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KATRI SAAVALAINEN

Evaluation of the Mechanisms of Gene Regulation on the Chromatin Level at the Example of Human *Hyaluronan Synthase 2* and *Cyclin C* Genes

Doctoral dissertation

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Author's address: Department of Biosciences
University of Kuopio
P.O. Box 1627
FI-70211 KUOPIO
Tel. +358 163 572
E-mail: katri.saavalainen@uku.fi

Supervisors: Professor Carsten Carlberg, Ph.D.
Department of Biosciences
University of Kuopio

Dos. Sami Väisänen, Ph.D.
Department of Biosciences
University of Kuopio

Thomas W. Dunlop, Ph.D.
Department of Biosciences
University of Kuopio

Reviewers: Professor Jörg Reichrath, M.D.
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Saarland University Hospital
Homburg, Germany

Professor Moray Campbell, Ph.D.
Department of Pharmacology and Therapeutics
Roswell Park Cancer Institute
Buffalo NY, USA

Opponent: Dos. Jukka Hakkola, Ph.D.
Department of Pharmacology and Toxicology
University of Oulu

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Abstract

Gene expression is controlled by regulating both protein and chromatin modifications involved in the initiation of transcription. The regulation of gene expression consists of a series of events finally leading to the initiation of transcription. These events include several protein associations and dissociations in the regulatory regions of genes in addition to many protein-protein interactions and chromatin modifications. The main players in this system are the RNA polymerase II (Pol II), for polyadenylated messenger RNAs and numerous transcription factors (TFs), co-regulators and chromatin modifying enzymes.

The main objective of this study was to gain more information on the mechanisms of gene regulation on the chromatin level using as an example of two genes, human *hyaluronan synthase 2* (*HAS2*) and human *CYCLIN C*. The human *HAS2* gene is regulated by the TFs retinoic acid receptor (RAR), signal transducer and activator of transcription (STAT), nuclear factor κB (NF-κB) and cAMP-response element binding protein 1 (CREB1). In addition, we demonstrated that the responses are mediated via response elements (REs) for corresponding TFs located within the first 2,250 bp of the human *HAS2* promoter and that each RE recruits a different pattern of transcriptional regulators in response to different stimuli. We showed that the human *CYCLIN C* gene was also regulated by RAR and CREB1. The co-regulator recruitment to REs for RAR and CREB1 (RAREs and CREs, respectively) and to the vitamin D receptor (VDREs) was unique for each RE is response to different stimuli. The different TFs were also shown to interfere with each other both on mRNA and chromatin level. This interference suggests integration of different signaling pathways via the use of common co-regulators.

In conclusion, the studies presented in this doctoral thesis provide new information about the regulation of two important genes, human *HAS2* and *CYCLIN C*, and more detailed understanding of the mechanisms of the gene regulation on the chromatin level.

National Library of Medicine Classification: QU 470, QU 475, QU 56, QU 83

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Medical Subject Headings: Genes; Gene Expression Regulation; Chromatin; Receptors, Cytoplasmic and Nuclear; Transcription Factors; Response Elements; Hyaluronic Acid; Glucuronosyltransferase; Cyclins; Epidermal Growth Factor; Keratinocytes; STAT Transcription Factors; Cyclic AMP Response Element-Binding Protein; NF-kappa B; Receptors, Retinoic Acid; Vitamin D Response Element

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Abbreviations

$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
AP-1	activating protein 1
AR	androgen receptor
cAMP	cyclic AMP
CAR	constitutive androstane receptor
CBP	CREB-binding protein-binding protein
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CoA	co-activator
CoR	co-repressor
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CRE	cAMP response element
CREB 1	cAMP response element-binding protein 1
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate
DR	direct repeat
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERR	estrogen-related receptor
FBS	fetal bovine serum
FXR	farnesoid X receptor
GCNF	germ cell nuclear factor
GR	glucocorticoid receptor
HaCaT	human immortalized keratinocyte cell line
HAS	hyaluronan synthase
HAT	histone acetyl transferase
HDAC	histone deacetylase

HEK293	human embryonic kidney epithelial cell line
HMT	histone methyl transferase
HNF	hepatic nuclear factor
HOX	homeobox containing protein
IGF-1R	insulin growth factor receptor 1
IKK	IκB kinase
IL	interleukin
LBD	ligand-binding domain
LRH	liver receptor homolog
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MED	mediator
MCF-7	human breast adenocarcinoma cell line
MR	mineralocorticoid receptor
NCoR1	nuclear receptor co-repressor1
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NGFIB	nerve growth factor inducible
NR	nuclear receptor
p300	E1A-binding protein
PARP-1	poly-ADP-ribose polymerase 1
PEI	polyethylenimine
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipase Cγ
PML	promyelocytic leukemia
Pol II	RNA polymerase II
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PXR	pregnane X receptor
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element

Rb	retinoblastoma protein
RE	response element
REK	rat epidermal keratinocytes
RPLP0	acidic riboprotein 0
RTK	receptor tyrosine kinase
RXR	retinoid X receptor
SF-1	steroidogenic factor 1
SHP	small heterodimer partner
SMRT	silencing mediator for retinoid and thyroid hormone receptors
Sp1	specificity protein 1
SRC-1	steroid receptor co-activator 1
SREBP	sterol response element binding protein
STAT	signal transducer and activator of transcription
TBP	TATA-binding protein
TF	transcription factor
TGF	transforming growth factor
TNFα	tumor necrosis factor α
TR	thyroid hormone receptor
TRAP220	thyroid-hormone-receptor-associated protein of 220 kDa
TSS	transcription start site
VDR	vitamin D receptor
VDRE	vitamin D response element

List of original publications

This dissertation is based on the following publications referred to in the text by their Roman numerals (I-IV):

I. **Saavalainen K., Pasonen-Seppänen S., Dunlop T.W., Tammi R., Tammi M.I. and Carlberg C.** The human hyaluronan synthase 2 gene is a primary retinoic acid and epidermal growth factor responding gene. (2005) J Biol Chem **280**: 14636:14644.

II. **Sinkkonen L., Malinen M., Saavalainen K., Väisänen S. and Carlberg C.** Regulation of the human cyclin c gene via multiple vitamin D_3-responsive regions in its promoter. (2005) Nucl Acids Res **33**:2440-2451.

III. **Saavalainen K., Tammi M.I., Bowen T., Schmitz M.L. and Carlberg C.** Integration of the activation of the human hyaluronan synthase 2 gene promoter by common cofactors of the transcription factors RAR and NF-κB. (2007) J Biol Chem **282**:11530-11539.

IV. **Saavalainen K., Tammi M.I. and Carlberg C**. The genes *hyaluronan synthase 2* and *cyclin C* demonstrate convergence in CREB and retinoid signaling but differential co-factor preference. (2007) *Submitted*.

Table of contents

1. Introduction

Every cell is continuously exposed to stimuli from the extracellular environment. In order to survive in this changing environment, cells need to respond to those stimuli in a highly co-ordinated and accurate way. All cells of an organism are of same origin, the fertilized egg, so they contain the same genetic material, their genomic DNA, which stores and transmits the genetic information. In humans, the genome is formed by more than 20,000 genes and a lot of repetitive sequences. The genes are the templates for producing the functional units of a cell, mostly proteins. Individual cell types are characterized by which proteins it produces, i.e. which genes are first transcribed into mRNA and then translated into proteins. Therefore, although the genomic material is the same in each cell, cells are unique how they recognize and respond to environmental changes. The changing environment requires that a cell changes its protein synthesis, that is regulates the gene expression, which is the process that transfers the information from the genomic DNA via transcription and translation to proteins. This means that gene transcription also has to be a dynamic process.

The regulation of gene expression is under tight control. At its start, gene transcription is a result of well-co-ordinated actions of several regulatory proteins. In addition to a wide range of protein co-factors, a proper chromatin conformation is also required for transcription initiation. The activation state of chromatin is regulated by histone-modifying enzymes and chromatin-remodeling complexes. A host of proteins necessary to transcription initiation are assembled on to Pol II, the general TFs, co-activators (CoAs), co-repressors (CoRs), chromatin remodelers, histone acetyltransferases (HATs), histone deacetylases (HDACs) and histone methylases (HMTs). Activation of transcription requires the binding of specific TFs to their REs following the opening of chromatin, the binding of co-regulators and finally the assembly of the pre-initiation complex with Pol II to initiate RNA synthesis.

Nuclear receptors (NRs) are ligand-activated TFs that respond to both hormonal and nutritional signals by changing their target gene´s expression. Common to their ligands is that they are all lipophilic and thus able to cross the cellular membranes. RARα, β and γ are NRs that mediate the effects of the metabolites of vitamin A on the gene expression. CREB, STAT and NF-κB are TFs that mediate the actions of hydrophilic compounds and proteins, such as Forskolin, epidermal growth factor (EGF) and tumor necrosis factor α (TNFα), respectively, through different cell surface receptors and signaling cascades.

This study addresses the mechanisms of gene expression on the chromatin level by studying the expression of two different genes, human *HAS2* and *CYCLIN C*, by different kinds of signaling routes aiming to characterize both the general properties and the individual differences.

2. Review of literature

2.1 Gene regulation

Every cell of a human individual contains the same genomic material, its genome, in the form of 2 x 22 plus either an XX or XY pair of sex chromosomes. A chromatin subregion being necessary to produce a functional unit of the cell, a protein, is called a gene. Genes are the hereditary units of a cell. They contain regulatory regions determining, under which conditions the protein is produced. Transcribed regions dictate the structure of the proteins and/or other functional sequence regions. In order to fulfill its tasks DNA is not acting alone in the cell, instead it is wrapped around a protein core, to form the basic unit of chromatin, the nucleosome. In this environment the genome interacts with a wide range of regulatory proteins. In addition, all cellular life can recognize and most likely responds to molecules in the extracellular environment. The responses of individual cells to those stimuli make a cell unique. The differences between our cells arise from the fact that not all of our genes are expressed in every cell and in every condition. Thus understanding how gene expression is regulated becomes extremely important in understanding the behavior of our cells.

The regulation of gene expression involves a multitude of events leading finally to the beginning of gene transcription from a gene. The process consists of a series of spatially and temporally regulated events including several protein associations and dissociations in the regulatory regions of the genes. Gene expression is controlled mostly at the level of transcription initiation but also to a great extent on the chromatin level by controlling the activation status of chromatin. Below I will discuss in general and at the example of two genes, human *HAS2* and human *CYCLIN C*, the regulatory regions, proteins involved and chromatin architecture. This should give an overview on the events responsible for the regulation of gene expression.

2.1.1 The human genome

The whole set of genetic material, which includes of the genes of an organism or a cell is known as its genome. The human genome was published in 2004 (International Human Genome Sequencing Consortium, 2004). Still, now in 2007 some of the genes are thought to be missing and the exact genomic structure is estimated to be correct for only 50 % of the predicted genes (Guigo et al., 2006). The human genome consists of 3.08 Gb of DNA of which only 1.2 % is coding for proteins. A large proportion (approximately 50 %) of the human genome consists of repetitive sequence and it is thought to have a less significant role. The remaining sequence contains the genes and the regions regulating gene expression. Humans have in total approximately 20,800 protein-coding genes, which can be classified according to their biological function. This labeling is the function of the Gene Ontology (GO) database. Nucleic acid binding proteins, TFs and other proteins maintaining and regulating the

genetic information form the largest group consisting of approximately 13.5 % of all genes. The second largest group, 12.2 % of all genes, consists the proteins responsible for signal transduction including signaling molecules, receptors, kinases and other regulatory molecules. Surprisingly, there are a large number of genes responsible for regulation of gene expression, which emphasizes the importance of understanding its mechanisms. Another argument supporting the importance of getting more information on the details of gene regulation comes from the comparison of human genome with the genomes of other species. On the level of genes and proteins humans are very similar to other mammals. For example, the genome-wide divergence of the human and chimpanzee is only 1.06 % (Chimpanzee Sequencing and Analyzing Consortium, 2005). Even though the differences at genomic level are very small, the differences at phenotype level become apparent. The variation is due to develop as a sum of small changes. It can be assumed that the gene regulation part has here a huge impact.

2.1.2 Chromatin

The eukaryotic genomic DNA is orderly packed in the nucleus (Horn, 2002). Nucleosomes form the basic unit of the packing. Each nucleosome consists of 147 bp of DNA wrapped around the protein core (consisting of an octamer of histones). The nucleosomes are separated from each other usually by 10 to 60 bp of so-called linker-DNA. This structure forms the classical "beads-on-a-string" structure of DNA packing. Furthermore, nucleosomes are often packed into a 30 nm fiber, which can become even more compact by fiber-fiber interactions. In general, the density of chromatin packing affects its transcriptional activity. The more densely packed the chromatin is, the less transcriptional active it is. This is because chromatin condensation leads to a highly ordered chromatin structure that presents a physical obstacle for gene transcription by limiting the access of TFs and the Pol II core machinery to the target promoter DNA. The accessible open chromatin is called euchromatin and inaccessible, densely packed, chromatin is named heterochromatin.

Nucleosome protein cores consist of eight subunits, called histones (two each of H2A, H2B, H3 and H4) (Kornberg and Thomas, 1974). As early as mid 1960s, histones were shown to be post-translationally modified in multiple ways (Allfrey et al., 1964). Those modifications are now known to strongly affect the chromatin activation status of the nucleosomes. Covalent modifiers of histones include enzymes that catalyze the acetylation (HATs), deacetylation (HDACs), methylation (HMTs), phosphorylation (histone kinases) and ubiquitination (Cheung *et al.*, 2000; Grunstein, 1997; Turner 2002; Zhang and Reinberg, 2001). Histone modifications can either recruit (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Zeng and Zhou, 2002) or exclude (Zegerman *et al.*, 2002, Carmen *et al.*, 2002; Nishioka *et al.*, 2002) additional factors affecting chromatin structure and function. Specific modifications have been shown to correlate with specific transcriptional states forming a so-called histone code. For example, hypoacetylation of histones and trimethylation at lysine 9 (K9) of histone 3 are linked to condensed and transcriptionally inactive heterochromatin (Nakayama *et al.*, 2001; Jenuwein *et al.*, 2001). The trimethylation at K9 of histone 3 acts as a binding site for the heterochromatin-associated protein-1, which causes formation and propagation of heterochromatin. Additionally, sumoylation of H4 can also recruit heterochromatin-associated protein 1 (Shioo and Eisenmann, 2003). General hyperacetylation of histones and methylation at K4 of histone 3 are linked to accessible and transcriptionally more active euchromatin (Schubeler *et al.*, 2004). Even though there are some general features of active and inactive

chromatin, there are also exceptions. Kurdistani and co-workers published in 2004 microarray results showing that depending on the acetylated residue, the level of acetylation may correlate with gene expression either positively, negatively or not at all (Kurdistani *et al.*, 2004).

2.1.3 Regulation of gene expression

The importance of understanding the mechanisms of gene regulation at the molecular level has become even more evident during the last few years. The major mechanism for regulating gene expression is to control the transcription (Kleinjan and van Heyningen, 2005). Transcription of a eukaryotic gene precedes by multiple events including decondensation of the locus, nucleosome remodeling, histone modifications, binding of transcriptional activators and CoAs to enhancers and promoters and finally, recruitment of the basal transcriptional machinery to the core promoter (Smale and Kadonaga, 2003). A vast majority of protein-coding genes are organized into multiple exons, which are then spliced into mRNA that is then translated into protein. The exon splicing is preceeded by transcription through the whole gene containing area from the transcription start site (TSS) to the transcription termination site. The minimal or core promoter containing the 5' promoter element, contiguous with the TSS, is required to assemble the protein complex necessary for transcription (Levine and Tjian, 2003). A host of proteins necessary to transcription initiation are assembled into the Pol II, the general TFs, CoAs, CoRs, chromatin remodelers, HATs, HDACs, kinases and HMTs (Malik and Roeder, 2000; Näär *et al.*, 2001; Näär *et al.*, 1998; Jones and Kadanaga, 2000, Jenuwein and Allis, 2001). All eukaryotic cells express these proteins contributing to the initiation of every Pol II primary transcript. The total number of co-regulators and proteins of the transcriptional machinery is between 200 and 300.

2.1.3.1 TFs

TFs are the proteins that regulate the choice of gene specific initiation sites of transcription. There are 2,000 to 3,000 TFs in mammals (Brivanlou and Darnell, 2002). Two domains characterize them: a DNA-binding domain (DBD) that binds gene-specific regulatory sites and another domain possessing transcriptional activation potential, the transactivation domain. These two activities can also be divided between two partner proteins, one possessing the DNA-binding activity and the other the transactivation potential.

The action of the most TFs involves firstly a cascade of transcriptional controllers of TF genes and secondly signals from outside that activate the TFs. The complete set of these regulatory events and the number of TFs are unique for each gene.

Classification of DNA binding TFs

The classification of TFs presented here is mainly based on the review from Brivanlou and Darnell (2002). TFs can be divided into positively and negatively acting factors. Binding of a positively acting TF to the regulatory region of its target gene induces the initiation of target gene transcription. Negatively acting TFs repress the gene transcription. The positively acting TFs can be divided into several classes (Fig. 1). First, they can be constitutively active or regulatory. The constitutively active TFs are discussed below. The regulatory TFs are either developmental (cell-specific) or signal-dependent.

Developmental or cell-specific TFs accumulate in specific cells during their development. The accumulation is regulated by sequential waves of regulated transcription of genes encoding these TFs (Driever and Nüsslein-Volhard, 1989; Xanthopoulos *et al.*, 1989; Johnston and Nüsslein-Volhard, 1992). These proteins enter the nucleus automatically upon being made and contribute directly to transcription. This group includes a series of helix-loop-helix factors (MyoD, Myf5 and myogenin) appearing in sequence in the control of muscle differentiation (Yun and Wold, 1996) and a series of factors required for β cell differentiation in pancreatic islands (Duncan *et al.*, 1998). However, developmental TFs are not strictly cell-, tissue- or region-specific.

The expression of signal-dependent TFs may be either developmentally restricted or they can be present in most or even all cells, but are inactive until cells are exposed to appropriate intra- or extracellular signals (Brivanlou and Darnell, 2002). Signal-dependent TFs can further be divided into the NR superfamily (discussed in more detailed in section 2.1.4), TFs activated by internal signals and those activated by cell surface receptor-ligand interactions.

TFs that are regulated by internal signals form the most recently recognized group. The proteins of this group are activated by a variety of signals. Internal sterol concentrations regulate the proteolysis of a precursor to release sterol RE binding protein (Brown and Goldstein, 1999). Internally generated lipid compounds activate steroid-like receptors (Forman *et al.*, 1995; Kliewer *et al.*, 1995). Also genome damage that demands DNA repair enzyme function and thus increases p53 concentration is an example of internal signal activating TFs (Levine, 1997).

TFs that are activated by cell surface receptor-ligand interactions can be further divided into constitutive nuclear factors activated by serine phosphorylation and into latent cytoplasmic factors (Brivanlou and Darnell, 2002). For example, liganded G protein-coupled cell surface receptor proteins and receptor tyrosine kinases (RTKs) regulate transcription through a multitude of serine kinase cascades that finally phosphorylate serine of the abundant resident nuclear factors. The substrate proteins include the Ets family, the c-Jun/c-Fos/ATM family and the CREB family (discussed in more detailed in section 2.3.1.1).

