cells (Chiu et al., 1989) and in NIH 3T3 (M. Iordanov, unpublished). JunB has been shown to be a negative regulator of cJun in the regulation of the collagenase I promotor (Chiu et al., 1989). JunB has also very high affinity to cFos, both *in vitro* and *in vivo* (Kovary & Bravo, 1991b). Thus, the biological meaning of the c-*fos* induction, as well as the functions of cFos and of the cFos-containing heterodimers in response to cAMP simulation is completely unknown. It is hard to believe that cFos, whose transcriptional activation by cAMP is ensured via at least three CREs and one nonhomologous cAMP responsive element (+18/+38) in its promotor region (Berkowitz et al., 1989; Fisch et al., 1989; Härtig et al., 1991), would not have specific functions in cAMP-stimulated cells. Whatever these functions might be, however, one might expect them to be *different* from those of the "classical" AP-1 complex, as the example with the collagenase I gene clearly shows. It is, then, possible to imagine that glucocorticoids, by decreasing the amount of cFos/JunB heterodimers and increasing the probability of cJun/cJun homodimer formation, would interfere with these specific cFos functions. Furthermore, if the result of the combined PKA and GR activations *vs* activation of PKA alone were preferential formation of one or another homo- and heterodimeric AP-combination, this might have effects on the GR-AP-1 interference itself. It has been shown that different AP-compositions are differentially (and, sometimes, oppositely) affected by GR. For instance, in the regulation of the composite promotor element of the proliferin gene, cFos/cJun is repressed, while cJun/cJun is activated by the GR (Miner & Yamamoto, 1992) and similar results have been observed in the regulation of a simple AP-1-dependent promotor (Teurich & Angel, 1995). Another example is provided by c-*jun* itself, whose promotor is mainly regulated by an AP-1-like activity consisting of cJun/ATF-2 heterodimers (van Dam et al., 1993). Since neither the basal (fig. 25), nor the induced (by TPA or serum, not shown) levels of the endogenous c-*jun* expression are repressed by glucocorticoid application in NIH 3T3 cells, this suggests that cJun/ATF-2 is, probably, also not a target for interference by the activated GR.

5.8. Mechanisms of the CREB-GR Interference in the Regulation of c-*fos*

The information about the mechanisms of glucocorticoid-induced repression of the cAMP-induced c-*fos* transcriptional activation, obtained in this thesis (4.2.1-4.2.6, figures 27-34), may be summarized in the following models (**fig. 37**).

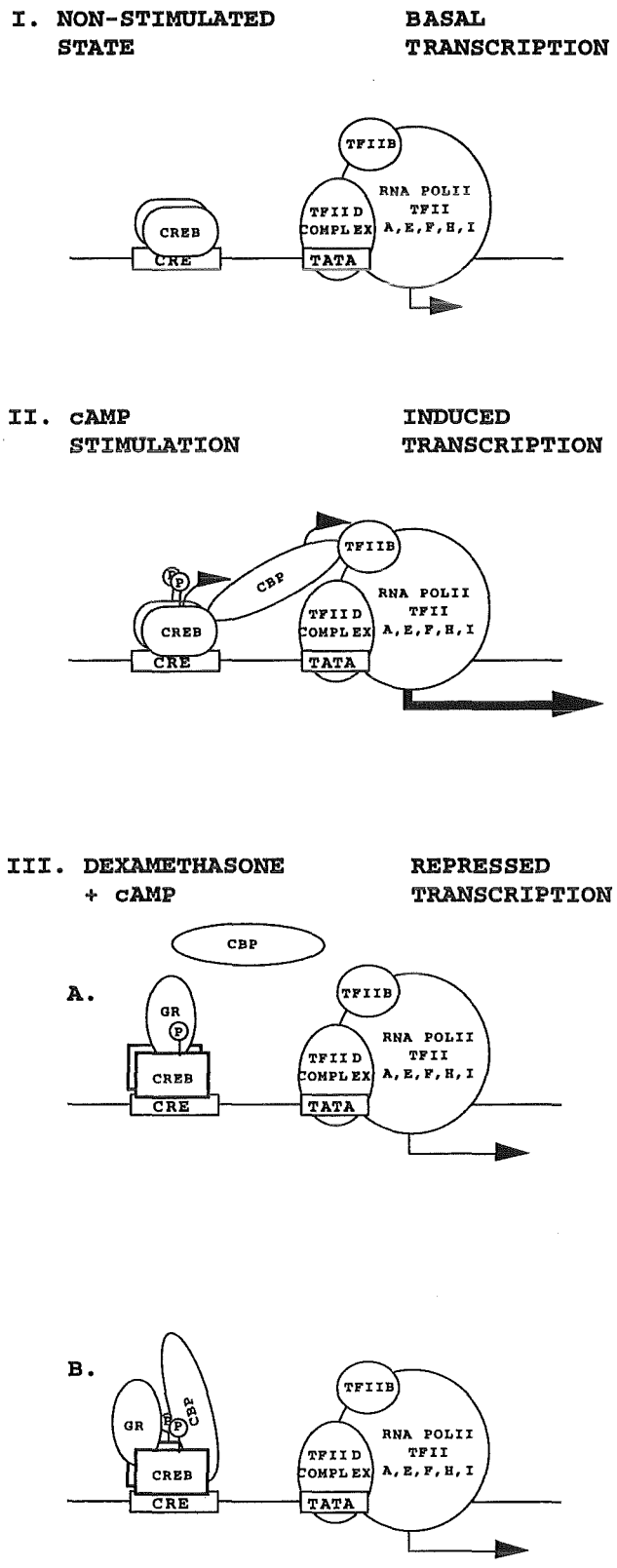


Fig. 37: Hypothetical models for the interference of GR with the function of CREB in the regulation of the c-*fos* promotor. See explanations in the text.

In quiescent NIH 3T3 cells, the level of c-*fos* transcription is very low although the major promotor elements determining the c-*fos* promotor activity are already occupied by their respective transcription factors (fig. 37, I). These include the SRE (Herrera et al., 1989; König, 1991), the CRE (Runkel et al., 1991 and fig. 31) and the TATA element (M. Iordanov, unpublished). Activation of PKA leads to serine 133 phosphorylation of CREB and, thereby, to formation of a functional CREB-CBP-basal transcriptional machinery complex. This, in turn, enhances the transcriptional activity of the promotor (fig. 37, II). Activation of the glucocorticoid receptor does not interfere with the DNA-binding (fig. 31) or with the PKA-mediated phosphorylation of CREB (figures 32 and 33). It also does not negatively affect the ability of the C-terminal portion of CBP to contact TFIIB and, thus, to enhance transcription (fig. 34). Hence, the activated GR probably prevents the formation of the CREB-CBP-basal transcriptional machinery complex via GR-induced conformational changes in the CREB molecule (fig. 37, III). The conformationally altered CREB may either be unable to bind CBP (fig. 37, IIIA) or may participate in an abortive GR-CREB-CBP complex (fig. 37, IIIB).