Latent cytoplasmic factors reside in an inactive form in the cytoplasm until they get activated by proteins, which bind to cell surface receptors. These factors can get activated directly after receptor-ligand interaction by association of the TF with the activated receptor and following receptor phosphorylation. The factors can also undergo a more tortuous route of activation involving cytoplasmic serine phosphorylations and either promotion or inhibition of proteosomal proteolysis. Also Ca^{2+} or phosphoinosite fluctuations can cause TFs to release and move to the nucleus. Examples of latent TFs include STATs (discussed more detailed in section 2.3.2.1.1), the Rel/NF-κB family (discussed more detailed in section 2.3.3.1), the Wnt proteins (Hoppler and Kavanagh, 2007) and Notch (Weinmaster 2000).

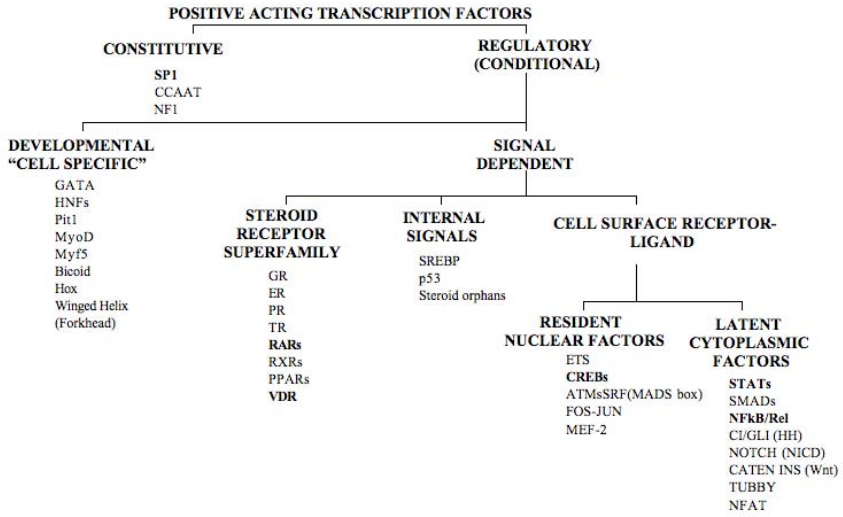

Fig. 1. Classification of DNA binding TFs (according to Darnell and Brivanlou, 2002). Positive acting TFs can be divided into constitutive active TFs and regulatory TFs. Regulatory TFs are either developmental of signal dependent. Signal dependent TFs form three distinct groups: NR superfamily of TFs, TFs activated by internal signals and those activated by cell surface receptor-ligand interactions. The latter group of TFs falls into two distinct groups: resident nuclear factors and latent cytoplasmic factors. The TFs studied in this thesis are marked in bold.

2.1.3.2 Regulatory DNA sequence elements

A prerequisite for the regulation of gene expression is first activation of and then binding of DNA-binding TFs to the regulatory regions of a gene (Levine and Tjian, 2003). In order to get a complete understanding of the regulation of gene expression, not only TFs, chromatin modifiers and co-regulators but also the putative regulatory elements, on which they act must be found out. The regulatory regions of genes contain sequence-specific binding sites for TFs. In general, a binding site or a RE is a short DNA sequence, usually 5 to 15 bp in length. The ability of a TF to bind certain DNA sequence is mainly determined by the structure of its DBD. However, the sequence of certain RE is not always very strict. A TF can bind REs that vary in their sequence by some nucleotides. However, some of the nucleotides are more critical than the others for the binding. Lack of a critical nucleotide in the RE sequence makes the binding of a TF impossible.

Array-based assays and gelshift assays can be used to screen the ability of TFs to bind certain DNA sequences. However, the *in vitro* data they provide is not always corresponding to the situation *in vivo*. The progress in the field of bioinformatics has offered us ways to predict transcriptional regulatory mechanisms from sequence, gene expression and binding data (Siggia, 2005; Tavazoie *et al.*, 1999; Birnbaum *et al.*, 2001; Bussemaker *et al.*, 2001; Segal *et al.*, 2003; Gao *et al.*, 2004; Haverty *et al.*, 2004; Ho Sui *et al.*, 2005; Beer and Tavazoie, 2004). In spite of the great advance in this field during the previous years, bioinformatics still has its limitations in

identifying the regulatory regions and the regulators involved in the process giving the traditional methods still a big role in finding out the regulatory mechanisms.

Simple eukaryotic regulatory regions

As the number of genes between unicellular and metazoan eukaryotes is not too much different, the differences are supposed to arise from the regulation of gene expression. The regulation in eukaryotic genes derived from organism with multicellular bodies in general is far more complicated, as are also their eukaryotic regulatory regions. The regulatory unit of a gene from a unicellular eukaryote is simpler (Fig. 2) and the complexity of multi-protein transcription complexes that regulate gene expression has increased as a result of the need to coordinate and communicate information between different cells in a single body as well as dedicating specific functions to discrete cell populations known as organs. A simple eukaryotic gene promoter usually contains a TATA box that serves as a binding site for TATA-binding protein (TBP) (Struhl *et al.*, 1998). Binding of TBP to the TATA box is generally enough for transcription initiation. The gene regulation in prokaryotes is largely dependent on the regulation of the binding of TBP. This depends on upstream activating sequences composing of two or three closely situated binding sites for one or two sequence-specific TFs (de Bruin *et al.*, 2001). The majority of genes from simple eukaryotic organisms such as yeast contain just one upstream activating sequence located within a few hundred base pairs of the TATA box.

Regulatory regions from metazoan eukaryotes

Genes from these organisms are composed of highly structured regulatory DNAs directing complex gene expression patterns in many different cell types (Levine and Tjian, 2003). The core eukaryotic promoter consists of approximately 75 bp surrounding the TSS. In addition to the TATA box, also initiator element and the downstream promoter element can recruit the TBP containing the TFIID initiation complex (Smale and Kadonaga, 2003). Many genes contain binding sites for proximal regulatory factors located just 5' of the core promoter. These factors, including GC-box binding specificity protein 1 (Sp1), rather than acting as classical activators or repressors, might serve as tethering elements recruiting distal enhancers to the core promoter (Su *et al.*, 1991). The core promoter is usually not enough to fully recapitulate a complex eukaryote gene´s transcription. These genes contain other regulatory elements, enhancers, silencers and insulators that are scattered over distances of roughly 100 Kb, on average, in mammals. A typical eukaryotic gene is likely to contain several enhancers that can be located both in 5' and 3' regulatory region, as well as in introns (Levine and Tjian, 2003). An enhancer is typically approximately 500 bp in length and contains about ten binding sites for at least three different sequence-specific TFs. Each enhancer is responsible for a subset of the total gene expression pattern usually mediating expression within a specific tissue or cell type. In addition, a single transcription unit can be regulated by multiple enhancers, as they can integrate different regulatory inputs, such as those produced by multiple signaling pathways. Different enhancers can work independently to direct composite patterns of gene expression when they are linked within a common *cis*-regulatory region. A *cis*-regulatory element is an element regulating the expression of genes located on the same strand. An example of this is the expression of the seven stripes of *even-skipped* in the *Drosophila* embryo that is dependent on five separate enhancers acting in an autonomous fashion (Small *et al.*, 1993; Fujioka *et al.*, 1999).

Insulators were first identified at defined boundaries between open and condensed chromatin domains (West *et al.*, 2002). They are usually 300 bp to 2 kB in length and they contain clustered binding sites for large zinc finger proteins, such as Su(Hw) and CCCTCC binding factor (Burgess-Beusse *et al.*, 2002; Bell *et al.*, 2001). Insulators are known to contain both enhancer blocking and heterochromatin barrier activities (West and Fraser, 2005). They selectively block the long-range integration of a distal enhancer with a proximal target promoter when, positioned between them. They can also protect a gene from silencing by acting as a barrier to prevent the spread of the repressive heterochromatin from and adjacent transcriptionally silenced gene neighbors (Schedl and Broach, 2003).

In addition to enhancers and insulators also DNA regulatory elements, called silencers take part into gene regulation. Silencers are elements that contain binding sites for repressors and thus prevent Pol II to start transcription.

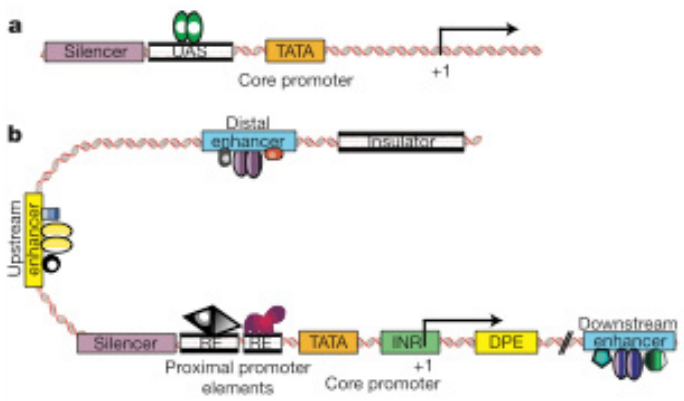

Fig. 2. Gene regulatory regions from simple and complex eukaryotes (adopted from Levine and Tjian, 2003). The simple eukaryotic regulatory unit consists of TATA box and usually one UAS and sometimes a repressive silencer element (a). The regulatory unit from a metazoan eukaryote is much more complex. The core promoter may contain in addition to TATA box also initiator element (INR) and downstream promoter element (DPE). Metazoan eukaryotic genes may contain multiple enhancers and silencers. At the same time, proximal promoter elements serve as binding sites for constitutive nuclear factors.

2.1.4 The NR superfamily

NRs typically function as ligand-activated TFs that regulate the expression of target genes involved in metabolism, development and reproduction (McKenna et al., 1999). The human genome contains genes for 48 members of this TF family. The superfamily contains the classic endocrine receptors that mediate actions of steroid hormones, thyroid hormones and the biologically active forms of the fat-soluble vitamins A and D and also so-called orphan NRs, whose ligands, target genes and physiological functions were initially unknown. Today natural ligands are known for about half of these orphan NRs and these are now referred to as adopted orphan NRs (Fig. 3).

All characterized NR ligands are lipophilic compounds and therefore capable of diffusing through cellular membranes. The classical endocrine members of the NR superfamily include the glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), androgen (AR), progesterone (PR), thyroid hormone (TR), retinoic acid (RAR) and vitamin D (VDR) receptor (Chawla *et al.*, 2001). They bind to their ligands with high affinity and their dissociation constant (K_d) values vary from 0.01 nM to 10 nM.

The group of adopted orphan NRs contains receptors for fatty acids (peroxisome proliferator-activated receptors (PPARs)), oxysterols (liver X receptors (LXRs)), bile acids (farnesoid X receptor (FXR)) and xenobiotics (pregnane X receptor (PXR), and constitutive androstane receptor (CAR)). The group now includes the retinoid orphan receptors, which are supposed to respond to stearic acid and cholesterol, hepatocyte nuclear factor (HNF) 4 α and γ, which are receptors for palmitic acid and receptors for anti-estrogens, estrogen-related receptors (ERRs) (Carlberg, 2004). The heterodimerization partner of many NRs, the retinoid X receptor (RXR), belongs to this group as well. The natural ligand for RXR is 9-*cis* RA. The adopted orphan NRs bind their ligands with lower affinity and their K_d values vary from 1 μM to 10 μM (Chawla *et al.*, 2001).

The remaining members of the NR family, for which still no ligand has been identified, are called true orphan NRs. According to phylogenic analysis the first NRs seem to have been true orphans and a ligand has been adopted later in evolution (Carlberg, 2004). This suggests that some of orphan NRs have yet to acquire a ligand. Orphan NRs are important TFs being pharmaceutical drug targets because of their association with human diseases. For example, small heterodimeric partner (SHP) is associated with obesity and nerve growth factor-induced clone B (NGFI-B) with schizophrenia.

2.1.4.1 Structure of NRs

The structure of NRs is extremely important in determining their functional properties. NRs share a common structural organization. The majority of them contain a variable amino (N)-terminal domain, a highly conserved DBD, a hinge region and a discrete ligand-binding domain (LBD) (Mangelsdorf *et al.*, 1995). The N-terminal domain is the least conserved part of the receptor. Most NRs carry a ligand-independent transactivation function in this part of the receptor (Tenbaum and Baniahmad, 1997). It serves as one contact point for co-regulators and often contains residues that can be phosphorylated affecting the activity of the receptor. The central part of the receptor contains the DBD and a hinge region the latter of which permits flexibility whilst the former allows simultaneous receptor dimerization and DNA binding. The DBD consists of two highly conserved zinc finger structures that target the receptor to specific DNA sequences, the REs. The hinge region may in some cases contain a nuclear localization signal or it can participate in co-regulator interactions. The LBD is in the carboxy (C)-terminal part of a NR. It is conserved to a lesser extent among different NRs and interacts directly with a ligand. The LBD encompasses a dimerization interface and a ligand-dependent activation function or helix 12. Helix 12 is crucial in the transition between inactive and active states of the receptor.

	Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Ligands:	High-affinity, hormonal lipids	Low-affinity, dietary lipids	Unknown
	TR α, β	PPAR α, δ, γ	DAX
	RAR α, β, γ	LXR α, β	SHP
	VDR	FXR	RevErbA α, β
	ER α, β	PXR	TR2 α, β
	GR	CAR	TLX
	MR		PNR
	PR	ROR α, β, γ	COUP-TF α, β, γ
	AR	RXR α, β, γ	NGFI-B α, β, γ
		HNF-4 α, γ	SF1
		ERR α, β, γ	LRH
			GCNF

Fig. 3. The NR superfamily (according to Carlberg, 2004). The 48 members of the human NR superfamily can be sorted into the three groups, endocrine receptors, adopted orphan receptors and orphan receptors.

2.1.4.2 Principles of the NR function

NRs act as TFs in the cell nucleus. They exert their effects via sequence-specific binding to the regulatory regions of their target genes. This occurs mainly by ligand-dependent mechanisms although ligand-independent regulation of transcription is known. NRs can activate or repress target genes by binding directly to REs or by binding to other classes of DNA-bound TFs (Glass and Rosenfeld, 2000). Certain NRs, such as GR, need to be complexed with their ligand to translocate into the nucleus and subsequently bind DNA (Hermanson *et al.*, 2002). Other receptors, such as VDR, the RARs and the TRs can bind to DNA in their unliganded state and associate with CoR complexes that actively repress transcription. For all NRs the role of the ligands in the transcriptional process is to modulate the receptor's functionality (Tenbaum and Baniahmad, 1997). Ligand-binding induces a conformational change to the LBD of the NR and causes an exchange of protein-protein interaction partners. Within the NR-LBD the most C-terminal helix, helix 12, acts as a molecular switch (Nettles and Greene, 2003). In the agonistic conformation, helix 12 forms one border of a hydrophobic CoA binding cleft, allowing recruitment of a LXXLL (L, leucine; X non standard amino acid) motif, which is present in many transcriptional co-factors. In the absence of ligands or in the presence of antagonists, helix 12 adopts another conformation that favours NR interaction with CoRs.

2.1.4.1.1 NR DNA binding

NRs bind selectively to DNA in the regulatory regions of their target genes. The DBD of all NRs contains two α-helices perpendicular to each other with their hydrophobic faces towards the protein core (Shaffer and Gewirth, 2002; Carlberg, 2004). One of these α-helices, the recognition helix, is located behind the first zinc finger and inserts directly into the major groove of a hexameric DNA sequence. This DNA sequence is referred to as the core binding motif. Since the recognition helix is highly conserved within the NR family, multiple NRs recognize closely related hexameric DNA motifs. Most NRs interact preferably with the consensus sequence RGKTCA (R = A or G, K = G or T). GR, PR, AR and MR, exceptionally, prefer the sequence RGAACA.

Most NRs bind to DNA either as a homodimer or as a heterodimer with RXR. Some of the NRs, such as the RORs, steroidogenic factor 1 (SF-1), liver receptor homologue (LRH), NGFIBs, RevErbA, *Drosophila* homologue of tail-less and photoreceptor-specific NR can bind to DNA as a monomer. The steroid receptors ER, GR, MR, PR and AR and the orphan receptors RXR, HNF4, ERRs, TR2, chicken ovalbumin upstream promoter (COUP) TF and germ cell nuclear factor (GCNF) form homodimers, while RARs, TRs, VDR, PPARs, LXR, FXR, PXR and CAR bind to DNA as heterodimers with RXR (Carlberg, 2004).

The hexameric core binding motif bound by NRs can be organized in three different configurations: direct repeats (DRs), inverted repeats or everted repeats. The number of nucleotides between the motifs varies as well. As the number of different NR REs is clearly less than the number of different dimeric NR complexes, every RE type is used by more than one NR. The types of REs used by NRs are shown in Fig. 4.

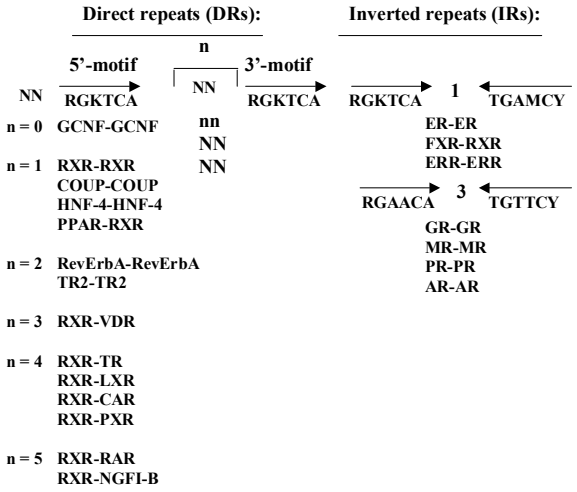

Fig. 4. The types of REs used by NRs (according to Carlberg, 2004). The most preferred RE for each type of homo- or heterodimeric NR complex is shown. NN represents 5' flanking sequences.

2.1.4.1.2 Co-factors

An essential prerequisite for the direct modulation of a gene by certain NR ligand is the location of the activated NR close to the basal transcriptional machinery. This happens by binding a RE and by interacting with other proteins involved in the gene transcription making a connection to the basal transcriptional machinery. In addition to the connection of NR with the basal transcriptional machinery, also the chromatin must be accessible for NR or any other TF and Pol II. Co-factor proteins are factors than enable the efficient gene transcription regulated by NRs.

2.1.4.3 Classification of NR ligands

NR ligands can be divided into two main group, first agonists and second inverse agonists and antagonists. Agonist binding to NR stabilizes the receptor in an active conformation and promotes the association of the LBD with CoAs. In addition, agonists disable the interaction of the receptor with CoRs. For example, all-*trans* RA serves as an agonist for RAR and the natural ligand for VDR, $1\alpha,25(OH)_2D_3$, is an agonist for VDR. Inverse agonists and antagonists support the interaction of the receptor with CoRs and stabilize the receptor in an inactive conformation. Synthetic compounds, such as C-1 phenyl substituted retinoids (Klein *et al.*, 1996) and ZK168281 (Bury *et al.*, 2000) function as antagonists for RAR and VDR, respectively. Inverse agonists for RAR include the synthetic compound AGN193013 (Klein *et al.*, 1996) while the two-side chain $1\alpha,25(OH)_2D_3$ analog Gemini can act as an inverse agonist for VDR (Bury *et al.*, 2000; Macias-Gonzalez *et al.*, 2003).

In the simple mode, NR agonists are thus compounds that when bound to the receptors activate them and trigger the response in the cell. Antagonist binding fails to activate the receptor and actually blocks the activation by agonists through competing with the same binding site. Many of NR ligands may work as agonists in one tissue but as antagonists in others (Weatherman *et al.*, 1999). That makes the terms agonist and antagonist not a perfect way to describe the ligands utilized in NR signaling.