This model applies only to the situation where the interference between CREB and GR is not mediated by a third partner protein. However, even in the case of a third partner-mediated GR-CREB interference, similar conformational changes in the CREB molecule are plausible. How can the hypothesis of GR-induced conformational changes of CREB be proved? One possibility is to investigate the susceptibility of *in vitro* transcribed and translated CREB to mild protease digestion in the presence of activated GR. In such a case, upon a conformational change, certain cryptic protease cleavage sites may be exposed to the action of the protease, or protection of susceptible (in the absence of GR) cleavage sites may occur.

How do the mechanisms of CREB repression by GR compare to those that are already known to apply to AP-1? The interference of GR with the function of both AP-1 and CREB:

- is mediated through the binding site of the interfered transcription factor (TRE for AP-1 and CRE for CREB) and does not require DNA binding of GR;
- does not occur via a GR-induced removal of the factor from its binding site;
- does not occur via a GR-dependent block of the signal transduction pathways leading to the activation of either AP-1 or CREB by phosphorylation (for AP-1 data are available only on cJun, S. Gebel, unpublished)
- occurs in the presence of an antiglucocorticoid (RU486)-loaded receptor.

The following questions have to be answered, however, in order to further extend this analogy or to define differences between AP-1 and CREB:

1. Is the interference of GR with the function of CREB a function of the monomeric form of GR, as it has been suggested for the GR-AP-1 interference (Heck et al., 1994)?

2. Is the repression of CREB by GR mediated by the basic region/leucine zipper of CREB, as it has been shown for AP-1 (Miner & Yamamoto, 1992; Teurich & Angel, 1995)?

With regard to the findings described in this thesis it is safe to assume that AP-1 and CREB, two related basic region/leucine zipper transcriptional activators, probably obey similar rules in the mechanisms of their repression by the activated glucocorticoid receptor.

6. MATERIALS AND METHODS

The protocols used in this work were taken from the laboratory manual of Maniatis et al. (1989) unless otherwise stated. All general chemicals were from *Carl Roth GmbH + Co.*, Karlsruhe, *Merck,* Darmstadt or *Sigma Chemie GmbH*, Deisenhofen, and were of a highest quality.

6.1. GENERAL METHODS

Phenol/chlorophorm extraction of nucleic acids

In order to remove proteins from nucleic acids, an equal volume of phenol/chlorophorm/isoamylalcohol (25:24:1) was added to the nucleic acid solution and mixed in. The two phases were separated by centrifugation. The upper (aqueous, nucleic acid-containing) phase was gently transferred to a new reaction tube and the same procedure was repeated with chlorophorm/isoamylalcohol (24:1).

Ethanol (or 2-propanol) precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration was brought to 200 mM with 3 M Na-acetate (pH 4.8-5.0), and 2.5 volumes of ethanol or 1 volume of 2-propanol were added. After 30 min to overnight incubation at -20°C or 5-15 min at -80°C (only ethanol precipitation), the precipitate was pelleted by centrifugation at 10000xg for 15-20 min. The pellet was washed with 80% ethanol to remove the salt and was then dried.

Determination of nucleic acid concentration.

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. An OD_{260} = 1 is equvalent to 50 µg/ml of double stranded DNA or 40 µg/ml RNA or 20 µg/ml single stranded oligonucleotide. The OD_{280} is used as an indication of the purity of the nucleic acid; it should be approximately 50% of the OD_{260}.

Restriction endonuclease digestion of DNA

Usually 2-3 units of a restriction enzyme for each µg DNA were used. DNA was digested at a concentration of 1µg/10µl in a buffer recommended by the supplier. The reaction was carried out for 2 hours to overnight at 37°C (unless otherwise recommended by the supplier) and was stopped by a phenol/chlorophorm extraction. The DNA was precipitated with ethanol. The quality of the digest was controlled by agarose gel electrophoresis.

Size separation of nucleic acids by agarose gel electrophoresis

The required amount of agarose (SeaKem, Biozym Diagnostik, Hameln, final concentration between 0.8 and 2%) was dissolved in 50 ml electrophoresis buffer (TBE: 90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Ethidium bromide was added at a

concentration of 0.3 µg/ml. The molten gel was poured into a horizontal (13.5x8 cm) chamber. Combs with the appropriate number and size of the teeth were used to make the loading slots. The gel (when set) was covered with 200 ml electrophoresis buffer and run at 35-45 mA (50-100 V) at room temperature for the required time. Samples were loaded onto the gel in loading buffer (10 mM EDTA, 10% glycerol, 0.1% SDS, 0.02% bromphenol blue). DNA was visualised by transillumination with 302 nm ultraviolet radiation.

Isolation of DNA fragments from agarose gels (for preparation of DNA probes used in Northern blot hybridizations)

Plasmid DNA, bearing an insertion of the gene of interest, was digested with appropriate restriction enzymes, separated in an agarose gel and visualized under UV illumination. A cut was made in the agarose with a scalpel below the fragment to be isolated. A strip of DE81 DEAE-cellulose paper (Schleicher & Schuell, Dassel) was inserted into the slit and electrophoresis was continued until the DNA fragment has run onto the paper. The latter was removed from the gel and washed briefly with distilled H_2O. DNA was eluted by incubation of the paper (torn into small pieces) in 400 µl 1.5 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA for 30 min at 65°C. After two times extraction with phenol/chlorophorm, the DNA was precipitated with ethanol, dried and dissolved in an appropriate volume of H_2O.

Radioactive labeling of DNA

a. Multiprime labelling

[α-^{32}P] dCTP (Amersham Buchler GmbH, Braunschweig, 370 MBq/ml, 10 mCi/ml) and a multiprime labelling kit (Prime-It® II, Stratagene) were used to label 25-100 ng DNA fragment of interest according to the accompanying instructions. The radioactively labelled DNA was separated from unincorporated nucleotides using a Nacs Prepac™ column (Life Technologies, Inc., Gaithersburg, MD, USA) following the manufacturer´s instructions.

b. 5´end labelling of oligonucleotides

Single- or double-stranded oligonucleotides (1-5 pmol, TIB-Molbiol, Berlin or MWG-Biotech, Ebersbach) were incubated with 10 U T4 polynucleotide kinase (Promega) and 30 µCi [γ-^{32}P] ATP (Amersham Buchler GmbH, Braunschweig, 370 MBq/ml, 10 mCi/ml) in 10 µl kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine) at 37°C for 30 min. The kinase reaction was stopped by adding 1 µl 0.5 M EDTA. The radioactively labelled oligonucleotides were separated from unincorporated nucleotides using a Nacs Prepac column following the manufacturer´s instructions.