2.1.4.4 RAR-mediated gene regulation

The RAR sub-family of NRs mediates the actions of retinoids derived from dietary vitamin A and from the provitamin β-carotene of plant origin (Altucci and Gronemeyer, 2001). There are three subtypes of RAR (α, β and γ) that are encoded by different genes. In addition, each subtype expresses several isoforms that differ in their N-termini due to alternative mRNA splicing and different promoter use. RAR transduces the retinoid signal together with RXR (of which there are also three sub-types: α, β and γ) acting as RAR-RXR heterodimer (Kastner *et al.*, 1997). Both all-*trans* RA and its 9-*cis* isomer activate RARs. RXRs are activated only by 9-*cis* RA. Binding of a ligand to RAR induces RAR-RXR heterodimer formation. RARs and RXRs alone bind poorly to DNA, but the effective DNA binding required for receptor function is achieved when a RAR-RXR heterodimer is formed. In the absence of agonists or in the presence of antagonists, RAR target genes are repressed, whereas agonistic ligand binding causes transcriptional activation. The basic regulatory mechanism of ligand activated RARs is further complicated by the interaction of CoAs and CoRs. Agonist binding causes a conformational change in the LBD of RAR that leads to replacement of CoRs with CoAs. CoAs interact with HATs leading to loosening of the chromatin. Further interactions with the mediator (MED) complex enable contact to the basal transcriptional machinery and subsequent gene transcription. Poly-ADP-ribose polymerase 1 (PARP-1) has quite recently been shown to be important in RA-mediated gene expression (Pavri *et al.*, 2005). Pavri and co-workers demonstrated that PARP-1 is a co-regulator of RAR-mediated gene expression acting in concert with the MED complex. It binds MED via its BRCT domain and this interaction is critical in determining whether the gene will be transcribed or not. In the absence of PARP-1 the RAR-MED-complex is unable to switch MED to its active form abrogating gene activation, while the presence of PARP-1 enables MED activation.

Retinoid regulation of the target gene transcription rate and the duration of the response are further complicated by covalent modifications of the retinoid receptors by phosphorylation triggering CoA association and ubiquitinylation involved in receptor degradation.

2.1.4.4.1 RAREs

RAR-RXR heterodimers modulate the frequency of transcription initiation of target genes by binding to RAREs in the promoter region of their target genes. The sequences bound by RAR-RXR heterodimers are rather pleiotropic (Glozak *et al.*, 2003). Although there is a loose consensus sequence of two directly repeated RGKTCA half-sites separated by five nucleotides (DR5-type REs), the spacing, relative position and number of these repeats are highly variable. RARs have been shown to bind and activate transcription via half-sites separated by up to 150 nucleotides (Kato *et al.*, 1995). RAR-RXR heterodimers also bind to DRs separated by 1 (DR1-type REs) or 2 nucleotides (DR2-type REs). RXRs can bind to DR1-type REs as RXR-RXR homodimers. RAREs can be located also within distant control regions. In addition to their location close to the TSS of their target genes, RAREs have been identified in introns or kilobases downstream of genes (Glozak *et al.*, 2003). There is also some evidence of the ability of DR2-type REs in repetitive Alu sequences to function as RAREs (Laperriere *et al.*, 2007).

2.1.4.4.2 RAR interference with other signaling pathways

RARs are known to interfere with a wide variety of other signaling pathways making the understanding of retinoid signaling even more complicated (Pfahl and Chytil, 1996). Promoters of RAR target genes contain in addition to the cognate RAREs also some other TF binding sites. Therefore RARs can interact with other TFs either in a synergistic or an antagonistic way. For example, RARs co-operate with Sp1/Sp3 TFs in the transactivation of the *CYP26* gene. Similarly, in the presence of cytokines, STAT5 co-operates with RARs to achieve maximum transcription of some RA responding genes (Si and Collins, 2002). Such synergistic effects are likely to result from the co-operative recruitment of co-regulators or increased chromatin remodelling (Bastien and Rochette-Egly, 2004). Retinoids are thus also able to antagonize the activation of genes by interfering with other TF signaling systems. The best-studied example of this action is the TF activator protein 1 (AP-1), which is a heterodimer of the proteins Jun and Fos. AP-1 activates the genes of growth factors, inflammatory peptides, oncogenes and tumor promoters, which usually results in an increased cell proliferation. In the presence of their respective ligands, RARs as well as RXRs can block AP-1 activity and thus directly interfere with many cell proliferation signals. However, the mechanistic basis remains elusive. Several distinct mechanisms have been proposed. These include the competition for limiting amounts of CoAs, disruption of the Jun-Fos dimerization or exclusion of some components (e.g. kinases) from the AP-1 complex. Retinoids have also been shown to inhibit the phosphatidylinositol 3 kinase/Akt pathway (Bastien and Rochette-Egly, 2004; del Rincon *et al.*, 2003) and to interfere with CREB signaling (Aggarwal, 2005). Retinoids activate CREB in a non-genomic way, through activation of protein kinase C, ERK and RSK in the absence of the influence of RAR-RXR heterodimers.

2.1.4.4.3 The role of RAR in normal physiology and disease

The natural ligands for RARs, the retinoids can be both detrimental and beneficial. They are modulators of cellular proliferation and differentiation as well as effectors of morphogenic changes. The production of retinoids needs to be tightly controlled for proper organogenesis. All-*trans* RA regulates the expressions of *homeo-box containing* (*HOX*) genes specifying positional information during morphogenesis (Kessel and Gruss, 1991; Marshall *et al.*, 1992; Marshall *et al.*, 1994; Ogura and Evans, 1995) and several *HOX* genes, e.g. *HOXB1*, contain RAREs in the 5' or 3' region (Ogura and Evans, 1995). Lack of vitamin A is known cause severe malformations and too high concentrations are teratogenic. It has been shown that nearly all of the congenital malformations caused by vitamin A deficiency are due to the absence of RAR or RXR functions.

The development of central nervous system is strongly influenced by RA, which is known to provide information for several processes during vertebrate development including positional information necessary for the proper functioning of the nervous system (Durston *et al.*, 1989; Maden *et al.*, 1991; Maden and Holner, 1992). Null mutations in retinoid receptors were subsequently shown to lead to abnormal neural development (Mendelssohn *et al.*, 1994). At the molecular level the regulation happens most likely by regulation the *HOX* gene expression patterns (Kessel and Gruss, 1992; Ogura and Evans, 1995).

RA also regulates the development of several other organs. The molecular mechanisms of many of these events are not established yet. RA appears to have a role in cardiac muscle morphogenesis as RAR null mutations combined with RXR mutation led to various defects often blocking the development (Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994). Similarly, RA is supposed to influence the development of pulmonary system (Chytil, 1984). The expression of RAR and RXR in lung tissues is influenced by all-*trans* RA (Hag *et al.*, 1991). Likewise, exogenous RA can induce the branching of the trachealveolar system. Again, several *HOX* genes influenced by RA have been identified in the lungs of newborns (Bogue *et al.*, 1994).

Retinoids have also an important role in adult physiology. Retinoids and their cognate receptors are required for the proper functioning of certain organs, such as the brain (de Urquiza et al., 2000; Krezel et al., 1998), liver (Imai et al., 2001; Wan et al., 2000), the reproductive system (Kastner et al., 1996) and the immune system, where they are involved both in cell-mediated and humoral immunity (Chun et al., 1992; Dennert and Lotan, 1978).

Because of their importance in the control of normal physiology natural retinoids and a large number of synthetic retinoids are used in the treatment of medical conditions (reviewed in Soprano *et al.*, 2004). All-*trans* RA, 13-*cis* RA and some synthetic retinoids are used in the therapy of several dermatoses (Peck and DiGiovanna, 1994). For example, *acne vulgaris* and photodamaged skin are often treated successfully by a topical application of retinoids. The medicine for acne, Adapalene, activates selectively RARβ and -γ (Bernand *et al.*, 1992; Delescluse *et al.*, 1991). Natural and synthetic retinoids have also shown promise for the prevention and treatment of cancers. The presence of retinoids can program cells to differentiate or even enter programmed cell death and can therefore interfere with tumorigenesis at several levels. Differentiation therapy has impressive beneficial effects, such as in the treatment of acute pro-myelocytic leukaemia, which is caused by genetic defects leading to

dysregulated RAR signaling. The origin of acute pro-myelocytic leukemia is a chromosomal translocation that fuses the pro-myelocytic leukemia gene *PML* and the *RARα* gene. PML-RARα has several effects including disturbance in the regulation of p53 (PML acts as a CoA for p53) (Guo *et al.*, 2000). Also many other cancers are being treated with retinoid-based therapies, examples of these include Kaposi's sarcoma, squamous-cell carcinoma and T-cell lymphoma. Retinoids can also be combined with other therapies. For example, retinoids and the anti-estrogen/estrogen antagonist tamoxifen are used together to treat breast cancer.

Although retinoids have shown to be promising in the treatment of several conditions the broad clinical use of retinoids has been hindered by their undesired side effects ranging from mild skin rashes to severe nausea, bone fractures and embryonic malformations (reviewed in Pfahl and Chytil, 1996). Nowadays many synthetic retinoids are available for experimental exploitation. There are selective agonists and antagonists for every receptor subtype (for both RARs and RXRs). These synthetic retinoids have the advantage of having not as many side effects as the naturally occurring retinoids.

2.1.4.5 VDR-mediated gene regulation

The biologically most active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ mediates its effects by acting via its receptor VDR (Chatterjee *et al.*, 2001; Sutton *et al.*, 2003). $1\alpha,25(OH)_2D_3$ binds to VDR with high affinity (K_d = 0.1 nM) inducing the formation of a VDR-RXR heterodimer. RXR was long thought to be a non-permissive or silent companion of VDR, but later on more evidence imply also RXR having an active role in $1\alpha,25(OH)_2D_3$-mediated gene regulation (Bettoun, 2003). In its unliganded state VDR is associated to CoR proteins (Burke and Baniahmad, 2000) that link it to enzymes with HDAC activity leading to chromatin condensation (Polly *et al.*, 2000). Thus, the actions of VDR resemble those of RAR and TR. $1\alpha,25(OH)_2D_3$ binding to VDR causes a change in the conformation of its LBD resulting in the replacement of CoRs by CoAs (Leo and Chen, 2000). This then leads to interactions with HATs and to subsequent loosening of the chromatin. Like RAR, VDR then interacts with the MED complex that bridges it to the basal transcriptional machinery.

2.1.4.5.1 VDREs

VDR-RXR heterodimers need to bind to a VDRE in order to modulate transcription. VDR recognizes DNA sequences with the consensus RGKTSA (R = A or G, K = G or T, S = C or G). As RXR also contacts DNA, simple VDREs are often formed by a direct repeat of two hexameric core binding motifs spaced by 3 nucleotides (DR3-type VDRE) (Carlberg, 1996). VDR-RXR can also bind to two hexameric motifs arranged as a DR spaced by 4 nucleotides (DR4-type VDRE) (Quack and Carlberg, 2000) or as an everted repeat with nine intervening nucleotides (ER9-type VDRE) (Nayeri *et al.*, 1995). Individual VDREs have been shown to be able to induce $1\alpha,25(OH)_2D_3$-mediated transactivation on their own. The presence of multiple VDREs in a certain promoter suggests that they act synergistically. For example, a DR3-type VDRE at a core of a VDRE cluster on human *25-hydroxyvitamin D_3-24-hydroxylase* (*CYP24*) gene promoter is the most potent human VDRE known so far (Chen and DeLuca, 1995). CYP24 is an enzyme that begins the degradation of $1\alpha,25(OH)_2D_3$ by hydroxylation it at carbon 24.

2.1.4.5.2 The role of VDR in normal physiology and in disease

Vitamin D_3 is taken up to the cells from diet or synthesized in skin with the help of UV light. Its bioactive metabolite, $1\alpha,25(OH)_2D_3$, is an endocrine hormone present everywhere in the body because of the circulation. Furthermore, the VDR is ubiquitously expressed enabling $1\alpha,25(OH)_2D_3$ to exert its effect throughout different tissues. $1\alpha,25(OH)_2D_3$ is predicted to have hundreds of responding genes.

The best-characterized processes regulated by $1\alpha,25(OH)_2D_3$ are the regulation of mineral homeostasis and skeletal integrity. In mammals, the maintenance of bone integrity depends on the regulated absorption and distribution of bone forming mineral. VDR target genes are involved in the calcium and phosphorous intake via the intestine. These target genes involve intracellular transporter proteins *calbindins D-9K* and *D-28K* and the calcium pump *PMCA* (Bouillon *et al.*, 2003). The increase in the activity of these genes increases the intake of minerals. VDR has also target genes involved in bone remodeling. Bone resorbing osteoclasts lack VDR mRNA, but the bone-forming osteoblasts express the VDR. Osteoblasts express known $1\alpha,25(OH)_2D_3$-responsive genes, such as *osteocalcin*, which is associated with the mineralized matrix of bone and enables osteoclast adhesion, acts as a component of elastic fibers in skin and aorta and also modulates immune reactions (Reinholt *et al.*, 1990; Baccarani-Contri *et al.*, 1995; Weber *et al.*, 1996).

VDR has been shown to have important roles in the control of cell growth and differentiation, both in normal and malignant tissues. Already in 1981, $1\alpha,25(OH)_2D_3$ was shown to arrest the proliferation of murine myeloid leukemia cells and induce their differentiation into macrophages (Abe *et al.*, 1981). Since then research concerning on the role of VDR in various cancer models and the modulation of cellular differentiation has emerged.

Many genes responsible for the regulation of cell cycle have been shown to be VDR target genes making it obvious that the anti-proliferative effects of $1\alpha,25(OH)_2D_3$ are mediated by those genes. As discussed in section 2.2.2, the progression of the cell cycle is controlled by cyclin-dependent kinases (CDKs) and cyclin complexes. The initiation phase is tightly controlled by CDK4/6 complex that binds cyclin D. This complex when activated phosphorylates and thus inactivates the retinoblastoma protein (Rb). This leads to TF E2F release from its inhibitor Rb and initiates the transcription of genes needed for the progression into the S phase (Harbour and Dean, 2000). The activity of CDK4/6 complex is inhibited by the specific INK4 family of CDK inhibitors including the proteins $p16^{INK4A}$ (Kamb *et al.*, 1994; Serrano *et al.*, 1993), $p15^{INK4B}$ (Hannon and Beach, 1994), $p18^{INK4C}$ (Guan *et al.*, 1994) and $p19^{INK4D}$ (Chan *et al.*, 1995). CDK complexes are also inhibited by other CDK inhibitors, such as $p21^{CIP1/WAF1}$ (Harper *et al.*, 1993; El-Deiry *et al.*, 1993) $p27^{KIP1}$ (Toyoshima and Hunter, 1994) and $p57^{KIP2}$ (Lee *et al.*, 1995; Matsuoka et al., 1995), which belong to the family of CIP/KIP CDK inhibitors. Both, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$genes have been shown to be upregulated by $1\alpha,25(OH)_2D_3$ (Liu *et al.*, 1996; Wang *et al.*, 1996). The $1\alpha,25(OH)_2D_3$ response of the *p21* gene is mediated by VDR together with another TF, p53 involved in cell cycle regulation (Saramäki *et al.*, 2006). In addition to $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, also many other CDK inhibitors, cyclins and CDKs are under VDR regulation (Jensen *et al.*, 2001).

VDR is also known to regulate both proto-oncogenes and tumor suppression genes. A proto-oncogene, *c-fos* and a tumor suppressor, *E-cadherin* have both been reported to be under the control of $1\alpha,25(OH)_2D_3$ (Mathiasen *et al.*, 1993; Pálmer *et al.*, 2001). VDR affects also growth factor signaling, such as transforming growth factor (TGF) β (Wu *et al.*, 1999; Pálmer *et al.*, 2003) and insulin-like growth factor (IGF)-1 (Peng *et al.*, 2004; Drivdahl *et al.*, 1995) signaling.

VDR is involved in the regulation of immune responses. It has been reported to have immunosuppressive action leading to suppression of inflammatory cytokines most likely because of VDR/AP-1 interaction (Alroy *et al.*, 1995). VDR can also block the interaction of the TFs Fos, Jun and nuclear factor of activated T-cells (NF-AT) (Towers *et al.*, 1999). Because of the immunosuppressive nature of VDR ligands, they have been used beneficially in several autoimmune diseases, such as multiple sclerosis (D'Ambrosio *et al.*, 1998), rheumatoid arthritis (Andjelkovic *et al.*, 1999) inflammatory bowel disease and type I diabetes (Gregori et al., 2002; Cantorna et al., 1998). VDR can also interfere with prostaglandin synthesis by totally blocking IL-1β-stimulated prostaglandin E_2 (Tetlow *et al.*, 1999), as well as it affects antigen-presenting cells. In mouse experiments, vitamin D could inhibit dendritic cell differentiation (Griffin *et al.*, 2001). Due to its effects on antigen-presenting cells, $1\alpha,25(OH)_2D_3$ and its analogs are used in graft rejection therapies. In addition, $1\alpha,25(OH)_2D_3$ treatment has been shown to induce tolerogenic dendritic cells, which protects against allograft rejection (Adorini *et al.*, 2003).

2.1.5 Signaling via cell surface receptors

In contrast to lipophilic NR ligands, there are numerous hydrophilic signaling compounds that cannot pass membranes and need to exert their effects via their cell surface receptors. These signaling molecules include peptide hormones, neuropeptides, neurohormones as well as growth factors (Brivanlou and Darnell, 2002). Their binding to a receptor on the surface of the cell thus initiates a chain of intracellular reactions, a so-called signal transduction cascade, finally leading to nucleus resulting in changes in gene expression.

Cell surface receptors include guanosine nucleotide binding protein (G protein)-coupled receptors, RTKs, cytokine receptors, nonreceptor protein-tyrosine kinases and several other receptors linked to other enzymatic activities.

2.1.5.1 G protein-coupled receptors

G protein-coupled receptors form the largest group of cell surface receptors that transmit signals to intracellular targets via the intermediary action of G proteins. The genes for those receptors account for approximately 5 % of the genes in human genome (Morris and Malbon, 1999). The ligands are chemically diverse including neurotransmitters, hormones, phospholipids, odorants and photons (Gavi *et al.*, 2006). The receptors are characterized by seven transmembrane α-helices. Ligand binding to the extracellular domain of these receptors induces a conformational change that allows the cytosolic domain of the receptor to bind a G protein that associates at the inner surface of the plasma membrane. Receptor-G protein interaction activates the G protein which then dissociates from the receptor and carries the signal to an intracellular target, which is often an enzyme

or an ion channel. One important G protein target is adenylyl cyclase, an enzyme making the important second messenger cyclic AMP (cAMP).

Forskolin

Forskolin is a diterpene that is produced by the Indian and East African Coleus plant, *Plectranthus barbatus*. It is now in extensive use in clinical studies and also in treatments of several conditions. Forskolin resensitizes cell receptors by activating the enzyme adenylyl cyclase and thus increasing the cAMP levels in the cell. This effect accounts for its versatile role in the body. Forskolin works as a vasodilator (Bubolz *et al.*, 2005). In addition, it has been studied extensively as a weight loss aid. Godard and co-workers showed that in a clinical study Forskolin significantly increased lean mass, bone mass and also testosterone levels (Godard *et al.*, 2005). Forskolin can also be used to treat glaucoma, reduce urinary tract infections and enhance the ability of antibiotics to kill bacteria that would normally survive. It increases skin's natural resistance to burning under UV light and stimulates tanning response when applied topically. It is also used to promote nerve repair by increasing cAMP concentrations. It has shown to be able to activate the proliferation of Schwan cells. In addition, various experimental studies are underway in using Forskolin as an adjunct treatment for diseases such as Parkinson's or nerve damage caused by trauma or accident.