Preparation of nuclear extracts (modified from Dignam et al., 1983)

HeLa tk$^-$ cells were grown to confluency in 10 or 15 cm petri dishes, serum starved (0.5% FCS) for 24 hours, then treated as described in the text. Cell medium was removed, the cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 0.7 mM $CaCl_2$, 6.5

mM NaH_2PO_4, 1.5 mM KH_2PO_4, pH 7.6) and were harvested in 5 ml ice-cold PBS. All following steps were carried out at 4°C. The cells were centrifuged at 250xg. The volume of the cell pellet was determined (packed cell volume = PCV). The cells were resuspended in 3xPCV buffer A [10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml of each aprotinin, leupeptin, and pepstatin (the last three protease inhibitors were from Sigma, München)] and incubated on ice for 15 min. The cells were lysed by pushing 5x through a 26G needle and the nuclei were pelleted by centrifugation at 5600xg for 45 sec. The supernatant was used, if required, as a *cytoplasmic extract.* The nuclei were resuspended in 1.5xPCV buffer C (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 200 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml of each aprotinin, leupeptin, and pepstatin) and rotated 30 min. The nuclear membranes were pelleted by centrifugation at 5600xg for 5 min. The supernatant (*nuclear extract*) was dialysed for 2 hours against 500x volume buffer D (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 200 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). The dialysed extracts were aliquoted in small volumes (20-50 µl) and stored in liquid N_2. The dialysis step was omitted when nuclear extracts for "in gel" kinase assay were prepared.

Preparation of Poly A^+ RNA

Medium was removed from one 15 cm petri dish and the cells were lysed immediately in 10 ml STE (20 mM Tris-HCl pH 7.4, 10 mM EDTA, 100 mM NaCl, and 0.5% SDS) containing 300 µg/ml proteinase K (Sigma, München). The high molecular weight DNA was sheared for 15 sec with an Ultra-Turax™ homogenizer and the mixture was incubated at 37°C for 30 min. After bringing the final concentration of NaCl to 500 mM, 50-100 mg oligo-dT cellulose (type VII, Pharmacia Biotech) in HSB (20 mM Tris-HCl pH 7.4, 10 mM EDTA, 300 mM NaCl, and 0.2% SDS) was added. The poly A^+ RNA was allowed to bind to the oligo-dT cellulose overnight, rotating the mixture on a rotator. The oligo-dT cellulose was then washed three times with HSB and the poly A^+ RNA was eluted from the cellulose four times with 1 ml H_2O. The RNA concentration was determined from 400 µl of the eluent, then the remainder was precipitated with ethanol for 5-15 min at -80°C, centrifuged at 10000xg, and, after washing once with 80% ethanol, the RNA pellet was resuspended in H_2O, usually at a concentration 1 µg/µl. The RNA solution was stored up to several months at -80°C.

Transformation of bacteria (modified from Hanahan, 1983)

a. Preparation of competent E. coli.

A single colony of *E. coli* DH5α was innoculated into 50 ml SOB^+ (20 g/l Bacto Trypton, 2 g/l Yeast Extract, 0.5 g/l NaCl, 0.186 g/l KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 7.0) medium and grown to an OD_{600} 0.3-0.35. After incubation on ice for 10 min, the bacteria were sedimented by centrifugation at 3600xg for 15 min at 4°C. The bacteria were resuspended in 5 ml TFB (100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM Hexamine $CoCl_3$, 10 mM K-MES pH 6.2, 15% glycerol) and incubated on ice for a further 15 min. The cells were

centrifuged again and resuspended in 2 ml TFB. They were either directly transformed or frozen in liquid nitrogen in aliquots.

b. Transformation.

Five ng supercoiled plasmid DNA or 5-10 µl ligation mix were added to 200 µl of competent cells. After incubation on ice for 30 min, the cells were heat-shocked at 37°C for 3 min and were returned to ice for 2 min. Two ml SOC medium (20 g/l Bacto Trypton, 2 g/l Yeast Extract, 0.5 g/l NaCl, 0.186 g/l KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0) medium was added and the bacteria were incubated at 37°C with shaking for 45 min. The cells were pelleted by a short centrifugation (3 min, 1000xg), resuspended in 200 µl SOC and plated out onto LB-agar plates (10 g/l Nutrient Broth, 5 g/l Yeast Extract, 15 g/l Bacto Agar), supplemented with an appropriate antibiotic. The plates were incubated overnight at 37°C.

Large scale plasmid preparation from bacteria

250-500 ml LB medium supplemented with the relevant antibiotic were innoculated with a single bacterial colony and incubated with shaking at 37°C overnight until the bacteria had reached stationary phase. The bacteria were pelleted by centrifugation at 3600xg at 4°C for 10 min, resuspended in 10 ml solution I (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 400 µg/ml RNase A). After 20 min at room temperature the cells were lysed with the addition of 10 ml solution II (200 mM NaOH, 1% SDS). The mixture was neutralized with 10 ml solution III (3 M potassium acetate pH 5.5). After an additional 15 min on ice, the cell fragments and the chromosomal DNA were sedimented by centrifugation at 13200xg for 20 min at 4°C. The supernatant was applied to a pre-equilibrated *Qiagen-tip 500* column (Qiagen Inc.) and the plasmid DNA was eluted following the manifacturer's instructions. Usually up to 1 mg plasmid DNA was obtained and stored as an approximately 1mg/ml solution in H_2O at -20°C.

6.2. CELL CULTURE AND TRANSFECTIONS

Cell lines:

a. NIH 3T3 - mouse embryonal fibroblasts (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS, Gibco, Eggenstein), 100 U/ml penicillin, and 100 µg/ml streptomycin.

b. HeLa tk^- - human cervical carcinoma cells (devoid of thymidine kinase activity) were maintained in DMEM supplemented with 8% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

c. JEG-3 - human chorionic carcinoma cells (glucocorticoid receptor-deficient, a generous gift from Prof. M. Beato, Marburg) were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cell culture

All cells were maintained in a 37°C incubator (Forma Scientific, Labotect GmbH, Göttingen) with 6% CO_2. NIH 3T3, HeLa tk^-, and JEG-3 cells were cultured in 9 cm petri dishes (Greiner Labortechnik, Flickenhausen) with 10 ml culture medium. Just before the cells became confluent, they were trypsinized and either reseeded at a lower density or a desired number of cells was plated out for an experiment.