2.1.5.1.1 CREB

cAMP-response element-binding protein 1 (CREB1) is an important TF implicated in a large number of biological processes, including long-term neuronal plasticity, cell survival, circadian rhythms, adaptation to drugs and hormonal regulation of metabolism (Lonze and Ginty, 2002). To get activated, CREB1 needs to be phosphorylated. This is done by protein kinase A (PKA) and by a variety of other kinases, such as calcium/calmodulin-dependent protein kinases, SNF1-like kinase and ribosomal S6 kinase 1, at the same critical serine residue, Ser-133. The phosphorylation is essential for the recruitment of the transcriptional CoA, CREB-binding protein (CBP) or its paralog p300 (Chrivia *et al.*, 1993). CBP induces transcription via its acetylase activities and by interacting with the core transcriptional machinery (Vo and Goodman, 2001). Recently, it has been noticed that other phosphoserine-133-independent mechanisms are required for full activation of CREB (Johannessen and Moens, 2007). These include multiple phosphorylations by a diversity of kinases on other phosphoacceptor sites in CREB1.

The CREB-CBP pathway includes first the binding of CREB to the CRE in target genes following the recruitment of the CoA CBP (Cha-Molstad *et al.*, 2004) (Fig. 5). The first CRE (TGACGTCA) was identified in the promoter of somatostatin gene (Gonzalez *et al.*, 1989). Nowadays it is predicted that there may be as many as 10,000 CREB-binding sites in the mammalian genome (Euskirchen *et al.*, 2004). The binding has also been demonstrated to be cell type specific (Cha-Molstad *et al.*, 2004). Cell type specificity might be due to the fact that epigenetic differences in chromatin from one cell type to another determine the population of genes capable of binding CREB and, in turn, the pattern of gene responses to the wide variety of pathways that activate CREB1 function. CREB1 binding to CRE is supposed to be constitutive, not regulated and CREs in most genes are occupied by CREB under basal conditions. (Hagiwara *et al.*, 1993; Richards *et al.*, 1996; Gonzalez and

Montminy, 1989). Thus, the regulation of CREB-CBP pathway happens mainly via regulating the recruitment of CBP.

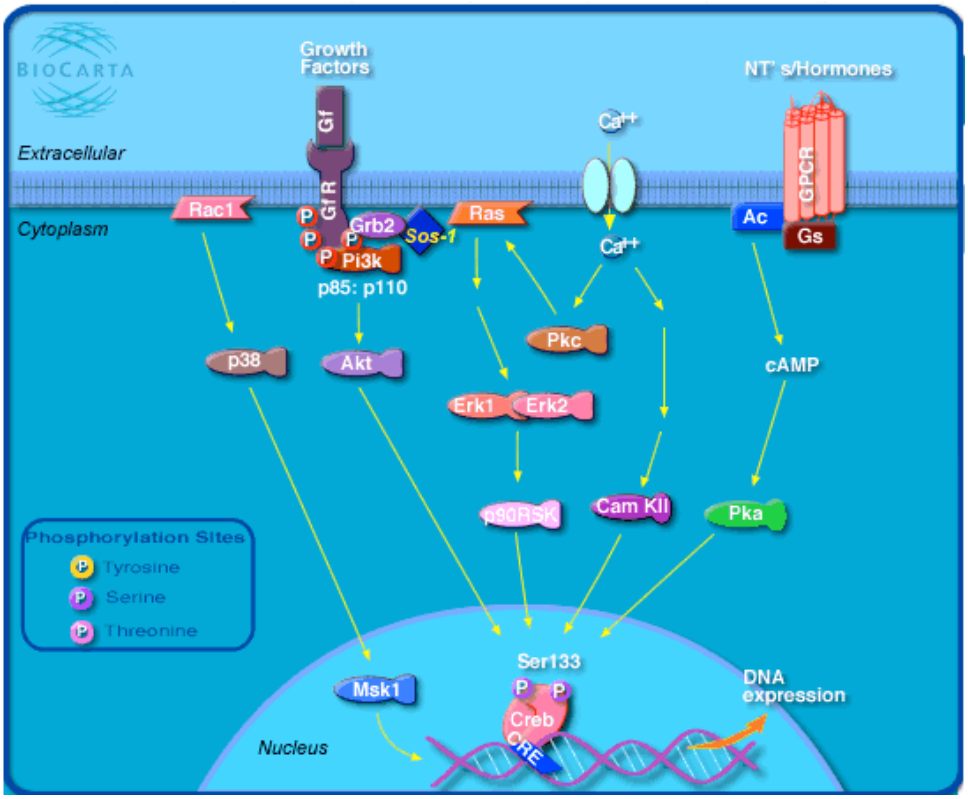

Fig. 5. Overview of CREB signaling (www.biocarta.com). The main feature of CREB signaling is phosphorylation of CREB and binding to CRE to induce target gene transcription.

2.1.5.2 Receptor tyrosine kinases

The receptors for most growth factors belong to the group of RTKs (Pawson and Scott, 1997). Examples of RTKs include the cell surface receptors for insulin, IGF receptor 1 and epidermal growth factor (EGFR) (Gavi *et al.*, 2006). These receptors are directly linked to intracellular enzymes, which phosphorylate their substrate proteins on tyrosine residues. Ligand-binding first induces receptor dimerization leading to autophosphorylation of the receptor. The auto-phosphorylation in the catalytic domain plays a regulatory role by increasing the receptor protein kinase activity. The phosphorylation outside of the catalytic domain creates specific binding sites for additional protein that transmit intracellular signals downstream of the activated proteins. The best characterized of these downstream signaling molecules are Src homology 2 (SH2) domain containing proteins.

2.1.5.2.1 EGF

EGF is an important growth factor regulating cell growth, proliferation and differentiation. There is a family of mammalian polypeptide growth factors that exhibit significant sequence identity with EGF (Holbro and Hynes, 2004). All these EGF-related peptide growth factors are encoded by distinct genes and contain an EGF-like

domain that confers binding specificity. They are responsible for activating the four different EGFRs, also called ErbB receptors. The group of EGF-related growth factors includes in addition to EGF, TGF-α, amphiregulin, betacellulin, heparin-binding EGF, epiregulin and neuregulins 1-4. All the peptides of this group are synthesized as glycosylated integral membrane precursor proteins with extracellular domains containing an EGF-like mature peptide sequence. The precursor of EGF contains approximately 1200 amino acid residues. The sequence of this precursor includes in addition to the sequence of EGF, eight EGF-like units and, near the C-terminus, a hydrophobic sequence that is characteristic of an integral membrane protein. The membrane-associated precursor of EGF is biologically active (Dobashi and Stern, 1991) as is the mature, soluble peptide that is released from the extracellular domain by proteolytic cleavage (Pandiella *et al.*, 1992).

EGF signaling

EGF and all the EGF-related peptides act via binding to the cell surface membrane receptor, EGFR. EGFR belongs to the group of RTKs, which are the primary mediators of the signals regulating complex biological processes, such as cell growth, differentiation, motility and death (Hackel *et al.*, 1999) (Fig. 6). EGF binding to EGFR induces the formation of a dimer and, as a consequence, the intrinsic kinase domain of the receptor is activated. This results in autophosphorylation at multiple tyrosine residues within the cytoplasmic tail of the protein (Holbro and Hynes, 2004). These phosphorylated residues serve then as docking sites for a variety of signaling molecules, whose recruitment leads to the activation of many downstream signaling pathways. Among the most strongly activated and best-studied signaling cascades, that EGFR initiates from the plasma membrane, are those that go through RAS, phospholipase Cγ (PLC-γ) and STAT proteins (Wells and Marti, 2002).

Proteins with SH2 domains and PTB domains binding to EGFR start the signaling cascade going via the RAS pathway. After binding to EGFR SH2-containing and growth factor receptor-bound protein 2 adaptor proteins attract the GTP-exchange factor son-of-sevenless. This activates RAS (Lowenstein *et al.*, 1992) and in turn RAS activates other small GTPases (Cdc42, Rho and Rac), which have a critical role in the control the cytoskeleton. RAS also initiates a kinase cascade activating the extracellular PLC-γ signal-regulated kinases 1 and 2 (ERK1 and 2) and the mitogen-activate protein kinase (MAPK) pathway through Raf and MAPK/ERK kinase (MEK). ERK can then activate the intracellular-limited protease calpain II or it can translocate to the nucleus and act as a TF (Wells and Marti, 2002).

PLC-γ is one of the best-characterized substrates of the EGFR. PLC-γ binds to phosphotyrosines of EGFR to align itself for cross-phosphorylation and activation. It hydrolyses phosphoinosite 4,5-bisphosphate to produce inositol trisphosphate and diacylglycerol (Wells and Marti, 2002). Inositol trisphosphate acts as a second messenger molecule and liberates stored calcium from the endoplasmatic reticulum and thereby activates calcium-dependent enzymes and processes. Diacylglycerol is an activator of protein kinase C (PKC). PKC plays important roles in regulating several processes involved in cell growth and differentiation.

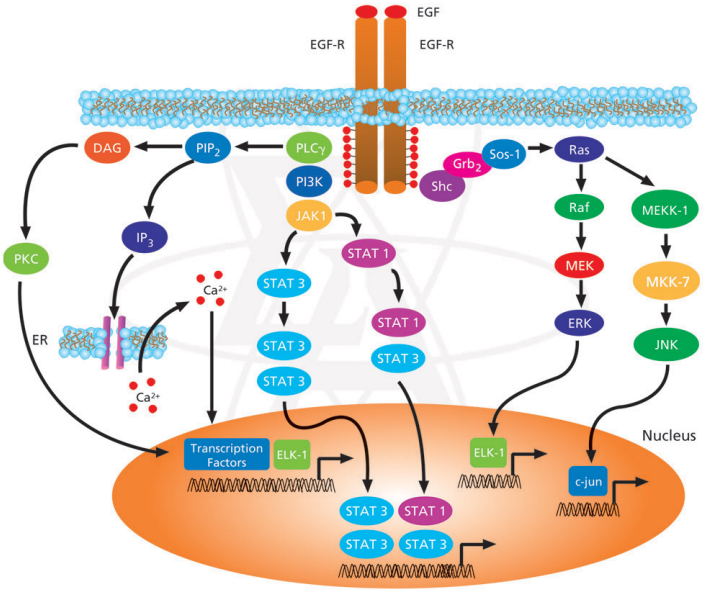

Fig. 6. Overview of EGF signaling (www.sigma-aldrich.com). The different pathways of EGF signaling starting from the binding of EGF to its receptor and leading to transcription of target genes.

2.1.5.2.1.1 STAT

STATs are a family of latent cytoplasmic proteins that acts as TFs (Benekli *et al.*, 2003) and participate in many signaling systems. STAT target genes influence growth, survival, apoptosis, host defence, stress and differentiation functions, depending on the signaling pathway and the target tissue (Levy and Darnell, 2002). In total seven STAT genes have been identified in mammals in three chromosomal clusters (Benekli *et al.*, 2003).

More than 35 different polypeptide ligands are known to activate one or more different STATs. Thus STATs are involved in a wide range of biologic events (Darnell, 1997). Three of the known STATs have a narrow activation profile: STAT2 is activated only by interferon α; in lymphocytes STAT4 is activated only by interleukin 12 (IL-12) and interferon α; and STAT6 is activated only by IL-4 and IL-13. In contrast, STATs 1, 3, 5A and 5B are activated by many different ligands. STAT activation involves cytoplasmic recruitment to activated cell-surface receptors, tyrosine phosphorylation, receptor release, dimerization and nuclear translocation followed by site-specific DNA binding to control target gene expression (Murray, 2007). STATs are the only known TFs that become activated from a latent state by tyrosine residue phosphorylation (Brivanlou and Darnell, 2002). Ligands that can induce STAT activation can be associated with Janus kinases that tyrosine-phosphorylate the liganded receptor and then the associated STAT. STATs can also be activated by RTKs, such as EGFR, by non-RTKs, such as Src and Abl, and through G protein-coupled cell surface receptors (Bromberg and Darnell, 2000). There is not much known about the specificity of STAT activation. For example, EGF is known to induce the activation

of STAT1, STAT3 and STAT5 (Zhang *et al.*, 2003). However, the activation is different in different cell types. STAT3 has been demonstrated to play critical roles in EGF signaling in normal and malignant cells.

Once STATs are phosphorylated, they form dimers through reciprocal SH2-phosphotyrosine interaction. They form either homo- or heterodimers depending on intermolecular SH2-phosphotyrosine interactions (Darnell, 1997) and enter the nucleus. The mechanism of the nuclear entry of the tyrosine-phosphorylated dimers is not exactly known, but it has been suggested to happen through binding of the dimer to an importin (Sekimoto *et al.*, 1997). Nuclear entry is then followed by specific DNA binding and participation in the control of gene expression. STAT dimers with a two-fold symmetry bind in the nucleus to a palindromic DNA motif (Darnell, 1997). This palindromic core motif, $TTCN_{2-4}GAA$, is recognized by all the different STATs (Ehret *et al.*, 2001). Activation of STAT target genes requires CoA recruitment by STAT transactivation domains and interaction with acetyltransferases, minichromosome maintenance proteins and additional factors (Levy and Darnell, 2002).

2.1.5.2 Cytokine receptors and non-RTKs

Cytokine receptors do not posses any intrinsic enzymatic activity but they act by stimulating intracellular protein-tyrosine kinases with which they are non-covalently associated. Ligand binding to receptor induces dimerization of the receptor and activates the non-RTKs. The activated kinases then phosphorylate the receptor that can then recruit downstream signaling molecules containing SH2 domains. Cytokine receptor/non-RTK combination can thus work analogously to the RTKs, The family of cytokine receptors contains the receptors for most cytokines and also receptors for some polypeptide hormones (e.g., the growth hormone receptor). Non-RTKs are made of nine distinct families including Src, Jak, Abl, Fak, Fps, Csk, Syk, Pyk2, Btk (Gavi *et al.*, 2006).

2.1.5.2.1 NF-κB

NF-κB is a TF involved in the control of a large number of normal cellular and organ level processes, such as immune and inflammatory responses, development, cellular growth and apoptosis. In addition, it is persistently active in a number of disease states, including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases and heart disease (Kumar *et al.*, 2004). It belongs to the family of Rel proteins of which the most common member is a heterodimer consisting of p50 and p65 proteins (Schmitz and Baeuerle, 1995). The activity of NF-κB is regulated by inhibitory IκB proteins (Beg and Baldwin, 1993) (Fig.7). In most cells, NF-κB is present as a latent, inactive IκB-bound complex in the cytoplasm. IκB masks the nuclear localization signal in NF-κB preventing it entering to the nucleus. In response to external stimulus IκB gets phosphorylated by specific kinases, called IκB kinases (IKK). Phosphorylation leads to degradation of IκB following NF-κB translocation to the nucleus, DNA binding and contacts with MED and CoA proteins. NF-κB can be activated by a wide variety of signals including bacterial and viral antigens, UV irradiation, free radicals and several cytokines including in total nine different ILs and both TNF α and β. The consensus sequence for NF-κB DNA binding is GGGRNNYYCC, (Y = C or T, N = any base).

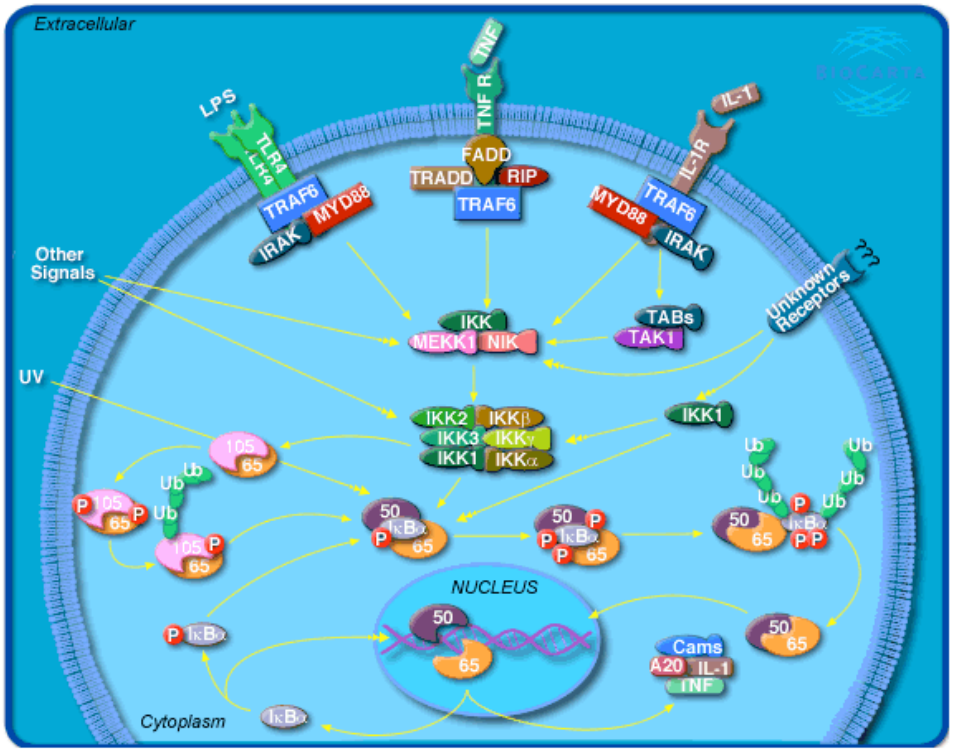

Fig. 7. Different pathways of NF-κB signaling (www.biocarta.com). The different pathways leading to degradation of IκB and activation of p50/p65 heterodimer are depicted.

2.1.5.3 Receptors linked to other enzymatic activities

Majority of the enzyme-linked receptors stimulate protein-tyrosine phosphorylation. However, a number of receptors are associated with other enzymatic activities. The group includes protein-tyrosine phosphatases (Andersen *et al.*, 2004), protein-serine/threonine kinases (reviewed in de Garcer *et al.*, 2007) and guanylyl cyclases. Protein-tyrosine phosphatases remove phosphate groups from phosphotyrosine residues. They often have negative regulatory roles in cell signaling pathways by terminating the signals initiated by protein-tyrosine phosphorylation. Still many of them have a positive role in cell signaling. For example the surface molecule CD45, which is expressed on the surface of T and B lymphocytes belongs to the protein-tyrosine phosphatases. The group of protein-serine/threonine kinases contains TGF-β and some related polypeptides. Ligand-binding to these receptors leads to the association of two distinct polypeptide chains to form heterodimers in which the receptor kinases cross-phosphorylate each other. The activated receptors then activate further downstream signaling molecules. The receptor guanylyl cyclases have catalytic intracellular domains that catalyze the formation of a second messenger cyclic GMP. For example, nitric oxide acts by stimulating guanylyl cyclases.

2.1.6 Constitutively active nuclear factors

In addition to regulatory TFs there is a group of site-specific DNA binding proteins present in the cell nucleus of all cells all of the time (Brivanlou and Darnell, 2002). The group includes Sp1 (Briggs *et al.*, 1986), CCAAT

binding protein (Chodosh *et al.*, 1988; Johnson and McKnight, 1989), nuclear factor 1 (Driever and Nüsslein-Volhard, 1989) and many others. These constitutively active nuclear factors have transcriptional activating potential. Still, they have not been implicated in changing the rates of individual gene transcription in a chromosomal context. They are supposed to facilitate the transcription of many chromosomal genes, possible genes that are constitutively transcribed, such as structural proteins tubulin and actin, and ubiquitous metabolic enzymes, such as glyceraldehyde phosphate dehydrogenase. These TFs are also able to participate in enhanceosomes together with TFs that are being regulated.

2.1.6.1 Sp1

Sp1 belongs to the constitutively active TFs (Turner and Crossley, 1999). It is ubiquitously expressed and it binds to GC-rich REs and therefore regulates a large number of so called housekeeping genes that have GC-rich promoters (Black *et al.*, 2001). Sp1-like family factors regulate transcription by interacting with CoAs, like CBP, involving histone acetylation (Song *et al.*, 2002; Zhang *et al.*, 2001).

2.2 Target genes

The total number of transcriptional regulators and signaling pathways regulating gene expression in humans is huge. It is also known that the same stimulus affects different genes differently. Even though Métivier and co-workers provided a model of the general features of transcriptional activation on chromatin level (Métivier *et al*., 2003) it can be speculated that there is not a general model that applyes to all genes in all situations. As well, each gene contains many REs for different TFs. That makes it interesting to speculate that not only each gene has a unique pattern of transcriptional regulators but also each RE on the regulatory regions of the gene might be unique in how it recruits transcriptional co-regulators.

In order to get a broader understanding of the events of transcriptional regulation, two totally different genes, human *HAS2* and *CYCLIN C*, were selected as candidate genes. In addition to different functions of the proteins encoded by the two target genes, also the expression levels of *HAS2* and *CYCLIN C* genes differ *HAS2* having a much lower basal expression level than *CYCLIN C*. Studying the regulation of two such different genes should give more information of the general mechanisms of gene expression than studying just one gene.

2.2.1 *HAS2*

HAS2 is an enzyme making hyaluronan. Hyaluronan (also called hyaluronic acid or hyaluronate) is a linear high molecular mass non-sulfated polysaccharide (Tammi *et al.*, 2005). It is composed of repeating disaccharide of D-glucuronic acid and N-acetylglucosamine: $[\beta1,4\text{-GlcUA-}\beta1,3\text{-GlcNAc-}]_n$ (Fig. 8). The number of disaccharides usually varies leading to molecular weights of hyaluronan polymers between 2,000 and 25,000 Da. Hyaluronan is a major constituent of the vertebrate extracellular matrix. It has multiple physiological and cellular roles due to its unique biophysical and interactive properties (Toole, 2002). Its charge characteristics and polymeric properties contribute in many ways to tissue homeostasis and biomechanics. The interactions with other extracellular macromolecules (such as aggregating proteoglycans) are crucial to the assembly and integrity of

extracellular and pericellular matrices. Its interactions with cell surface receptors, such as CD44 and hyaluronan-mediated motility receptor, influence the cell behaviour in many morphogenetic and physiological systems. Hyaluronan is involved in maintenance of tissue architecture, cell proliferation, migration, differentiation, angiogenesis, wound healing and also tumorigenesis (Tammi *et al.*, 2002). In addition, increased levels of hyaluronan in tissues and body fluids can be detected in several inflammatory conditions, such as lung fibrosis, rheumatoid arthritis, myocardial infarction and transplant rejection as well as in some invasive tumours.

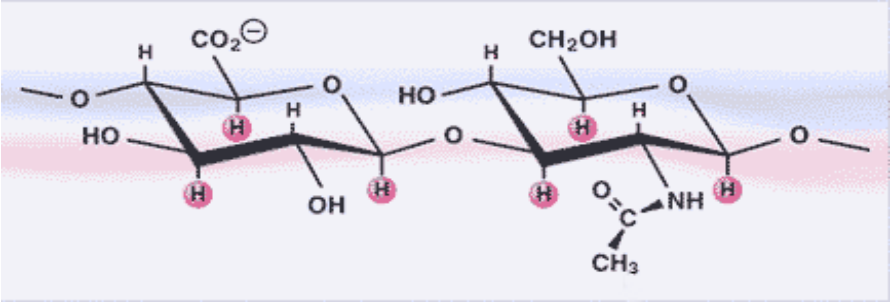

Fig. 8. Hyaluronan structure (http://www.glycoforum.gr.jp/science/hyaluronan/HA23/HA23E.html). The basic unit of hyaluronan is a disaccharide consisting of D-glucuronic acid and N-acetyl-D-glucosamine. These are linked by a glucuronidic β (1-3) bond. The disaccharide units are then polymerized by hexosaminidic β (1-4) linkages.

2.2.1.1 Hyaluronan synthases

Hyaluronan is highly metabolically active. Its half-life in humans ranges from less than one day to several days (Weigel *et al.*, 1997). Hyaluronan is synthesized at the plasma membrane by monomeric enzymes called hyaluronan synthases (HASs), which are located at the plasma membrane. Three different genes that produce HAS enzymes have been identified in mammalian cells and are called, *HAS1*, *HAS2* and *HAS3*. In the human genome *HAS1* is on chromosome 19, *HAS2* on chromosome 8 and *HAS3* on chromosome 16. The three Has enzymes are related to each other but have distinct enzymatic properties, which suggests a different physiological role for each synthase (Itano *et al.*, 1999). They differ from each other in stability, elongation rate of hyaluronan and apparent K_m values for the two substrates (UDP-GlcNAc and UDP-GlcA). They are also expressed differentially in several cell types and show an idiotypic response to growth factors (Jacobson *et al.*, 2000). The Has enzymes have extremely short half-lives of 2 to 4 h. Moreover, Has enzyme activity has been shown to be 6.5-times higher in the exponential growth phase than in the stationary growth phase of cells (Zhang *et al.*, 2000).

2.2.1.2 Regulation of hyaluronan synthesis

Understanding the regulation of hyaluronan synthesis is important because of the important roles of hyaluronan in normal physiology and in disease. The mechanisms that regulate hyaluronan synthesis in normal tissues and in pathological processes, such as tumor metastasis and inflammatory foci are not fully understood. Several groups have found that growth factors, such as platelet-derived growth factor-BB, TGF-β1, IGF, fibroblast growth factor, follicle-stimulating hormone and EGF regulate hyaluronan synthesis in various cells of mesenchymal

origin (Jacobson *et al.*, 2000; Pienimäki *et al.*, 2001). The growth factors mediate their stimulatory effects on hyaluronan synthesis partly through the activation of PKC and PKA (Suzuki *et al.*, 1995; Klewes and Prehm, 1994). The influence of growth factors is specific for each cell type and may also depend on the stage of the cellular differentiation. The expression of the *HAS* genes has been shown to be higher in sub-confluent cultures compared with growth-arrested cultures (Jacobson *et al.*, 2000). Also some NR ligands, such as all-*trans* RA and glucocorticoids, have been observed to modulate its synthesis rate (Tammi *et al.*, 1989; Zhang *et al.*, 2000).

Of the three Has enzymes, Has2 is thought to be the main regulator of the hyaluronan synthesis. The proximal promoter region of its gene shows lower levels of constitutive activity compared to *HAS1* and *HAS3* proximal promoters making it more potent target of regulation (Monslow *et al.*, 2004).

2.2.2 *CYCLIN C*

Cyclin C belongs to the cyclin protein superfamily the members of which are the main regulators of the eukaryotic cell cycle (Johnson, 1999). The regulation of cell cycle happens by sequential assembly and activation of the family serine-threonine protein kinases, the CDKs (Villiard *et al.*, 2001). Cyclins are needed to activate the CDKs. Cyclin binding to CDKs introduces a conformational change that leads to the activation of the enzyme. In addition cyclins activate kinases that phosphorylate CDKs to make them fully active (Murray, 2004). Mammals have in total 18 cyclin proteins and nine CDKs. Many of the cyclins are very similar to each other and capable of activating the same CDKs.

Cyclin C has a role in many processes as it is known to activate two different kinases, CDK3 and CDK8. Cyclin C/CKD3 complex causes the arrested G_0 phase cells to go trough G_0 to G_1 transition. This transition is possible as CDK3 phosphorylates the well-known tumor suppressor Rb (Weinberg, 1995). The actions of the other Cyclin C complex, Cyclin C/CDK8, are better understood. Cyclin C/CDK8 complex regulates the transcriptional activity of a cell. The complex has been demonstrated to be associated with the Pol II basal transcriptional machinery (Rickert *et al.*, 1996). The complex is also known to be a subunit of the MED complex. MED proteins form big complexes consisting of seven to eighteen subunits differing among the subunit composition (Rachez *et al.*, 2001; Bourbon *et al.*, 2004). Cyclin C is involved in high molecular mass MED complexes (Wang *et al.*, 2001). The MED complexes containing CDK8/Cyclin C were shown to be able to inhibit transcription (Sun *et al.*, 1998; Akoulitchev *et al.*, 2000). The inhibition is mediated through two different kinase activities of CDK8. CDK8 can phosphorylate the C-terminal domain of Pol II leading to the inhibition of transcription. When the CTD of Pol II is phosphorylated, it cannot form a transcription pre-initiation complex (Lu *et al.*, 1991). CDK8 can also phosphorylate the general transcription initiation factor II H (TFIIH) inhibiting the transcription-activating role of Cyclin H/CDK7. There is also recent evidence of CDK8 as a part of MED complex being a positive regulator of transcription (Furumoto *et al.*, 2007). In addition to being a general regulator of transcriptional activity in cell, Cyclin C is also linked to tumorigenesis (Li *et al.*, 1996) and to Alzheimer's disease (Ueberham *et al.*, 2003). The *CYCLIN C* gene is known to be regulated by $1\alpha,25(OH)_2D_3$ (Polly *et al.*, 2000; Verlinden *et al.*, 2000).

2.2.2.1 Cell cycle

Each cell replicates its DNA and divides into two daughter cells during cell cycle (Johnson, 1999). The mammalian cell cycle consists of four distinct phases that need to take place in a well-defined order and each need to be completed successfully until the next phase can start. The starting point of the cycle is the G_1, or gap1 phase. During G_1 phase cell needs to decide on the basis of different signals, whether to continue proliferation or to exit cell cycle into G_0 phase during which proliferation does not continue. If the different mitogenic signals surpass growth inhibitory signals the cell commits for another cell cycle and starts to prepare the entry into the S (or synthesis) phase during which DNA is replicated. The S phase is followed by the G_2 phase during which the integrity of DNA is checked and the cell prepares the final fourth phase, mitosis (M). During mitosis the cell divides into two daughter cells, which have to do the decision between proliferation and differentiation.

2.3 General features of co-regulator function

CoA and CoR complexes are acting as sensors that integrate signaling inputs to generate precise and complex programs of gene expression. Co-regulators contain enzymatic activities that are required for modification of the integral components of the co-regulator complexes and are also involved in modifications of the components of the basal transcriptional machinery and chromatin at target gene promoters. Many CoAs and CoRs are components of multi-subunit co-regulator complexes possessing a multitude of enzymatic activities. The complexes can be divided in two classes. Complexes of the first class consist of enzymes that can covalently modify histone tails such as acetylating/deacetylating activities (HATs and HDACs), methylating/demethylating enzymes (HMTs (Jenuwein and Allis, 2001)), protein kinases, protein phosphatases, poly(ADP)ribosylases, ubiquitin and SUMO ligases. The second class contains the components of a family of ATP-dependent remodelling complexes such as the SWI/SNF complex that alters the structure of the nucleosome in an ATP-dependent manner most likely by modifying the histone-DNA interface that often leads to nucleosome sliding (Peterson, 2002).

Co-regulator complexes are thought not to be TF-specific. Many co-factors are known to mediate the actions of numerous different classes of TFs (Glass and Rosenfeld, 2000). In addition, several TFs are able to use distinct combinations of co-factors depending on the cell type, promoter, DNA-binding site and the actions of various signaling pathways and ligands (Hermanson *et al.*, 2002).

The signal-dependent interactions of co-regulators with sequence-specific TFs are controlled at several levels, including the expression levels of co-regulators, post-translational modifications of co-regulators and their targets and for NRs there is control by ligand binding. Covalent modifications including phosphorylation, acetylation, sumoylation, ubiquitination and poly(ADP)ribosylation of DNA-binding factors (Rochetty-Egly *et al.*, 1997) and CoAs such as CBP and E1A-binding protein (p300), are critical aspects of regulation (Yaciuk and Moran, 1991; Banerjee *et al.*, 1994; Chakravarti *et al.*, 1999). For example, CREB activates target genes in part through direct interactions with the KIX domain of the CoA CBP. This interaction has been shown to be dependent upon phosphorylation (Radhakrishnan *et al.*, 1997; Impey and Goodman 2001; Mayr *et al.*, 2001). Similarly, CoRs,

including NCoR1 and silencing mediator for retinoid and thyroid hormone receptors (SMRT/NCoR2) are modulated by phosphorylation (Hong and Privalsky, 2000) and sumoylation.

There is known to be signal-dependent, temporal-specific factor exchange (Cosma, 2002). The first example came from studies of the HO locus of budding yeast, in which an ordered recruitment of SWI5 an SBF, the SWI/SNF complex, the SAGA complex and finally the Ash1 repressor was reported. This ordered exchange both defines the sequence in recruitment of enzymatic machinery necessary to achieve activation of specific transcription units and also provides a temporally chancing complement of potential sensors for responding changes in the signaling milieu of the cells. It is now widely accepted that the active exchange cycles of sequence-specific TFs and associated co-regulators are required for sustained transcriptional responses in signaling inputs in metazoan organisms.

Signal-dependent turnover has been correlated with transcriptional activation for several NRs. Proteasome-mediated degradation and ligand-dependent transactivation are reported to be linked and act continuously, in the case of both RAR (Kopf et al., 2000) and VDR (Masuyma and MacDonald, 2000; Li et al., 1999). Also some CoAs such as receptor associated CoA3 can be targeted for degradation by ubiquitinylation/proteasome pathways (Morris *et al.*, 2003; Gillette *et al*., 2004; Li *et al.*, 2006).

The dynamics of these processes are still to some extent unclear and a common model of the recruitment of transcriptional regulators and chromatin modifiers still does not exist. Métivier and co-workers have studied the dynamics of co-factor recruitment to human *pS2* gene promoter in response to estrogens (Métivier *et al*., 2003). They concluded transcriptional activation to be a cyclic process involving both activating and repressive epigenetic processes. They defined the concept “transcriptional clock” that is supposed to direct and achieve the sequential and combinatorial assembly of transcriptional regulators on a promoter. The initial cycle of ERα recruitment is transcriptionally unproductive process that is required for promoter commitment. Nucleosome remodeling complex SWI/SNF is recruited to *pS2* promoter following by the recruitment of HMTs, HATs and then the components of basal transcriptional machinery. This step finally leads to proteosomal degradation of ERα. The degradation step happens at the end of each cycle and is followed by histone deacetylation prior to the initiation of following cycles. The cycle following the initial cycle is transcriptionally productive. This cycle starts by the recruitment of p68 RNA helicase following combinatorial sequestering of HMTs, p160 and HATs that ultimately form a large complex on the promoter that then directs and achieves further posttranslational modifications of histones. This is followed by the recruitment of TR-associated protein (TRAP)/MED complex which promotes the phosphorylation of Pol II and transcriptional initiation.

2.3.1 CoAs

There are different groups of CoAs differing from each other by their structure and function. Some of the CoAs serve as adaptors between NRs or some other TFs and the basal transcriptional machinery. Many of the CoAs have enzymatic activities, such as HAT activity or HMT activity, which target histones for acetylation or methylation, respectively. HAT activities containing CoAs include the members of the steroid receptor CoA (SRC) family, SRC-1, transcription intermediary factor 2 and receptor-associated CoA3. In addition to them also

p300 and CBP are CoA possessing HAT activity. HMTs include CoA-associated arginine methyltransferase I and protein arginine methyltranferase I. CoAs can also serve as bridging factors that make a connection from even a far distant NR to the basal transcription machinery. For example, TRAP220 serves as a bridging factor for many NRs.

Nucleosome remodelling factors are protein complexes that weaken the tight wrapping of DNA around the histone octamers facilitating the sliding of histone octamers to neighboring DNA segments (Becker and Hörz, 2002). For example, brahma-related gene 1 is a CoA that forms a part of nucleosome remodelling complex. It is known to be a central regulator of the cell cycle and tumor suppression due to its direct interactions with Rb and several cyclins and CDKs (Shanahan *et al.*, 1999; Bochar *et al.*, 1999).

Some CoAs are involved in the ubiquitin proteasome pathway. These CoAs include receptor potentating factor 1, E6-associated protein, ubiquitin conjugating enzyme 7, suppressor of Gal4-thyroid hormone interacting protein 1, MB67-interacting protein 224 and TBP-1. There are also a variety of CoAs that are involved in splicing control and some that can be classified as signal-integrating CoAs. The former group includes among others CoA activator and CoA of AP-1 and ERs and the latter includes proteins such as the transducer of regulated CREB activity 2.

2.3.2 CoRs

CoRs are proteins whose interaction with NRs lowers the transcription rate of their target genes (Burke and Baniahmad, 2000). Several NRs, including RAR, VDR and TR, bind to DNA of their target genes already in the absence of ligand and actively repress transcription. Those NRs often bind to DNA in the complex with either NR CoR 1 (NCoR1) or SMRT (NCoR2). Both NCoR1 and SMRT contain transferable repression domains and are supposed to work as CoRs by recruiting a complex containing Sin3, HDACs and also some other proteins (Hörlein *et al.*, 1995; Chen and Evans, 1995). The roles of NCoR1 and SMRT have expanded also to many other transcriptional repression events than that of NRs (Xu *et al.*, 1998). They can also function as CoRs for homeodomain repressors Rpx and Msx-1 (Woloshin *et al.*, 1995) and they can repress the transcription of genes that are a result of Notch-Delta pathway activation (Kao *et al.*, 1998).

NcoR1 and SMRT localize mainly in the nucleus. Still, it has been suggested that they can be post-translationally modified which in turn can lead to nuclear-cytoplasmic shuttling of these CoRs. SMRT is known to be phosphorylated by MAPK-directed pathway and in the case of NCoR1, phophorylation of an associated protein TAB2 by IKK kinase induced nuclear exit (Hong *et al.*, 2001; Baek *et al.*, 2002).

In addition to NCoR1 and SMRT, the group of CoR proteins include also other proteins: thyroid hormone receptor interactor 15 (Alien), small unique NR CoR and homolog of mouse hairless (Hairless) that is involved specifically in the regulation of hair development.

3. Aims of this study

When this thesis was started the regulation of the human *HAS2* gene in general and in particularly on chromatin level, was not understood in detail. There was practically no information concerning the chromatin level events affecting *HAS2* gene expression. Also the regulation of another important gene, human *CYCLIN C*, was not characterized in detail. In addition, there was still a lot to clarify concerning the mechanisms of gene expression on the chromatin level in general.

Therefore the specific aims of this study were:

1. To investigate the regulation of human *HAS2* gene expression in response to all-*trans* RA, EGF, TNFα and Forskolin both on mRNA and chromatin level.

2. To study the chromatin composition of the $1\alpha,25(OH)_2D_3$ responsive regions on the human *CYCLIN C* promoter.

3. To investigate the regulation of human *CYCLIN C* gene by all-*trans* RA and Forskolin both on mRNA and chromatin level.