The trypsin treatment of the cells was performed as follows: culture medium was removed, cells were washed with 3 ml 0.25% trypsin (Difco Laboratories, Detroit, USA) and were then incubated with 1 ml trypsin for 3-5 min at 37°C. They were resuspended in 9 ml medium and replated at the desired density, usually $1x10^6$ cells per 9 cm dish.

Cell stocks were maintained in liquid N_2. Logaritmically growing cells were trypsinized, centrifuged at 250xg and resuspended in 1 ml culture medium (20% FCS, without antibiotica) containing 10% DMSO (Fluka Chemie AG, Buchs, Switzerland). After incubation on ice for 30 min, they were transferred to -80°C for several hours and finally to liquid N_2. Cells were thawed at 37°C, centrifuged at 250xg in 10 ml medium to remove the DMSO and plated out on petri dishes with fresh culture medium.

Transient transfection of cells

a. Calcium phosphate/DNA co-precipitation method (Graham & van der Eb, 1973)

Used to transiently transfect JEG-3 and NIH 3T3 cells. Cells were plated out at a density of $1x10^6$ per 9 cm petri dish one day prior to transfection. Between 10 and 15 µg of plasmid DNA per petri dish were co-precipitated with 125 mM $CaCl_2$ in HBS (25 mM HEPES pH 7.1, 140 mM NaCl, 0.75 mM Na_2HPO_4). The precipitate was left to stand at room temperature for 20 min, then added dropwise to the cell medium. After 6 hours (16 hours for NIH 3T3 cells) the precipitate was removed, the cells were washed twice with PBS and given fresh medium (0.5% FCS). In the case of NIH 3T3 cells, before washing with PBS, the cells were shocked by adding 4 ml 15% glycerol in PBS for exactly 2 min. The cells were subjected to further stimulation (UVC irradiation, cAMP, etc.) 24 hours post-transfection for an additional 24-36 hours.

b. DEAE-dextran method (Ausubel et al., 1987)

This method was used to transiently transfect HeLa tk^- cells. Cells were plated out at a density of $1x10^6$ cells per 9 cm petri dish one day prior to transfection. Between 10 and 15 µg of plasmid DNA per petri dish were mixed with 0.5 mg DEAE-dextran (Pharmacia Fine Chemicals AB, Upsala, Sweeden) in 1 ml TBS (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4). The medium was then removed, and

the cells were washed twice with TBS. One ml DNA mixture was added onto the cells and left at room temperature for 30 min with occasional shaking. Then, 9 ml fresh medium (0.5% FCS) was given to the cells which were incubated for an additional 4-6 hours at 37°C. Afterwards, cells were washed twice with TBS and given fresh medium (0.5% FCS). The cells were subjected to further stimulation (UVC irradiation, cAMP, etc.) 16 to 24 hours post-transfection for an additional 24-36 hours.

Treatment of cells

a. UVC irradiation of cells

The medium was removed from one 9 cm petri dish and the cells were washed once with PBS. The cells were irradiated from above with a UVC lamp with an emission peak at 254 nm (half-maximum peak width 2.3 nm; Vetter, Wiesloch). The UVC dose used in almost all experiments, 30 J/m^2, corresponds to 21 sec of irradiation at a distance of 32 cm between the cells and the UVC source. Higher UVC doses were achieved by shortening the distance between the cells and the UVC source and by increasing the time of UVC application. Immediately after the irradiation the cells were given the same medium and maintained for the required time at 37°C, 6% CO_2.

b. Chemicals and growth factors

TPA (Sigma Chemie GmbH, Deisenhofen, Germany) was stored as a 200 µg/ml stock solution in DMSO (Fluka Chemie AG, Buchs, Switzerland) at -20°C. Usually it was applied at a final concentration (f.c.) of 200 ng/ml. EGF (human, recompinant, from S. cerevisiae; Sigma, München), bFGF (human, recombinant, from E. coli; British Biotechnology Ltd., London), IL-1α (human, recombinant, from E. coli; British Biotechnology Ltd., London), and TNF-α (Saxon Biochemicals GmbH, Hannover) were stored as 10 µg/ml stock solutions in PBS/1% BSA at -20°C. Forskolin and IMX (both from Sigma Chemie GmbH, Deisenhofen, Germany) were stored as 10 mM and 0.5 M stock solutions in DMSO, respectively, at -20°C. In order to stimulate cAMP-dependent gene expression, the cells were treated either with forskolin alone (10-20 µM f.c.), or with a combination of forskolin and IMX (10 µM and 0.5 mM f.c.). Dibutyryl cAMP (Sigma Chemie GmbH, Deisenhofen, Germany) was stored as a 100 mM stock solution in DMSO at -20°C and was applied at a f.c. of 1 mM. A23187 and thapsigargin (both from Sigma Chemie GmbH, Deisenhofen, Germany) were stored as 50 mM and 1 mM stock solutions in DMSO, respectively, at -20°C and were applied at f.c. of 1 µM and 300 nM, respectively. Dexamethasone (Sigma, München) was stored as a 10^{-4} M stock solution in ethanol at -20°C and was applied at a f.c. of 10^{-7} M. Suramin (Germanin, Bayer, Leverkusen) stock solution (30 mM) in DMEM was freshly prepared and used only once. Tyrphostin-51 (Sigma Chemie GmbH, Deisenhofen, Germany) was stored as a 12.5 mM stock solution in DMSO at -20°C and was applied at a f.c. of 20 µM.

6.3. ANALYTICAL METHODS

Analysis of gene expression via chloramphenicol acetyltransferase (CAT) reporter gene assay (Gorman et al., 1982)

a. Preparation of Cell Extracts for CAT Assay

The medium was removed from one 9 cm petri dish and the cells were washed twice with PBS. The cells were harvested in 1 ml PBS with a rubber spatula, centrifuged for 2 min at 5600xg and resuspended in 100 µl 250 mM Tris-HCl pH 7.5. They were lysed by three cycles of rapid freezing (ethanol/dry ice bath) and thawing (37°C, 5 min). The cell debris was pelleted by centrifugation for 10 min at 5600xg and the supernatant (the protein extract) was stored at -20°C until needed or used immediately.