4. To compare the regulation of the two different genes, *HAS2* and *CYCLIN C*, on chromatin level.

4. Materials and methods

4.1 Materials

4.1.1 Ligands, inhibitors and recombinant proteins

The roman numerals used in the following sections refer to the four studies forming the theses. All-*trans* RA (I, III, IV) and EGF (I) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The EGFR kinase inhibitor 4-(3'-chloroanilino)-6,7-dimethoxyquinazoline, AG1478 (I), was from LC Laboratories (Woburn, MA). $1\alpha,25(OH)_2D_3$ (II) was kindly provided by Dr. L. Binderup (LEO Pharma, Ballerup, Denmark). Recombinant TNFα (III), IKK-2 inhibitor (III) and Forskolin (IV) were purchased from Calbiochem (Läufelfingen, Switzerland). Recombinant STAT1 (I) was from Lab Vision (Fremont, CA). $1\alpha,25(OH)_2D_3$ was dissolved in 2-propanol, TNFα in H_2O, whereas the other compounds were dissolved in DMSO. Further dilutions were made in DMSO or in EtOH as indicated in the original articles.

4.1.2 Cells

Table 1. Growth conditions for the cell lines used in the studies

Cell line	Medium	FBS (%)	Used in
HaCaT	DMEM	10	I, III, IV
REK	DMEM	10	I
HEK293	DMEM	10	IV
MCF-7	α-MEM	5	II

4.1.3 DNA constructs

Protein expression constructs

In vitro transcription/translation of the respective cDNAs and the overexpression of the respective proteins in mammalian cells were done using T7 RNA polymerase-driven mammalian expression vectors. The full-length cDNAs for human RARγ (Schräder *et al.*, 1993) and human RXRα (Mangelsdorf *et al.*, 1990) were sub-cloned previously into the T7/SV40 promoter driven pSG5-expression vectors (Stratagene, La Jolla, CA). The full-length cDNA for human CREB was subcloned into T7/SV40 promoter driven pFN2-expression vector and was purchased from Origene (Rockville, USA). The full-length cDNAs for human p50 and p65 proteins were subcloned into T7/SV40 promoter driven pcDNA3-expression vector and were kindly provided by Prof. M.L. Schmitz (Justus-Liebig-University, Giessen, Germany).

Reporter gene constructs

The core sequences of the REs and the promoter fragments are indicated in the original articles. They were fused to the *thymidine kinase* promoter driving the firefly *luciferase* gene (LUC) in a pGL3-(apart from *Cyclin C* promoter fragments in IV) derived Luciferase Reporter Vector (Promega, Madison, WI, USA), The nested set of *HAS2* promoter fragments (III and IV) was kindly provided by Dr. T. Bowen (Cardiff University, Cardiff, United Kingdom) (Monslow *et al.*, 2004). The *XbaI*-restriction site at nucleotide position 1742 of pGL3 had been removed from the original vector by mutagenesis. The individual REs were cloned as a two-copy constructs of the RE to the remaining *Xba*I cloning site. The fragments of the human *CYCLIN C* promoter (II) were cloned by PCR from human genomic DNA using primers with *SacI* (forward primer) and *XbaI* (reverse primer) restriction sites. The promoter fragments for human *CYCLIN C* promoter used in the fourth original publication were cloned by PCR from human genomic DNA using primers with *Kpn I* (forward primer) and *Nhe I* (reverse primer) restriction sites and fused to the *thymidine kinase* promoter driving the firefly *luciferase* gene in a pGL4-derived *luciferase* reporter vector.

4.2 Methods

4.2.1 *In silico* promoter analysis

The first 10 kb (I) or 2250 bp (III and IV) of the human *HAS2* promoter was screened *in silico* for putative RAR (I, III, IV), STAT (I), Sp1 (III), NF-kB (III) and CREB1 (IV) binding sites. The first 2250 bp (IV) of human *CYCLIN C* promoter was screened for putative RAR (IV) and CREB (IV) binding sites. Putative RAREs were identified by screening the promoter for two copies of RGKTCA consensus sequence in a DR5 arrangement. The maximal deviation from the consensus sequence was restricted to one position. Putative Sp1, NF-κB and CREB1 binding sites were screened by using the net-based program ConSite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) with a TF score cutoff of 85 %. The putative REs found on these criteria are described in the individual publications. The nucleotide positions referred to in the position of the found putative REs are respective to the indicated gene's TSS, with negative values assigned to sequence found upstream from the gene.

4.2.2 *In vitro* methods

4.2.2.1 *In vitro* transcription and translation

Human RARγ and RXRα proteins were generated by coupled *in vitro* transcription/translation using their respective pSG5-based full length cDNA expression constructs (Schräder *et al.*, 1993; Carlberg *et al.*, 1993) and rabbit reticulocyte lysate as recommended by supplier (Promega, Madison, WI, USA). The reaction set up is as follows:

25 µl rabbit reticulocyte lysate, nuclease treated
1 µl amino acid mix (1 mM)
1 µl RNase inhibitor (40 U/µl, Fermentas)
10 µl rNTPs (5 mM)
6 µl $MgCl_2$ (25 mM)

5 µl template DNA (0.4 µg/µl)

1 µl T7 RNA polymerase (20 U/µl, Fermentas)

The *in vitro* transcription/translation reactions were incubated for 90 min at 30 °C. Protein batches were quantified by test translation in the presence of [^{35}S]-methionine. For the experiments the specific protein concentrations were adjusted to 4 ng/µl (10 ng corresponds approximately to 0.2 pmol) after taking the individual number of methionine residues per protein into account.

4.2.2.2 Nuclear extracts preparation

For nuclear extract preparation, HaCaT cells were seeded into 175 m^2 culture bottles and grown overnight in DMEM supplemented with 5 % (w/v) charcoal-treated FBS to reach a density of 60 to 70 %. At the start of experiment the medium in the bottles was reduced to 10 ml. Cells were treated for 20 min with solvent or 20 ng/ml TNFα (Calbiochem, Läufelfingen, Switzerland) with or without 10 µM IKK-2 inhibitor (Calbiochem, Läufelfingen, Switzerland). Cells were harvested by mechanical scraping into 5 ml of PBS. Cell pellets were resuspended in 0.5 ml of low salt buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM dithiothretiol, proteinase inhibitors). Cells were then resuspended in 2-3 packed volumes of the same low salt buffer with 0.5 % NP40 and homogenized by pipetting on ice. The nuclear pellet was then resuspended in one packed cell volume of high salt buffer (10 mM Hepes, pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, proteinase inhibitors). Extraction was performed for 30 min on ice.

4.2.2.3 Gel shift assay

Gel shift assays were performed with 10 ng of *in vitro* translated proteins using the molar ratio 1:1 (4 µl RARγ and 1 µl RXRα), with 10 µg of the nuclear extracts or with 750 ng of recombinant STAT1 protein (1.5 µl from a 0.5 mg/ml stock, adjusted to 5 µl with H_20). The proteins were incubated at room temperature (RT) for 15 min in binding buffer containing 2.5 µl 1 M KCl, 4 µl 5 x gelshift buffer (10 mM Hepes, pH 7.9; 1 mM dithiothretiol, 25 ng/µl herring sperm DNA (Promega), 5 % glycerol) and the reaction volume was adjusted to 18 µl with H_20. Double-stranded oligonucleotides were labeled by a Klenow fragment DNA polymerase (Fermentas, Vilnius, Lithuania)-mediated filling in reaction in the presence of a nucleotide mixture containing radio-labeled [^{32}P]-α-dCTP. Constant amounts (1 ng) of [^{32}P]-labeled oligonucleotides (50 000 cpm) were incubated with the nuclear extracts for 20 min at room temperature (RT). Antibodies were added 15 min after the nuclear extracts to the DNA. Protein-DNA complexes were resolved by electrophoresis through 5 (STAT) of 8 % non-denaturing polyacrylamide gels in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.3)) and quantified on a Fuji FLA3000 (Fuji, Tokyo, Japan) reader using the ScienLab99 software package (Fuji).

4.2.2.4 Transfection

HaCaT and HEK293 cells were seeded into 6-well plates (I, III, IV) or into 96-well plates (IV) and grown overnight in DMEM supplemented with 5 % (w/v) charcoal-treated FBS to reach a density of 60 to 70 %.

Transfection with DOTAP

In DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate) transfection 10 µg (for 6-well plates) or 0.5 µg (for 96-well plates) of DOTAP was incubated for 15 min at RT with 1 µg or 0.04 µg of the reporter plasmid and 1 µg or 0.04 µg of pSG5-, pcDNA3-, or pFN2K-based expression vectors in a total volume of 100 µl or 20 µl for 6-well plates or for 96-well plates, respectively. After the 15 min incubation 900 µl (for 6-well plates) or 80 µl (for 96-well plates) of phenol red-free Dulbecco's modified Eagle's medium (DMEM) was added. Medium was replaced and the transfection medium was added. Ligands were added in phenol red-free DMEM supplemented 5 % FBS 4 h after transfection.

Transfection of siRNAs with Polyethylenimine (PEI)

PEI (Sigma-Aldrich) was used to transfect siRNAs into HEK293 cells (IV). siRNA mixtures used were composed of equal amounts of three different siRNA molecules against CBP, MED1 or NCoR1 in addition to control. 200 pmol of each siRNA molecule was incubated for 15 min with 50 µl of 150 mM NaCl (for 6-well plates). Simultaneously, 10 µg of PEI was incubated with 50 µl of 150 mM NaCl. After the 15 min incubation, both solutions were combined and the mixture was incubated for an additional 15 min. After that 900 µl of phenol red-free DMEM supplemented with 1 % glutamine was added per one well of a 6-well plate. Medium was replaced and the transfection medium was added. Ligands were added in phenol red-free DMEM supplemented with 5 % FBS 48 h after transfection. The silencing effect was checked by real-time PCR.

4.2.2.5 Luciferase reporter gene assay

In the reporter gene assays on 6-well plates (I, III) cells were washed twice with ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \bullet H_20$) and lysed with 200 µl of reporter gene lysis buffer (Roche) 16 h after ligand addition to transfected cells. The lysis was collected and the cells pelleted by centrifuging for 15 min with 20,000 x g at RT. In order to measure the luciferase activity, 150 µl of the lysate was combined with 100 µl of luciferase substrate (in Luciferase Reporter Gene Assay buffer, Canberra-Packard, Groningen, Netherlands) and measured with the Highpass filter. The total protein amount was measured by taking 10 µl of the lysate and combining it with 200 µl of Protein Assay buffer (Biorad, München, Germany). The absorbance was measured at 595 nm. A 96-well plate reader luminometer (Anthos Labtec Instruments, Wals/Salzburg, Austria) was used in the measurements in I and III.

In the 96-well plated method (IV), 16 h after ligand addition, the medium was removed from the cells and the 50 µl of luciferase substrate (Luciferase Reporter Gene Assay buffer, Canberra-Packard, Groningen, Netherlands) was added to the cells and measured with Victor^3 multi-label reader (PerkinElmer Life and Analytical Sciences, Shelton, USA). The total protein amount was measured by adding 50 µl of Protein Assay buffer (Biorad, München, Germany) to cells containing the luciferase substrate. The absorbance was measured at 595 nm with Victor^3.

4.2.3 *Ex vivo* methods

4.2.3.1 RNA extraction

Cells were seeded into 100 m^2 bottles (I) or 6-well plates (III, IV) and grown overnight to reach a density of 60 to 70 %. Ligand stimulation was done like indicated in the individual publications

Total RNA extraction (I)

Total RNA was extracted using Tri-reagent (Sigma-Aldrich). 1 ml of Tri-reagent was used for approximately 5 x 10^6 cells. 0.2 ml of chloroform was added to separated the mixture into 3 phases: an aqueous phase containing RNA, the interphase containing DNA and an organic phase containing proteins. After 2 min incubation at RT the mixture was centrifuged for 15 min at 4 °C with 12,000 x g. The supernatant containing RNA was taken into a new tube and RNA was precipitated by adding 0.5 ml isopropanol. After incubation for 10 min at RT the precipitated RNA was pelleted by centrifuging for 10 min at 4°C with 12,000 x g. The RNA pellet was washed with 1 ml of 75 % ethanol and dissolved in 100 µl sterile water.

mRNA extraction (I)

mRNA was extracted from the total RNA by using Oligotex mini mRNA kit (Qiagen, Hilden, Germany). To isolate mRNA 150 µl of RNase free water, 250 µl of binding buffer (20 mM Tris-HCl, (pH 7.5), 1 M NaCl, 2 mM EDTA, 0.2 % SDS) and 15 µl of Oligotex suspension were added for 100 µl of total RNA. Oligotex suspension contained oligodT particles suspended 10 % (w/v) in solution containing 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.1 % SDS, 0.1 % NaN_3. To allow Oligotex-mRNA complexes to form, samples were heated at for 5 min at 70°C and incubated for 10 min at RT. Complexes were then pelleted by centrifuging for 2 min with 14,000 x g at RT. The pellets were washed by resuspensing the Oligotex: mRNA pellets in 400 µl of washing buffer (10 mM Tris-HCl, (pH 7.5), 150 mM NaCl, 1 mM EDTA) and the suspension was removed to a spin column placed in an Eppendorf tube. After centrifugation for 1 min with 14,000 x g at RT, the spin column was transferred to a new RNase-free tube and the washing step was repeated. After centrifugation the spin column was transferred to a new RNase-free tube and mRNA was eluted by adding 100 µl of hot (70 °C) elution buffer (Buffer OEB: 5 mM Tris-HCl (pH 7.5)) onto the column and by centrifuging for 1 min with 14,000 x g at RT. The elution step was repeated. RNA yield and purity were determined by A_{260}/A_{280} ratio.

Total RNA extraction with the Mini RNA Isolation II kit (III, IV)

RNA was extracted from the cells directly with RNAeasy kit (HiSS Diagnostics, Freiburg, Germany). Cells were washed twice with ice-cold PBS. Cells were then lysed with 600 µl of ZR RNA buffer and collected into columns placed in Eppendorf tubes. The column was centrifuged for 1 min with 14,000 x g at RT. After that the RNA was washed twice with RNA wash buffer and the RNA was eluted with 50 µl of sterile water. Total RNA amount was measured with NanoDrop ND-1000 (NanoDrop, USA).

4.2.3.2 cDNA synthesis

cDNA synthesis was performed using 100 pmol of oligodT_{18} primer in the presence of reverse transcriptase (Fermentas). Sample reactions were prepared using 100 ng of mRNA (I, II) or 1 µg of total RNA (III, IV) as a template, 4 µl of 5 mM dNTPs, 2 µl of 0.1 M DTT, 8 µl of 5 x reverse transcriptase buffer (250 mM Tris-HCl

(pH 8.3), 250mM KCl, 20 mM $MgCl_2$, 50 mM DTT) (Fermentas), 2 µl of reverse transcriptase (20 U/µl), 1 µl of RNAse inhibitors (40 U/µl) and H_2O to a final reaction volume of 40 µl. cDNA was synthesized for 1 h at 37 °C followed by an inactivation for 5 min at 95 °C. The cDNA was diluted 1:10 in H_2O.

4.2.3.3 Real-time PCR

Real-time quantitative PCR was performed in an IQ-cycler (BioRad, Hercules, CA, USA) using the dye SybrGreen (Molecular Probes, Leiden, The Netherlands). The reaction set up is as follows:

2.2 µl 10 x reaction buffer (HotStart PCR buffer (Fermentas in I and II), FastTaq PCR buffer (Roche) in III and IV)
2.4 µl 25 mM $MgCl_2$
0.4 µl 10 mM dNTPs
1 µl SybrGreen (1:2500 dilution from the stock)
0.2 µl HotStart / FastTaq DNA polymerase (5 U/µl)
8 µl H_2O
1 µl primer mix (10 µM)

PCR primers and primer-specific annealing temperatures are described in the individual papers. PCR cycling conditions were:

1. Denaturation for 5 min at 95 °C
2. PCR amplification repeated for 40 cycles
   - Denaturation for 30 s 95 °C
   - Annealing for 30 s at primer-specific annealing temperatures
   - Elongation for 40 s at 72 °C
3. Final elongation for 10 min at 72 °C
4. Denaturation for 1 min at 95 °C

PCR product quality was monitored using post-PCR melt curve analysis at the end of the amplification cycles. Fold inductions were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is the $\Delta Ct_{(stimulant)}$- $\Delta Ct_{(solvent)}$, ΔCt is $Ct_{(test\ gene)}$-$Ct_{(control\ gene)}$ and Ct is the cycle at which the threshold is crossed.

4.2.3.4 Chromatin immunoprecipitation assay

Cell were seeded into 175 m^2 bottles and grown overnight to reach a density of 60 to 70 %. When starting the experiment 10 ml of the experiment medium was left into the bottles. The cells were then stimulated as indicated in the individual publications.

Nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1 % for 15 min at RT (I and II) or for 10 min (III and IV). Cross-linking was stopped by adding

1.5 ml of glycine to a final concentration of 0.150 M and incubating for 5 min at RT on a rocking platform. The medium was removed and the cells were washed twice with ice-cold PBS. The cells were then collected by scraping into ice-cold PBS supplemented with a protease inhibitor cocktail (Roche). After centrifugation (5 min at 4 °C with 700 x g) the cell pellets were resuspended in 1 ml of lysis buffer (1 % SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1). Lysis was allowed to continue for 10 min at RT. The lysates were sonicated to result in DNA fragments of 300 to 1000 bp in length (1000 to 1500 bp for *CYCLIN C* region 3, IV). Cellular debris was removed by centrifugation for 15 minutes at 4 °C with 20,000 x g and the lysates were diluted 1:10 in ChIP dilution buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors 16.7 mM Tris-HCl, pH 8.1).

Chromatin solutions were incubated with 5 µl (200 µg/ml) of specific antibodies and with 24 µg of sonicated salmon sperm DNA overnight at 4 °C with rotation. The antibodies used are described in the individual publications. For input samples, 50 µl of undiluted lysate was diluted with 450 µl of ChIP dilution buffer and processed like outputs from reverse cross-linking step onwards.

The immune complexes were collected with 60 µl of salmon sperm DNA/protein A agarose slurry (Upstate Biotechnology, Lake Placid, USA) (I, II, III) or with salmon sperm DNA/protein G agarose slurry (Upstate) (IV for 1 h) at 4 °C with rotation. The beads were pelleted by centrifugation for 1 min at 4 °C with 100 x g and washed sequentially for 4 min on a rotating platform with 1 ml of the following buffers: low salt wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), high salt wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1) and LiCl wash buffer (0.25 mM LiCl, 1 % Nonidet P-40, 1 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Finally, the beads were washed twice with 1 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The immuno-complexes were eluted by adding 500 µl of elution buffer (1 % SDS, 100 mM $NaHCO_3$) and incubating for 30 min at RT with rotation. After centrifugation, the supernatant was collected and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and incubation overnight at 65 °C (I, II and III). The remaining proteins were digested by adding proteinase K (final concentration 40 µg/ml, Invitrogen) and incubating for 1 h at 45 °C (I, II and III). The reverse cross-linking and protein digestion was done in the 4th article by adding 2 µl of proteinase K (final concentration 80 µg/ml, Fermentas) and incubating overnight at 65 °C. The DNA was extracted by adding 500 µl of phenol/chloroform/isoamyl alcohol (25/24/1) and precipitated with 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2 volumes of ethanol using glycogen as a carrier. The samples were dissolved in 60 µl of H_2O.