The protein concentration was determined by the method of Lowry et al. (1951) as follows: cell extract (5 µl) were mixed with 145 µl solution I (2% Na_2CO_3 in 100 mM NaOH). To this mixture 250 µl freshly prepared solution IV (100 µl 2% NaKTartrate, 10 ml solution I, 100 µl 1% $CuSO_4$) was added and the mixture was incubated at room temperature for 10 min. Then, 30 µl Folin and Ciocalteu´s phenol reagent (Merck, Darmstadt, 50% in H_2O) was added. After incubation for further 30-40 min at room temperature (in darkness), the extinction coefficient at 600 nm was measured. In parallel standard samples of BSA of known concentration were measured to obtain a standard curve from which the concentration of protein in the test sample was calculated.

b. CAT Assay

Between 50 and 200 µg protein extract were adjusted to 80 µl in 250 mM Tris-HCl pH 7.5. The reaction was initiated by the addition of 2 µl ^{14}C-chloramphenicol (Amersham Buchler GmbH, Braunschweig, 7.4 MBq/ml, 200 µCi/ml) and 20 µl 4 mM acetyl coenzyme A (Sigma, München, in 250 mM Tris-HCl pH 7.5) and was incubated at 37°C for 2 hours. The reaction was stopped by the addition of 500 µl ethyl acetate. The chloramphenicol was extracted from the aqueous phase with ethyl acetate by vortexing and centrifigation for 10 min at 5600xg. The ethyl acetate was evaporated in a vacuum centrifuge and the chloramphenicol was resuspeded in 18 µl ethyl acetate and dotted onto a TLC sheet (Macherey-Nagel, Düren). Chlorophorm/methanol (9:1) was used as a solvent in the chromatography. The acetylated chloramphenicol was separated from the unacetylated chloramphenicol within 45 min, after which the TLC sheet was air dried and exposed to Amersham Hyperfilm MP film at room temperature for 24-72 hours. The specific CAT activity (*pmol acetylated chloramphenicol per µg protein per hour*) was evaluated using a PhosphoImager Fujix BAS 1000 quantification and from the equation:

$$\frac{\text{pmol } ^{14}\text{C-chloramphenicol x cpm acetylated } ^{14}\text{C-chloramphenicol}}{\text{hours reaction time x µg protein extract x cpm input } ^{14}\text{C-chloramphenicol}}$$

More often the results were presented as *relative CAT activity* or *induction factor* :

RCA = IF = specific CAT activity (stimulated cells)/specific CAT activity (control cells)

c. plasmids used

- CAT reporter gene plasmids

p-711/+45*fos* CAT (Härtig et al., 1991)

p-711/Δ-65/-52/+45*fos* CAT (Härtig et al., 1991)

pGAL4 CAT (Chrivia et al., 1993; Kwok et al., 1994, a generous gift from Dr. R. Goodman, Vollum Institute, Portland, OR)

p-105/-79 HIV TATA CAT (Stein et al., 1989)

pRSV CAT (contains the CAT gene from the plasmid pRSVcat, Gorman et al., 1982, under the control of the RSV LTR from the plasmid pUC-RSV, a generous gift from Dr. P. Angel, Institut für Genetik, Forschungszentrum Karlsruhe, unpublished)

- expression vectors

pCREB (contains the full lenght CREB cDNA under the control of the RSV LTR, Gonzales & Montminy, 1989, a generous gift from Dr. G. Schütz, Deutsches Krebsforschungszentrum, Heidelberg)

pCREBM1 (contains the full lenght CREB cDNA with a point mutation serine 133 to alanine 133, under the control of the RSV LTR, Gonzales & Montminy, 1989, a generous gift from Dr. W. Schmid, Deutsches Krebs-forschungszentrum, Heidelberg)

pRSVGAL4 1-147 (contains the DBD from GAL4 under the control of the RSV LTR, Chrivia et al., 1993; Kwok et al., 1994, a generous gift from Dr. R. Goodman, Vollum Institute, Portland, OR)

pRSVGAL4 T1 (contains the DBD from GAL4 fused to the C-terminal part of CBP (amino acids 1678-2441) under the control of the RSV LTR, Kwok et al., 1994, a generous gift from Dr. R. Goodman, Vollum Institute, Portland, OR)

pRSVGAL4 T1SA (contains the DBD from GAL4 fused to the C-terminal part of CBP (amino acids 1678-2441), under the control of the RSV LTR with a point mutation serine 1782 to alanine 1782, Kwok et al., 1994, a generous gift from Dr. R. Goodman, Vollum Institute, Portland, OR)

pMLVGAL4 1-147 (contains the DBD from GAL4 under the control of the MLV LTR, Hurst et al., 1991, a generous gift from Dr. N. Jones, Imperial Cancer Research Fund, London, England)

pMLVGAL4 CREB (contains the DBD from GAL4 fused to the full lenght ΔCREB cDNA, under the control of the MLV LTR, Hurst et al., 1991, a generous gift from Dr. N. Jones, Imperial Cancer Research Fund, London, England)

pMtC (contains the full lenght cDNA coding for the catalytic subunit of PKA, under the control of the metallothionein I promotor, Melon et al., 1989, a generous gift from Dr. P. Sassone-Corsi, CNRS, Strasbourg, France)
pGR (contains the full length cDNA coding for the human glucocorticoid receptor under the control of the RSV LTR, Hollenberg et al., 1987, a generous gift from Dr. A. B. C. Cato, Institut für Genetik, Forschungszentrum Karlsruhe)

Genomic footprinting (Mueler & Wold, 1989)

a. *In vivo* methylation of DNA

At the indicated time after treatment of the cells with either dexamethasone, forskolin, or both (see fig. 31), the medium was removed, the cells were washed with PBS, and 0.5% dimethylsulfate (DMS, Fluka) in DMEM/10 mM Hepes pH 7.5 was added at room temperature for 2 min. After washing the cells two times with ice-cold PBS, they were harvested in 5 ml cold PBS. The cells were centrifuged (2°C, 400xg, 3 min) and then lysed in 4 ml 10 mM Tris-HCl pH 7.7, 400 mM NaCl, 2 mM EDTA, and 0.2% SDS. The lysates were incubated with 300 µg/ml proteinase K overnight at 37°C. The lysates were extracted twice with phenol/chlorophorm and precipitated with 2-propanol in the presence of 250 mM Na-acetate. The genomic DNA was dissolved in TE buffer and, in order to reduce the viscosity, digested overnight at 37°C with Eco RI restriction endonuclease (which has no recognition sequences within the promotor region studied). The probes were then subjected to RNase A digestion (50 µg/ml, 30 min, room temperature), extracted twice with phenol/chlorophorm, and precipitated with ethanol.

b. *In vitro* methylation of protein-free ("nacked") genomic DNA

15-20 µg genomic DNA (from cells *not* treated with DMS) were dissolved in 5-6 µl H_2O and mixed with 200 µl 0.5% DMS in 50 mM Na-cacodylate, 1 mM EDTA for 1 min at room temperature. The reaction was terminated by adding 50 µl DMS-stop solution (1.5 M Na-acetate pH 7.0, 1 M β-mercaptoethanol) and the DNA was precipitated with 750 µl ice-cold ethanol. The precipitate was dissolved in 250 µl 300 mM Na-acetate pH 7.0, re-precipitated with ethanol, washed with 80% ethanol, and dried.

c. Cleavage of the *in vivo* and *in vitro* methylated DNA with piperidine (Maxam & Gilbert, 1980)

The dried DNA was resuspended in 100 µl 10% piperidine (Fluka), incubated for 30 min at 90 °C, and, after chilling on ice and addition of 200 µl 300 mM Na-acetate, precipitated with 900 µl ethanol. The DNA pellet was washed 2-3 times with 80% ethanol.

d. Selective amplification and radioactive labelling of the piperidine-cleaved products

For the first-strand synthesis, 3 µg piperidine-cleaved genomic DNA together with 0.6 pmoles of primer 1 were denatured in 15 µl 40 mM Tris-HCl pH 7.7, 50 mM NaCl for 3 min at 95°C and then the primer was allowed to hybridize for 30 min at 45°C (in 0.5 ml reaction tube). Afterwards a mixture consisting of 7.5 µl 20 mM $MgCl_2$, 20 mM DTT, 1 µl 480 µM of each dATP, dCTP, dGTP, dTTP, and 1.5 µl 1:4 diluted Sequenase® Version 2.0 (13 u/µl, United States Biochemical) was added and mixed. The reaction was incubated for 15 min at 45°C and, after chilling on ice, 6 µl 310 mM Tris-HCl pH 7.7 were added and the reaction was incubated for additional 15 min at 67°C to inactivate the Sequenase.

For the ligation of the linker oligonucleotides, the probes were chilled on ice and given a 20 µl mixture of 17.5 mM $MgCl_2$, 42.3 mM DTT, 125 µg/ml BSA. Finally, 25 µl "ligation mix" [10 mM $MgCl_2$, 20 mM DTT, 3 mM ATP, 50 µg/ml, with 5 µl linker (20 pmoles linker/µgl in 250 mM Tris-HCl pH 7.7) and 3 Weiss units T4 DNA ligase (Promega) per 25 µl] were added and the reactions were incubated overnight at 15°C. The linker represented the following, hybridized in 250 mM Tris-HCl, pH 7.7, oligonucleotides:

5′-GCGGTGACCCGGGAGATCTGAATTC-3′ and

5′-GAATTCAGATC-3′.

The reactions were terminated by heating for 10 min at 70°C, precipitated with ethanol in the presence of 10 µg yeast tRNA, washed with 70% ethanol and dried.

For the PCR reaction the precipitates were dissolved in 59 µl H_2O. To the DNA solution 20 µl 5xTaq buffer (200 mM NaCl, 50 mM Tris-HCl pH 8.8, 25 mM $MgCl_2$, 0.05% gelatine), 5 µl primer 2 solution (2 pmoles/µl), and 5 µl long-linker solution (25-mer, 2 pmoles/µl) were added, mixed, and the mixtures were denatured for 5 min at 95°C. After quick chilling on ice, a 10 µl mixture of all four dNTPs (2.5 mM of each) and 4 µl 1:4 diluted (in 1xTaq buffer) recombinant Taq DNA polymerase (AmpliTaq™, Cetus-Perkin-Elmer Corporation, 5 u/µl) were added, the reactions were mixed and covered with 90 µl mineral oil.

The specific amplification of the cleavage products by PCR was done in a Coy TempCycler. For both coding and noncoding strands, 20 cycles were performed consisting of 1 min at 95°C, 2 min at 60°C, and 3 min at 75°C.

For the last primer extension a PCR step was applied, consisting of 2 min at 95°C, 2 min at 63°C, and 10 min at 75°C, using the 5′end labeled primer 3. The reactions were stopped by chilling on ice and 250 µl of 260 mM Na-acetate, 10 mM Tris-HCl pH 7.5 and 4 mM EDTA were added. After phenol/chlorophorm extraction and ethanol precipitation the probes were resuspended in 6 µl formamide loading buffer (80% vol/vol deionized formamide, 1 mM EDTA, 0.05% bromphenolblue, 0.05% xylenecyanol, pH 8.0). Two µl of each amplification reaction were resolved on a standard 8% sequencing gel (Maxam & Gilbert, 1980).

The mouse c-*fos* promotor-specific primers used were as follows:

coding strand:

1) 5′-TAGACACTGGTGGGAGCTGCAGAGC-3′,

2) 5′-AGCTGCAGAGCAGAGCTGGGTGGGA-3′,

3) 5′-AGAGCAGAGCTGGGTGGGAGCGCGG-3′;

non-coding strand:

1) 5′-CCTGGGGCGTAGAGTTGACGACAGA-3′,

2) 5′-CGACAGAGCGCCCGCAGAGGGCCTT-3′,

3) 5′-ACAGAGCGCCCGCAGAGGGCCTTGG-3′.

Northern blot hybridization (McMaster & Carmichael, 1977)

Five µg poly A^+ RNA (1 µg/µl) was diluted with 15 µl denaturing buffer [50 µl 20xPB (200 mM NaH_2PO_4, 10 mM Na_2HPO_4 pH 6.85), 500 µl DMSO, and 195 µl 30% deionized glyoxal]. The samples were heated to 55°C for 10 min and cooled rapidly on ice. Then, 4 µl RNA loading buffer (50% glycerol, 1xPB, 0.1% bromphenol blue) was added.

The RNA samples were then loaded on a 1% vertical agarose gel made in 1xPB. Electrophoresis took place at 100V at 4°C for 3 hours. The gel was stained with acridine orange (30 µg/ml) in 1xPB and the RNA was visualized under short-wave (260 nm) UV light. The RNAs were transferred to Hybond-N^+ membrane (Amersham Buchler GmbH, Braunschweig) as described in Maniatis et al. (1989), by the method of Southern (1975). After the transfer the RNA was UV cross-linked to the active groups of the filter using a UV Stratalinker 2400 (Stratagene Cloning Systems, La Jolla, CA, USA).

The hybridization procedure was carried out as follows. The filter was prehybridized for 2 hours at 65°C in 4xSSC, 0.02% BSA, 0.02% ficoll, 0.02% polyvinyl pyrollidone, 0.05% $Na_4P_2O_7$, 6.7 mM NaH_2PO_4, 10 mM Na_2HPO_4, and 0.1% SDS. To block the unspecific hybridization, small denatured salmon sperm DNA was added in the second hour of prehybridization to a final concentration of 20 µg/ml. The hybridization mix usually contained 1-3x10^6 cpm/ml of "random primer"-labeled DNA probe (denatured at 95°C for 10 min) in 4xSSC, 10 mM EDTA, 0.1% SDS, and 20 µg/ml salmon sperm DNA. Hybridization was carried out in a sealed plastic bag for 16 hours at 65°C. The filter was washed 4 times at 65°C in 2xSSC, 1xSSC, 1xSSC, and 0.5xSSC respectively, each containing 0.1% SDS, 0.05% $Na_4P_2O_7$, 6.7 mM NaH_2PO_4, 10 mM Na_2HPO_4. The filter was sealed in a plastic bag and exposed wet to Amersham Hyperfilm MP film at -80°C with an intensifying screen.