4.2.3.5 PCR of the chromatin templates

Immunoprecipitated DNAs were used as templates for PCR. Primer-specific temperatures and cycling numbers and the locations of the primers are described in the original publications. The amounts of reaction components are described earlier in section 4.2.3.3. The PCR was performed using the following program:

1. Denaturation for 5 min at 95 °C
2. PCR amplification repeated for 30 to 45 cycles according to primers
   Denaturation for 30 s at 95 °C

Annealing for 30 s at primer-specific annealing temperatures

Elongation for 30 s at 72 °C

3. Final elongation for 10 min at 72 °C

4. Denaturation for 1 min at 95 °C.

The products were resolved on 2 % TAE (200 mM Tris-HCl, pH 7.5, 100 mM acetate, 5 mM EDTA) gels. The gels were then imaged with Fuji FLA3000 reader using ImageGauge software, The intensities of the bands were measured using ImageJ 1.37V software (National Institutes of Health, USA) (III and IV). Output samples were normalized to their inputs and the non-specific IgG background was subtracted from the samples. The relative association levels were then calculated as % of input.

5. Results

5.1. All-*trans* RA and EGF -mediated regulation of the human *HAS2* gene (I)

EGF stimulates hyaluronan synthesis rate in epidermal keratinocytes in monolayer and organotypic cultures and all-*trans* RA in human skin organ cultures (Pienimäki *et al.*, 2001; Tammi *et al.*, 1989). In skin epidermis the narrow extracellular space surrounding keratinocytes contains a high concentration of hyaluronan. The concentration of hyaluronan is closely correlated with the proliferative activity and volume of the vital part of the epidermis. It is inversely related with the markers of differentiation suggesting that hyaluronan synthesis regulated by Has enzymes is an important component in the proliferative reactions of the epidermis and is also involved in the epidermal differentiation process (Pasonen-Seppänen *et al.*, 2003). Of the three Has enzymes, Has2 is the main regulator of hyaluronan production in epidermis. Therefore, in this study, *HAS2* mRNA levels in response to all-*trans* RA, EGF and the EGFR inhibitor AG1478 alone or in combinations were measured in REK and HaCaT cells (I, Fig 2A and 2B, respectively). In REK cells both all-*trans* RA and EGF stimulated *HAS2* mRNA expression approximately 4-fold after 2 h treatment and the combination of both stimuli resulted in a more than 8-fold induction. AG1478 application resulted in a slight but not significant induction of *HAS2* expression, whereas the combined treatment with all-*trans* RA and AG1478 showed a minor if any reduction of the all-*trans* RA response of the gene. In HaCaT cells all-*trans* RA and EGF stimulated human *HAS2* mRNA levels 8.3- and 33-fold, respectively. The combined treatment resulted even in a 63-fold induction. AG1478 reduced *HAS2* mRNA expression to 18 % of solvent-treated cultures and its combination with all-*trans* RA resulted only in 1.6-fold induction.

As both all-*trans* RA and EGF induced human *HAS2* mRNA levels significantly, the first 10 kB of the *HAS2* promoter were screened *in silico* for RAR and STAT binding sites (I, Fig. 3A). For RAREs the promoter was screened for RGKTCA sequence in a DR5 arrangement. By restricting the maximal deviation from this consensus sequence to one position four DR5-type REs were found, at positions -1,207 to -1,225, -1,218 to -1,236, -2,471 to -2,453 and -6,879 to -6,897. Allowing between two and six spacing nucleotides between a pair of the trimeric STAT recognition motif TTC, four putative STAT-REs were found. They were located at positions -379 to -387, -1,212 to -1,221, -1,864 to -1,871 and -7,014 to -7,025.

To test the capability of these putative REs to bind RAR and STAT, gelshift assays were performed. Of the four putative STAT-REs only the RE closest to the TSS (-379 to -389) showed a significant binding of recombinant

STAT protein (I, Fig. 3.B). The binding of *in vitro* translated RARγ and RXRα either alone or in combination was assessed on the four putative RAREs in reference to an idealized DR5-type RARE (I, Fig. 3C). None of the four candidate REs showed significant binding of RAR-RXR heterodimer *in vitro*. However, in reporter gene assays the RARE cluster formed by RAREs 1 and 2 was able to mediate all-*trans* RA-dependent reporter gene induction that was comparable with that of the idealized DR5-type RARE (I, Fig. 3D).

To investigate whether retinoid receptors and STAT proteins associate in live cells with the human *HAS2* promoter, ChIP assays were performed with all-*trans* RA and EGF stimulated HaCaT cells using antibodies against RARγ, STAT3 and pSTAT3. 60 min all-*trans* RA treatment induced RARγ association weakly to regions containing STAT-REs 1 and 2, the RARE cluster and RARE4 together with STAT-RE4 (I, Fig. 4). RARγ binding to region containing the RARE3 was increased hugely after 60 min exposure to all-*trans* RA. In the absence of all-*trans* RA STAT-RE1 containing region bound STAT3. Retinoid-treatment increased the binding there and also induced STAT3 and pSTAT3 binding to regions containing STAT-RE2 together with the RARE cluster and STAT-RE3. All-*trans* RA induced the association of STAT3 to RARE3 containing region and pSTAT3 to RARE4 and STAT-RE4 containing region. EGF stimulation only induced STAT3 and pSTAT3 binding to STAT-RE1 conforming its role as the mediator of EGF-induction on the *HAS2* promoter.

In order to get a clearer picture of the retinoid-response of *HAS2* promoter, ChIP assays were performed to check the association of RXR and co-regulators CBP, MED23, Pol II and pPol II. Additionally, the acetylated histone 4 levels, as a marker of activated, open chromatin were measured (I, Fig. 5). RXR was found only in regions containing STAT-RE2 together with the RARE cluster and in region containing STAT-RE3. CBP binding, in contrast, was detected in all regions studied, apart from the negative control region. MED23 binding was constant in regions containing STAT-RE3 and in region containing STAT-RE4 in addition to RARE4. Regions containing STAT-RE 1 and STAT-RE2 in addition to the RARE cluster showed increased MED23 binding after retinoid treatment. Pol II showed the strongest association in region containing STAT-RE1, the binding was retinoid-dependent, as is was in the region containing RARE4 and STAT-RE4. The region containing the RARE cluster in addition to STAT-RE2 and the region containing STAT-RE3 bound Pol II constantly but weakly. The functionally more important form of Pol II, pPol II, showed similar behavior as Pol II in the region containing STAT-RE1. Also the region containing RARE4 and STAT-RE4 showed strong retinoid-induced binding of pPol II. After all-*trans* RA treatment pPol II was also recruited to the region containing the RARE cluster in addition to STAT-RE2 and the region containing STAT-RE3. The chromatin activation status was measured by acetylated histone 4 associations and was found to be high for all the regions studied. It was induced by all-*trans* RA only in the RARE cluster and the STAT-RE2 containing region. The association patterns of the nuclear proteins studied and the acetylation levels suggested that the region containing the RARE cluster and STAT-RE2 is the most likely promoter region mediating the action of all-*trans* RA on *HAS2* gene induction.

5.2 Regulation of the human *CYCLIN C* gene by 1α,25(OH)$_2$D$_3$ (II)

The human *CYCLIN C* gene is known to be regulated by 1α,25(OH)$_2$D$_3$ (Polly *et al.*, 2000) However, the VDR binding sites and the mechanisms of 1α,25(OH)$_2$D$_3$-mediated response have not been characterized on the chromatin level. Screening of the promoter by ChIP assays identified in total four regions which bound VDR and its heterodimerization partner RXR constantly over a time period of 240 min. All those regions were shown to contain one functional VDRE. In order to get more details of the VDR complexes found on those VDRE containing regions, re-ChIP assays were performed with 1α,25(OH)$_2$D$_3$-stimulated MCF-7 cells using first an antibody against VDR, followed by antibodies against RXR, NCoA3, MED1 and pPol II, so that the chromatin templates that were enriched were associated with both VDR and its partner protein at the same time (II, Fig. 6). All the regions showed different patterns of partner proteins. The region from -1786 to -2176 recruited VDR-RXR complex in a 1α,25(OH)$_2$D$_3$-dependent manner. The complex was associated in this region 60 and 240 min after ligand treatment. The VDR-NCoA3 complex bound that region constantly, and the binding was increased after ligand stimulation. The VDR-MED1 complex was also constantly there showing the strongest association after 60 min 1α,25(OH)$_2$D$_3$. VDR-pPol II complex was weakly present without 1α,25(OH)$_2$D$_3$ treatment, but the binding of this complex increased notably after ligand treatment. The region form -5,217 to -5,746 bound VDR-RXR, VDR-NCoA3 and VDR-MED1 complexes with and without 1α,25(OH)$_2$D$_3$ treatment. VDR-RXR and VDR-MED1 complexes were slightly increased in a 1α,25(OH)$_2$D$_3$-dependent manner. The VDR-NCoA3 complex showed the strongest binding after 60 min 1α,25(OH)$_2$D$_3$ stimulation, as did the VDR-pPol II complex. The latter complex was not found on the promoter 240 min after 1α,25(OH)$_2$D$_3$ treatment. The region from -6,782 to -7,202 showed 1α,25(OH)$_2$D$_3$-dependent binding of all complexes. VDR-RXR and VDR-MED1 complexes were both present without 1α,25(OH)$_2$D$_3$ stimulation, VDR-RXR complexes showed the maximum binding after 240 min 1α,25(OH)$_2$D$_3$ treatment and VDR-MED1 after 60 min 1α,25(OH)$_2$D$_3$ treatment. VDR-NCoA3 and VDR-pPol II complexes associated with the promoter just after 1α,25(OH)$_2$D$_3$ stimulus, the VDR-NCoA3 complex showed its maximum binding after 60 min 1α,25(OH)$_2$D$_3$ treatment and the VDR-pPolII complex only after 240 min incubation with 1α,25(OH)$_2$D$_3$. The binding of VDR and its partner proteins to the region ranging from -7,825 to -8,383 was the least responsive to 1α,25(OH)$_2$D$_3$of all four regions. All complexes bound the region with and without any stimulus. The VDR-RXR complex was recruited in a 1α,25(OH)$_2$D$_3$-dependent manner, while the VDR-NCoA3 binding was the strongest after 60 min and diminished to the level of untreated samples. The binding of VDR-MED1 and VDR-pPol II complexes were even slightly reduced after 1α,25(OH)$_2$D$_3$ treatment. The differences in the patterns of partner protein recruitment indicate the individual character of the different VDREs of the *CYCLIN C* promoter.

5.3 Integration of RAR- and NF-κB–mediated activation of the human *HAS2* gene promoter (III)

The first publication of this thesis already indicated that the retinoid-responsiveness of human *HAS2* gene is complex. The second publication concerning the regulation of the human *CYCLIN C* gene on the chromatin level suggested that the chromatin level mechanisms of gene regulation are unique to each RE. In this study, the mechanisms of all-*trans* RA–mediated activation of the *HAS2* gene were studied in more detail. In addition, the roles of TFs NF-κB and Sp1 in the regulation of *HAS2* gene were assessed.

In silico screening of the human *HAS2* promoter revealed in addition to RAREs and STAT-REs also putative binding sites for constitutive nuclear factor Sp1 and NF-κB (III, Fig. 1). The net-based program ConSite identified with a TF score cutoff of 85 % three putative NF-κB REs at positions -375 to -384 (NF-κB RE1), -1,417 to -1,426 (NF-κB RE2) and -1,888 to -1,897 (NF-κB RE3) within the first 2,250 bp of human *HAS2* promoter. With the same cutoff of 85 % ConSite identified in total 12 putative Sp1 REs within the same promoter region.

To get information on whether the putative elements found *in silico* are functional and identify the patterns of co-regulators that they recruit, ChIP assays were performed with 0, 60 and 240 min all-*trans* RA- and 0 and 60 min TNFα-treated HaCaT cells (III, Fig. 2). The association of the MED protein MED1, the CoAs CBP and SRC-1, the CoR NCoR1 and the constitutive nuclear factor Sp1 was measured using specific antibodies for respective proteins. The different type of co-factors, MED1, CBP, SRC-1 and NCoR1 in addition to Sp1 showed each an individual pattern for their stimulation-dependent and -independent association with the *HAS2* promoter. All five proteins associated to the regions containing NF-κB REs 1 and 3. Interestingly, the RARE cluster containing region bound significantly only CBP and Sp1. All-*trans* RA increased the binding of CBP to the region containing the RARE cluster. Similarly, it increased the association of Sp1 to the NF-κB RE1 containing region, that of CBP and SRC-1 to the NF-κB RE2 containing region and CBP, MED1, Sp1 and surprisingly also NCoR1 binding to the NF-κB RE3 containing region. TNFα stimulated the association of SRC-1 to the NF-κB RE 1 containing region, of CBP and Sp1 to the NF-κB RE3 containing region and Sp1 binding to the NF-κB RE2 containing region, as well as MED1 binding to the RARE cluster.

The RARE cluster containing region demonstrated all-*trans* RA-dependent recruitment of co-factors (III, Fig. 2A). In order to induce transcription, this region must move closer to the TSS of the *HAS2* gene by DNA looping. Therefore, the chromatin compositions of both the RARE cluster and the TSS containing regions were analyzed in further detail (III, Fig. 3A). ChIP analysis were performed with 0, 60 and 240 min all-*trans* RA treated HaCaT cells using antibodies against pPol II, RAR and a known co-regulator involved in RA-mediated transcriptional responses, PARP-1. Furthermore, re-ChIP assays were performed with anti-RARγ antibodies in combination with antibodies for pPol II, SRC-1, MED1, CBP and NCoR1. pPol II binding was relatively constant to the TSS and only marginally detectable on the RARE cluster. Supporting the functionality of the

RARE cluster, RAR binding was clearly increased on both regions after all-*trans* RA treatment. Additionally, some weak but ligand-dependent association of PARP-1 to both regions could be detected. RAR-pPol II and RAR-SRC-1 complexes were detected on the RARE cluster in all-*trans* RA-dependent manner. Furthermore, the preassembled RAR-NCoR1 complex on the RARE cluster dissociated after ligand addition. Moreover, complexes of RAR with SRC-1, MED1, CBP and NCoR1 were detectable on the TSS, but their association was not affected significantly by all-*trans* RA.

ChIP results suggested functionality of *HAS2* promoter in response to retinoids, which was further supported by reporter gene assays. A nested set of *HAS2* promoter constructs was created in the *luciferase* reporter gene containing vector pGL3 (Monslow *et al.*, 2004). The 3' end of each promoter fragment was located at position +43, and the longest insert extended to position -2,118 bp relative to the TSS (III, Fig. 3B). The RARE cluster was contained in promoter fragments 11 and 12, which provided a 5.4- and 1.7-fold response to all-*trans* RA, respectively. Shorter promoter fragments showed a drastic loss of basal activity and all-*trans* RA inducibility. Still, promoter fragment 10 was an exception providing a 2.3-fold induction of luciferase activity. However, the basal and the stimulated absolute luciferase activity of this promoter fragment were both below that of the basal luciferase activity value of the constructs containing the RARE cluster.

As the regions containing the putative NF-κB REs showed responsiveness to TNFα in the recruitment of transcriptional co-regulators, the role of NF-κB in the TNFα-mediated activation of the H*AS2* promoter was further studied. The chromatin composition of all four NF-κB RE containing regions and of the TSS were investigated by ChIP analysis with HaCaT cells treated for 0 and 60 min with TNFα using antibodies against NF-κB p65 and pPol. Furthermore, re-ChIP assays were performed with anti-NF-κB p65 antibodies in combination with antibodies against pPol II, SRC-1, MED1, CBP and NCoR1. pPol II binding could be detected only at the TSS and in the region containing the NF-κB RE1. The binding was constitutive in both regions. The binding of NF-κB to the NF-κB RE1 containing region was TNFα-inducible, whereas its association to region encompassing NF-κB RE2 was weaker but also inducible, and finally, it bound constantly the region containing NF-κB RE3. The NF-κB-pPol II complex associated with the TSS and the region containing NF-κB RE3 in a clearly ligand-inducible manner, whereas the dominant binding of the complex to the NF-κB RE1 containing region was reduced after addition of the ligand. The region containing NF-κB RE2 did not bind significantly any of the complexes studied significantly. NF-κB-SRC-1 complex bound to the regions that contain NF-κB REs 1 and 3. This binding was found to be inducible by TNFα at first but showed decreased binding to the latter RE containing the site. NF-κB-MED1 and NF-κB-CBP complexes were found on the both regions possessing the same the putative NF-κB REs. The complexes showed the same profile of constant binding to the NF-κB RE1 containing region and an inducible association with the region containing NF-κB RE3. Finally, on these two NF-κB REs also some weakly binding TNFα-inducible NF-κB-NCoR1 complex were detected.

The functionality of the three putative NF-κB REs were further tested by reporter gene assays in HaCaT cells using the same set of luciferase reporter gene constructs as described earlier (III, Fig. 3B). As reporter gene assays traditionally accumulate signals and are not very suited for the measurements of transient stimulations,

such as a treatment with TNFα, these assays were focused on the effects of NF-κB on the basal activity of the *HAS2* promoter by overexpressing the subunits of NF-κB, p50 and p65 (III; Fig. 4B). Promoter fragment 12 containing all three NF-κB REs was induced 7.3-fold, when p50 and p65 were overexpressed. Under the same conditions promoter fragment 11 lacking NF-κB RE3 showed only a 4.5-fold induction and fragment 10 lacking both NF-κB REs 3 and 2 did not show any induction. When NF-κB RE1 was isolated from putative upstream regulatory elements (fragment 5), a drop of basal activity was observed, which could be restored by p50/p65 overexpression resulting in a 3.0-fold induction. Finally, the shortest promoter fragments 4 to 1 not containing any NF-κB REs showed no significant response to p50/p65 overexpression.

To assess, whether NF-κB binds directly to three putative NF-κB REs on the *HAS2* promoter, gel shift assays using HaCaT nuclear extracts were performed. The cells were treated for 60 min with 20 ng/ml TNFα, with and without 90 min co-treatment with 10 μM IKK-2 inhibitor (III, Fig. 4C). An idealized NF-κB RE served as a control, on which TNFα stimulation induced two complexes, which were previously identified as p50-p65 heterodimers (the lower complex) and p65 homodimers (upper complex) (Urban *et al.*, 1991). p50-p65 heterodimer binding could be observed on all putative NF-κB REs, while faint p65 homodimer binding could be observed in addition to the control RE only on NF-κB RE2. IKK-2 inhibitor treatment of the cells as well as incubation of the nuclear extracts with anti-p65 antibody reduced the binding of both types of NF-κB complexes to all four isolated NF-κB REs. In this direct comparison, NF-κB RE2 seemed to be the strongest binding element *in vitro*. However, in the functional ChIP assays, NF-κB RE2 did not associate with p65 indicating that greater complexities exist in the chromatin environment *in vivo*.

To support the assumption that *HAS2* gene is of larger impact than the other *HASes* in HaCaT cells, the basal activities of all these genes were monitored by real- time quantitative PCR in relation to the control gene *RPLP0* (III, Fig 5A). In fact, the data demonstrated that *HAS2* was the predominantly expressed member of the *HAS2* gene family, since it was found to be 30-fold higher expressed than the *HAS3* gene and even 10,000-fold more prominent than the *HAS1* gene. The basal *HAS2* expression was increased 7.4-fold by all-*trans* RA treatment, but only 1.8-fold through TNFα (III, Fig. 5B). The co-treatment with IKK-2 inhibitor abolished the TNFα-response of the *HAS2* gene. Interestingly, it also reduced the all-*trans* RA induction of the gene down to 3.2-fold.