The following DNA fragments were used as specific probes in Northern blot hybridizations:

a. v-*fos* (murine osteosarcoma virus)

A 1.0 kb *Pst1* fragment from the plasmid puc9/v-*fos* (Curran & Teich, 1982).

b. c-*jun* (human)

A 1.4 kb *Sma1/Hind3* fragment from the plasmid ph-cJ-1 (Angel et al., 1988)

c. *jun*B (mouse)

A 0.4 kb *BamH1* fragment from the plasmid pjunB (Chiu et al., 1989)

d. GAPDH (rat)

A 1.3 kb *Pst1* fragment from the plasmid pGAPDH-13 (Fort et al., 1985)

Immunoblot analysis of proteins (Western blot)

a. Separation of proteins by polyacrylamide gel electrophoresis (PAGE; Laemmli, 1970)

The polyacrylamide gel consisted of a 10% separating gel and a 5% stacking gel. For the preparation of the separating gel, 10 ml acrylamide/*bis*-acrylamide (30:0.8, Carl Roth GmbH + Co., Karlsruhe), 7.5 ml 1.5 M Tris-HCl pH 8.8, 0.15 ml 20% SDS, and 12.35 ml H_2O were mixed and polymerization was initiated by adding 250 µl 10% APS and 15 µl TEMED. The stacking gel consisted of 2.55 ml acrylamide/*bis*-acrylamide (30:0.8), 3.75 ml 0.5 M Tris-HCl pH 6.8, 75 µl 20% SDS, 8.7 ml H_2O, 75 µl 10% APS, and 15 µl TEMED.

The proteins (dissolved in Laemmli loading buffer: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 12% glycerol, 0.001% bromphenolblue, 715 mM β-mercaptoethanol) were denatured by heating for 3 min at 95°C, chilled on ice, and loaded onto the gel. The electrophoresis was carried out overnight at 150 V.

b. Staining of proteins with Coomassie Blue

A part of the gel which was not to be transferred onto a membrane was cut out and incubated with shaking for 15-60 min in Coomassie staining solution [1 g SERVA Blau G (SERVA Feinbiochemica), equivalent to Coomassie Brillant Blue G-250 (Bio Rad) in 46% methanol, 8% acetic acid, 46% H_2O]. The destaining was carried out for several hours in 50% methanol, 7.5% acetic acid, 42.5% H_2O.

c. Electrophoretic transfer of PAGE-separated proteins onto a membrane and detection with specific antibodies

The proteins were transferred onto a Immobilon™-P membrane (Millipore, filter type PVDF, pore size 0.45 µm, pre-wetted with methanol) using a BioRad Trans-Blot Cell transfer chamber following the manufacturer's instructions. All of the following steps were carried out at room temperature. After the transfer the membrane was incubated for 15 min with 10% low-fat milk in blocking buffer (PBS/0.3% Tween 20) in order to block the unspecific binding of the antibodies to the membrane. For detection of the protein of interest, the membrane was incubated for 1 hour with an appropriate dilution of the primary antibody (usually between 1:1000 to 1:15000) in 5% low-fat milk in PBS/0.3% Tween 20. After washing 3 times for 10 min with blocking buffer, the membrane was incubated for 1 hour with a 1:3000 dilution of the peroxidase-coupled secondary antibody (anti-rabbit or anti-mouse IgGs) in 5% low-fat milk in

PBS/0.3% Tween 20 and then washed 3 times for 10 min with blocking buffer. The specific proteins were detected by enhanced fluorescence using *Amersham ECL Western blotting detection reagents* (RPN 2106) following the manufacturer's instructions.

d. Antibodies used

Anti P-CREB (an affinity purified rabbit polyclonal serum which recognizes only *CREB phosphorylated at serine residue 133*, but does not cross-react with non-phosphorylated CREB, a generous gift from Dr. W. Schmid, Deutsches Krebsforschungszentrum, Heidelberg)

αIcp^{42} (a *p42 MAP kinase*-specific rabbit antibody, a generous gift from Dr. Ch. Marshall, Institute of Cancer Research, London, UK)

PY20 (an *phosphotyrosine*-specific mouse antibody, Affinity, Nottingham, England)

Anti rabbit immunoglobulins (polyclonal IgGs, recognize rabbit immunoglobulins, peroxidase-coupled, from goat, Dako, Glostrup, Denmark)

Anti mouse immunoglobulins (polyclonal IgGs, recognize mouse immunoglobulins, peroxidase-coupled, from rabbit, Dako, Glostrup, Denmark)

"In gel" kinase assay (modified from Geahlen et al., 1986)

Between 5 and 10 µl of nuclear extract, diluted 1:1 in 2xLaemmli loading buffer, were denatured by heating for 3 min at 95°C and resolved in 7.5% SDS-PAGE with or without co-polymerized peptide substrate (400 µg/ml) in the separating gel, using a Mighty Small™ Vertical Slab Unit (Hoefer) . The SDS was removed by washing the gels 2 times for 10 min in 250 ml buffer W (50 mM HEPES pH 7.4, 5 mM 2-mercaptoethanol) containing 20% ($^{v}/_{v}$) 2-propanol. After washing the gels 2 times for 30 min with 250 ml buffer W to remove the 2-propanol, the proteins were denatured by incubating the gels 3 times for 30 min with 150 ml buffer W/6 M guanidium-HCl. The proteins were allowed to renature by incubating the gels 3 times for 5 hours with buffer W containing 0.04% Tween 20 at 4°C. For the kinase reaction the gels were equilibrated in 250 ml buffer K (25 mM HEPES pH 7.4, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 90 µM Na_3VO_4) at 30°C. The kinase reaction was carried out in 30 ml buffer K for 1 hour at 30°C and was initiated by adding 18-25 µl ^{32}P-γ-ATP (180-250 µCi). The reaction was stopped by adding 5% TCA/10 mM $Na_4P_2O_7$ and the gels were washed for at least 12 hours with 3 changes of the same solution. The gels were then dried and exposed to Amersham Hyperfilm MP film (if required at -80°C with an intensifying screen).

Immunofluorescence

NIH 3T3 cells were plated out at low density onto round cover slips (Ø 10 mm) in 6 cm petri dishes, grown for 24 hours and then serum-starved for an additional 24 hours. Fifteen min post-induction with UVC, TPA, forskolin, EGF, or dexamethasone, the cells were washed with PBS and were fixed to the cover slips with 4% *p*-formaldehyde for 8 min at room temperature.

After washing 3 times with PBS, the cells were permeabilized with 0.2% Triton X100 for 10 min, and washed again 4 times with PBS. In order to prevent the unspecific binding of the antibody, the cells were incubated with 10% FCS in PBS for 10 min. After washing 3 times with PBS, the cover slips were transferred into 24-well tissue culture plates (one slip per well). The cells were then incubated with 30 µl appropriate dilution of the first antibody (usually 1:50 to 1:1000) in PBS/10% FCS for 30 min at room temperature. After washing 3 times with PBS, the cells were incubated with a 1:50 dilution of the rhodamine-coupled secondary antibody (anti-rabbit IgG, from goat, Dianova) for 30 min at room temperature. After washing 3 times with PBS and once with H_2O, the cover slips were mounted to microscopic slides using pre-warmed (50°C) glycergel (Dako). The microscopic analysis of the slides was carried out using a Diaplan fluorescent microscope (Zeiss) and photographed using Kodak Ektachrom P800/1600 film.

Free cytosolic Ca^{2+} measurement (Rijkers et al., 1990; Merritt et al., 1990)

HeLa tk^- cells were grown in DMEM, 8% FCS, in 96-well microwell plates (Nunc, Wiesbaden) when the *spectroscopic method* was used and in chamber slides (Nunc, Wiesbaden) when the *microscopic method* was used, serum-starved for 24 hours (DMEM, 0% FCS) and incubated for 1 hour with 2 µM *fluo*-3 in DMEM, 0% FCS at 37°C, 6% CO_2. After washing the cells 3 times with serum free-medium to remove the unincorporated dye and to reduce the background staining, the cell were treated with the indicated inducers and processed further for free cytosolic calcium quantification:

a. spectroscopic method

The free cytosolic calcium concentration was measured at room temperature in an automated spectrofluorometer (CytoFluor 2300, Millipore, Eschborn) The monochromator settings were 485 ± 20 nm for excitation and 530 ± 25 nm for emission.

b. microscopic method

For the video imaging the chamber slides were mounted onto an inverted microscope (Leica DMIL, Bensheim). The UVC irradiation was applied from above onto the cells covered with a 3 mm high serum-free medium layer. Images were obtained every minute through an objective (40/0.60) by means of a CCD video camera (Kappa, Gleichen) and processed by using a digital imaging analysis system (Quantimed 500, Leica). Qualitative alterations in Ca^{2+} concentrations were presented in pseudo colors.

7. ABBREVIATIONS

^{o}C	degrees Centigrade
A_{260}	absorption at wavelength 260 nm
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP-1	activator protein-1
APS	ammoniumperoxodisulfate
ATF	activating transcription factor
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
bZip	basic region/leucine zipper
c-	cellular (e.g. c-*fos*, cellular *fos* gene)
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
cDNA	copy DNA
COOH-	carboxy group
cpm	counts per minute
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CREBM1	dominant negative mutant of CREB
CREBtide	peptide representing amino acids 123-136 of CREB KRREILSRRPSYRK
CREM	CRE modulator
CTP	cytidine triphosphate
DBD	DNA-binding domain
DEAE	diethylaminoethyl
dex	dexamethasone
DMEM	Dulbecco´s modified Eagle´s medium
DMS	dimethylsulfate
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleoside triphosphate
DTT	dithiothreitol
ECL	enhanced chemoluminescence

EDTA	ethylenediamine-N,N-tetracetate
EGF	epidermal growth factor
EGF-R	EGF receptor
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
EPIF	extracellular protein synthesis-inducing factor
et al.	and others (lat. *et alii*)
f.c.	final concentration
FCS	fetal calf serum
FI	combined treatment of cells with forskolin and IMX
fig.	figure
g	gram *or* earth acceleration (9.18 m/s^2)
GAL4	yeast transcriptional activator GAL4
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GF	growth factor
GFR	growth factor receptor
GH	glucocorticoid hormone
GMP	guanosine monophosphate
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
GTP	guanosine triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethansulfonic acid
HIV	human immunodeficiency virus
IL-1α	interleukin 1α
IMX	*iso*-butylmethylxanthine
J	Joule
JNK	c-Jun N-terminal kinase
kb	kilobase (1 kb = 1000 bp)
kD	kilodalton (10^3 dalton)
KID	kinase-inducible domain
l	liter
LMPCR	ligation-mediated polymerase chain reaction
LTR	long terminal repeat
M	molar
mA	milliampere
MAPK	mitogen-activated protein kinase
μCi	microcurie
min	minute
μl	microliter (10^{-6} l)

ml	milliliter (10^{-3} l)
MLV	Moloney leukemia virus
μM	micromolar (10^{-6} molar)
mM	millimolar (10^{-3} molar)
mRNA	messenger RNA
NF-κB	nuclear factor-kappa B
NGF	nerve growth factor
NH_2-	amino group
nm	nanometer (10^{-9} m)
Ø	diameter
OD	optical density
P-ATF1	ATF-1 phosphorylated at serine 63
P-CREB	CREB phosphorylated at serine 133
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
pm	point mutation
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinyldifluoridene
RNA	ribonucleic acid
RSV	Rous sarcoma virus
RT	room temperature
Σ	combined treatment of cells with EGF, bFGF and IL-1α
SAPK	stress-activated protein kinase
SDS	sodium dodecylsulfate
SDS-PAGE	denaturing polyacrylamide gel electrophoresis
sec	second
SEM	standard error of the mean
SIS	v-*sis* transformed cell conditioned medium responsive element
SRE	serum responsive element
SRF	serum response factor
TA	combined treatment of cells with thapsigargin and A23187
TAD	transactivation domain
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TCF	ternary complex factor

TEMED	N,N,N´,N´-tetramethylethylenediamine
TFIIB	(basal) transcription factor IIB
TK	tyrosine kinase
tk	thymidine kinase
tk^-	thymidine kinase-deficient
TNF-α	tumor necrosis factor α
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
Tris	tris-(hydroxymethyl)-aminomethane
U	unit(s)
URE	UVR responsive element
UVA	ultraviolet A (315/320-400 nm)
UVB	ultraviolet B (280-315/320 nm)
UVC	short-wavelength ultraviolet radiation (200-280 nm)
UVR	ultraviolet radiation
V	Volt
v-	viral (e.g. v-*fos*, Finkel-Biskis-Jinkins virus *fos* gene)

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