Taken together, *HAS2* was shown to be the dominant *HAS* gene in HaCaT cells and a primary target of both all-*trans* RA and TNFα. Interestingly, a co-treatment of all-*trans* RA and IKK-2 inhibitor suggested a *HAS2*-specific interference of retinoid and NF-κB signaling.

5.4 Comparison of RAR- and CREB–mediated regulation of the human genes *HAS2* and *CYCLIN C* (IV)

We showed in the first three publications of this thesis that the regulation of both human *HAS2* and *CYCLIN C* genes is complicated. The chromatin compositions of different REs in response to a certain stimuli showed

individual characters of chromatin associated proteins. The finding that different signaling pathways could integrate by using the same co-regulators even further increased the complexity. In order to get a broader view on the mechanisms of gene regulation by different TFs and the integration of different signaling pathways on chromatin level the regulation of two different genes, *HAS2* and *CYCLIN C*, was investigated in this study (IV). Here we studied the regulation of the two different genes by the TFs CREB1 and RAR, and also their integration.

The basal expression levels of human *HAS2* and *CYCLIN C* genes were measured both in HEK293 and HaCaT cells (IV, Fig. 1A). The *CYCLIN C* gene was more expressed than the *HAS2* gene in both cell lines. In HEK293 cells, *CYCLIN C* mRNA levels were nearly 30-fold higher than that of the *HAS2* gene and in HaCaT cells they were still 25-fold higher. In the two different cell lines the two genes showed an individual pattern of inducibility by various stimulants. In HEK293 cells the basal *HAS2* mRNA expression was increased 2.1-fold by all-*trans* RA treatment compared to total 7.4-fold increase in HaCaT cells (IV, Fig. 1B). The cAMP inducer Forskolin increased *HAS2* expression 2.7-fold in HaCaT and 3-fold in HEK293 cells after 60 min stimulation and 4.6-fold and 7.9-fold after 24 h treatment, respectively. In HaCaT cells all-*trans* RA combined with 60 min Forskolin gave 6.2-fold induction of the *HAS2* gene, compared with a 3.6-fold induction in HEK293 cells. Surprisingly, 24 h Forskolin treatment reduced the all-*trans* RA effect down to 2.7-fold in HaCaT cells, but increased it to 13.3-fold in HEK293 cells. All-*trans* RA induced *CYCLIN C* mRNA levels only slightly, 1.6-fold in both cell lines. In HEK293 cells 60 min Forskolin treatment stimulated *CYCLIN C* mRNA levels 1.6-fold and the addition of all-*trans* RA potentates the induction to 2.9-fold. In HaCaT cells, neither 60 min or 24 h Forskolin treatment nor the combinations with all-*trans* RA affected the mRNA levels significantly. In HEK293 cells 24 h treatment with Forskolin induced *CYCLIN C* mRNA 2.3-fold and the combination with all-*trans* RA further 2.9-fold. A time course experiment with 1, 2, 4, 6 and 24 h time points showed that all-*trans* RA–mediated induction of the *HAS2* gene showed a similar trend in both cell lines (IV, Fig. 1C). The induction was close to its maximum already after 1 h not changing significantly until 24 h when no significant induction was seen. In HaCaT and HEK293 cells the Forskolin effect was highest after 24 h. In HaCaT cells the combination of all-*trans* RA and Forskolin induced *HAS2* mRNA the most after 1 and 2 h and the induction was diminished towards 24 h, whereas in HEK293 cells the induction by the combined treatment increased over time having its maximum at 24 h. The *CYCLIN C* gene was induced significantly in HaCaT only after 4 h treatment with all-t*rans* RA. Any of the other treatments did not result in a significant change in the mRNA levels. In HEK293 cells, all-*trans* RA induced *CYCLIN C* mRNA significantly only after 2 h and Forskolin only after 1 and 24 h. The combination of the both stimulants resulted in the maximal induction at 24 h.

As both *HAS2* and *CYCLIN C* genes were induced by Forskolin and all-*trans* RA, the first 2250 bp of both promoters were screened *in silico* for putative RAREs and CREs (IV, Fig. 2). Screening the promoter for two copies of RGKTCA consensus sequence in a DR2 and DR5 arrangement identified putative RAREs. The maximal deviation from the consensus sequence was restricted to one position. A cluster of two overlapping RAREs (position -1,208 to -1,237) have previously been identified and confirmed on *HAS2* gene. Two putative DR2-type RAREs were found on the *CYCLIN C* promoter at positions -537 to -550 and at -1,147 to -1,160. Putative CREB binding sites were screened by using the net-based program ConSite (http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) with a TF score cutoff of 85 %. Three putative CREs could be identified on the *HAS2*

promoter. They were located at positions -656 to -663, -1,731 to -1,751 and -1,878 to -1,889 relative to the TSS. On the *CYCLIN C* promoter four putative CREs at positions -586 to -595, -844 to -855, -1,970 to -1,981 and -2,234 to -2,244 were identified. The *CYCLIN C* promoter contains a lot of repetitive sequences. The CREs 2, 3 and 4 and the DR2-type RAREs are in repetitive sequence (CRE4 only partly). That means their role as functional REs should be considered critically.

The effects of Forskolin and all-*trans* RA on the recruitment of CREB, RAR, pPol II and transcriptional co-regulators: MED1, the CoAs CBP and SRC-1, the CoR NCoR1 were studied by screening the first 2250 bp of both the *HAS2* and the *CYCLIN C* promoters by ChIP assays with HEK293 cells that were treated for 0 and 60 min with 0.1 μM all-*trans* RA or 10 μM Forskolin (Figs. 9 and 10) (IV, Figs. 3A and 3B**)**. All co-regulators, the TFs and pPol II had a unique pattern for their stimulation-dependency and timing. RAR bound to the *HAS2* promoter ligand-independently and in both retinoid- and Forskolin-responsive way. Ligand-dependency was seen in the regions containing CRE1, the RARE cluster and CRE3. Surprisingly, to all these regions both Forskolin and all-*trans* RA induced the binding of RAR. CREB associated to all regions containing the putative CREs in addition to few other regions. Only at the TSS and in the region containing CRE1 there was some ligand-dependency seen. Again, both Forskolin and all-*trans* RA induced its binding more or less in a similar fashion. Additionally, the binding of pPol II, MED1, CBP and NCoR1 showed both ligand-dependent and -independent patterns. In addition, their association to the regions containing CREs was not merely Forskolin-dependent but also all-*trans* RA-dependent in some regions. Vice versa Forskolin was also found to induce the binding of RAR to the RAREs indicating interference of the signaling pathways. MED1 binding on the *HAS2* promoter showed more responsiveness to Forskolin than to all-*trans* RA, but other regulators did not make a difference between Forskolin or all-*trans* RA. On the *CYCLIN C* promoter the interference of Forskolin and all-trans RA signaling was also seen in a similar fashion as that observed on the *HAS2* promoter. RAR bound to regions containing the TSS and all CREs, in addition to the region containing the two DR2-type RAREs. CREB1 binding was significant only in the region covering CREs 1 and 2 and two DR2-type RAREs and in the region containing the CREs 3 and 4 after Forskolin treatment. pPol II associated with the three most closest regions to TSS (covering in total the TSS, CREs 1 and 2 and the two DR2-type RAREs) more or less constantly. MED1 associated significantly to the region containing the TSS, where its binding was strong even without any stimulus. In the region ranging from -1332 to -1845 and not containing any putative RE for the TFs studied all-*trans* RA recruited MED1 binding. CBP bound to the region covering CREs 1 and 2 and two DR2-type REs and in the region containing CREs 3 and 4. To both regions it associated in the absence of ligand, but the binding increased after Forskolin treatment and even further after all-*trans* RA stimulation. Without Forskolin or all-*trans* RA treatment NCoR1 was found at the TSS, in the region next to the TSS (from -108 to -363) and in the region containing CREs 3 and 4. In the region containing the TSS all-*trans* RA treatment caused the NCoR1 binding to disappear and in region 5 both Forskolin and all-*trans* RA diminished the binding, with Forskolin pushing even below the significance level.

As all putative CRE- and RARE-containing regions showed some responsiveness to both all-*trans* RA and Forskolin, re-ChIP assays were performed to confirm the role of RAR and CREB in the recruitment of co-regulators to both gene promoters. On the *HAS2* promoter the RAR-pPol II complex bound to the RARE cluster

in both Forskolin and all-*trans* RA-dependent manners verifying the functionality of the RARE cluster and supporting the idea that RAR and CREB may interfere each other. The RAR-CBP complex bound to the regions encompassing CRE1, the region containing the RARE cluster, the regions with CREs 2 and 3 and the region containing CRE3 after both Forskolin and all-*trans* RA stimulation indicating the CBP might be one link between Forskolin and retinoid signaling. The RAR-NCoR1 complex associated very weakly with the TSS in the absence of ligand disappearing after the addition of either Forskolin or all-*trans* RA. The CREB-pPol II complex was detectable more or less constantly in all regions covering the promoter from -244 to -1048 and containing CRE1 and in region having CREs 2 and 3. The CREB-CBP complex was found on the *HAS2* promoter only in two regions, in the region located at -244 to -481and in the region containing CREs 2 and 3. In both regions Forskolin increased the binding. The CREB-MED1 complex showed the highest Forskolin responsiveness to all CRE RARE containing regions.

Taken together, both ligands recruited the same co-regulators indicating functional interference but in parallel also showed some individual differences. The general features of the recruitment of transcriptional regulators to both *HAS2* and *CYCLIN C* promoter in response to different stimulations are depicted in the model (Figs. 9 and 10, respectively). All-*trans* RA seemed to regulate mainly NCoR1 association, while Forskolin affected in addition to NCoR1 also MED1 and CBP association. The REs differed in response of co-regulator binding after the same stimulus. There are indications that on CRE1 MED1 and NCoR1 seem to be the main regulators, whereas on CRE2 and CRE3 CBP together with NCoR1 may be central.

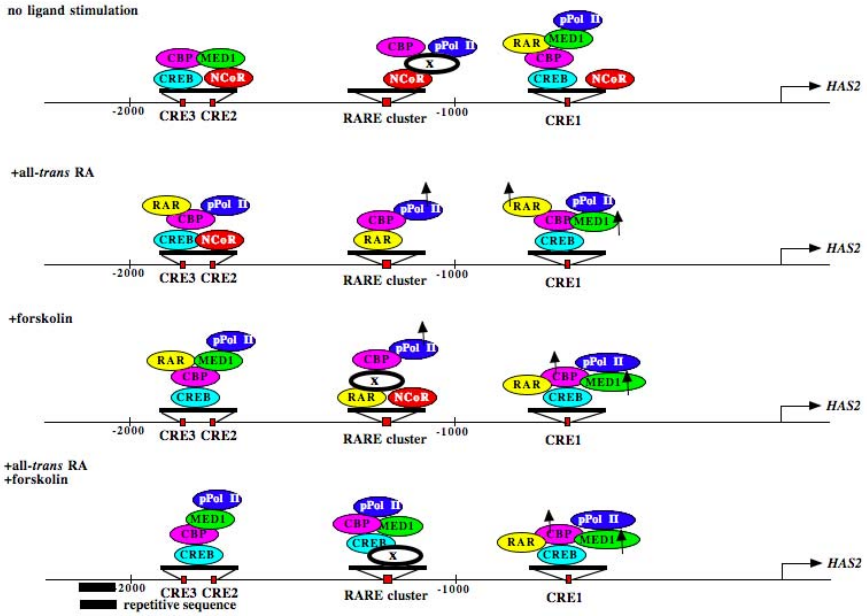

Fig. 9. Model describing the chromatin composition of CREs and the RARE cluster on the human *HAS2* promoter. CREs 1, 2 and 3 and the RARE cluster on the *Has2* promoter recruit each an individual pattern of co-factors, pPol II and TFs in response to all-*trans* RA, Forskolin or the combination of all-*trans* RA and Forskolin.

On the *CYCLIN C* promoter the RAR-pPol II complex associated to the region containing CREs 3 and 4 and to regions not bearing any putative REs (from – 108 to -363 and from -1332 to -1845) without any stimulus. In the region with CREs 3 and 4 its binding was constant. Surprisingly, the complex disappeared from the region covering the promoter from -108 to -363 after all-*trans* RA stimulation and from the region from -1332 to -1845 after both all-*trans* RA and Forskolin treatment. All-*trans* RA recruited the complex to the region with CREs 1 and 2. The RAR-CBP complex was found at the TSS and in the two regions with no putative REs and in the region with CREs 3 and 4. At the TSS and on the region containing CREs 3 and 4, the complex was recruited just after Forskolin and all-*trans* RA stimulation. Forskolin induced the dissociation of the complex from the two regions with no putative REs (from -108 to -363 and from -1332 to -1845). All-*trans* RA had the same effect in the region covering the area from -1332 to -1845. RAR-NCoR1 complexes were found merely also in that region, where the binding stayed more or less constant. The CREB-pPol II complex was found constantly and quite strongly in the regions covering the area from -108 to -363 and the one containing CREs 3 and 4. In the region containing the TSS the complex was found only after either Forskolin or all-*trans* RA treatment. CREB-CBP complexes associated with region 4 constantly, whereas in the region with CREs 3 and 4 it was found just after Forskolin stimulation. It also bound the TSS without ligand, but any stimulus decreased the binding. CREB-NCoR1 complexes bound very weakly to the region with TSS. In the region with CREs 3 and 4 the binding was strong without Forskolin or all-*trans* RA treatment, but both ligands diminished the binding nearly below the significant level.

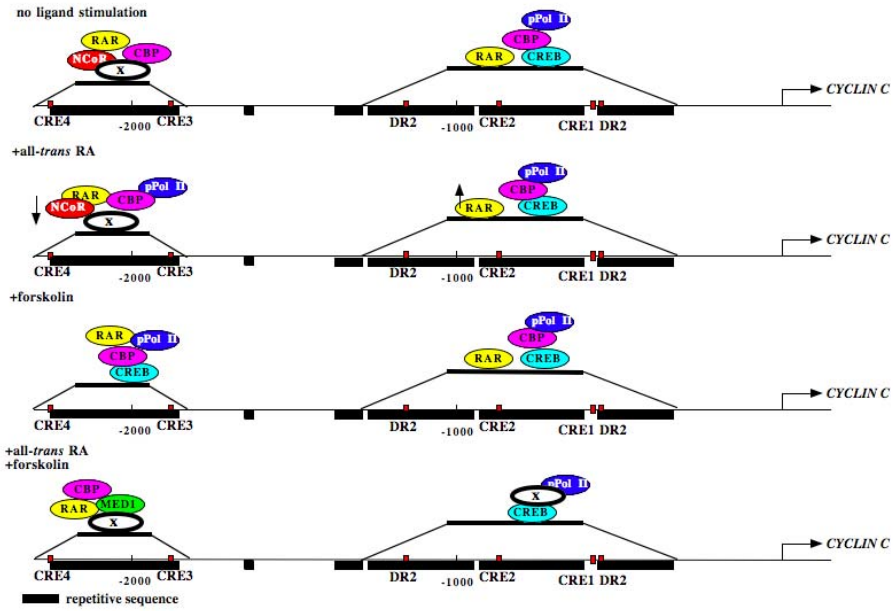

Fig. 10. The chromatin composition of two *CYCLIN C* promoter fragments. The chromatin composition of the region containing CREs 1 and 2 and two DR2-type RAREs is different from the region containing CREs 3 and 4. These two regions also recruit different patterns of transcriptional regulators in response to all-*trans* RA, Forskolin and their combination.

In summary, both Forskolin and all-*trans* RA recruited their TFs, pPol II and co-regulators to *CYCLIN C* promoter regions suggesting response to both Forskolin and all-*trans* RA (Fig. 10). The REs in repetitive sequences must be considered cautiously. That makes the only RE totally in unique sequence, CRE1, to be considered as the most critical RE. The role of the two putative DR2-type RAREs in region 3 as the mediator of the retinoid-response of the gene is not fully confirmed. Again, NCoR1 and CBP might be the functional co-regulators of the *CYCLIN C* promoter.

The functionality of both human *HAS2* and *CYCLIN C* genes in response to retinoids and Forskolin was further studied in reporter gene assays (IV, Fig. 4). For that purpose a nested set of *HAS2* promoter constructs was created in the *luciferase* reporter gene-containing vector pGL3 (Monslow *et al.*, 2004). The promoter fragments were the same than the ones used in the previous publication of this thesis (III). The 3' end of each promoter fragment was located at position +43, and the longest insert extended to position -2118 bp relative to the TSS. The RARE cluster was contained in promoter fragments 11 and 12. The CRE1 was contained in fragments 8 to 12 and CRE2 and CRE3 in fragment 12. Three *CYCLIN C* promoter fragments were created in the luciferase reporter gene-containing vector, pGL4. The 3' end of all promoter fragments was located at position +25. The shortest insert extended to position -149 relative to the TSS, the next fragment to -1,598 covering the unique CRE1, CRE2 and the two DR2-type RAREs, and the longest fragment extended to position -2,399 covering all four CREs and the two DR2-type RAREs. The effect of RAR on both promoters was studied by overexpressing the pSG5-based expression vectors for RARγ and RXRα in HEK293 cells and stimulating the cells with all-*trans* RA, Forskolin and their combination (Fig. 4A, IV). On the *HAS2* promoter the highest basal activity was seen in the largest fragment containing all three CREs and the RARE cluster. Fragment 11 containing the RARE cluster and CRE1 showed the second highest basal activity, though it was not significantly higher than the basal activity of the shorter fragments. Fragment 12 was induced by Forskolin 1.8-fold, by all-*trans* RA 2.2-fold and by their combination 1.3-fold, when RAR and RXR were overexpressed. Surprisingly, fragment 8 (containing CRE1) showed a significant 1.25-fold repression in response to all-*trans* RA. On the *CYCLIN C* promoter constructs, retinoid receptor overexpression did not have any effect on the activity of the promoter. However, the combined treatment of all-*trans* RA and Forskolin reduced the activity of the longest fragment 1.25-fold. Interestingly, the shortest promoter fragment, containing just the TSS, showed approximately 4-fold higher basal activity than the longer ones indicating that there are some repressive elements within the first 2,400 bp of the *CYCLIN C* promoter.

The effects of CREB on both promoters were investigated by overexpressing CREB1 in a pFN2K-based expression vector in HEK293 cells and stimulating the cells with all-*trans* RA, Forskolin and their combination (IV, Fig. 4B). The basal activity of the *HAS2* promoter started to arise from fragment 8 (CRE1) on the largest fragments showing the highest activity. On fragment 4, all-*trans* RA caused a 1.7-fold repression. Interestingly, fragment 5 was induced 3.8-fold in response to retinoids. Also fragment 6 was induced 2.8-fold. Fragment 8, the first fragment to contain CRE1, was induced 2.7-fold in response to Forskolin and 1.8-fold in response to all-*trans* RA. Fragment 9 was also induced 2.8-fold by Forskolin. Fragment 11, containing the RARE cluster in addition to CRE1 was induced 5.1-fold in response to all-*trans* RA and 3.1-fold in response to combination of Forskolin and all-*trans* RA. The largest fragment, containing now all three CREs and the RARE cluster was

induced by Forskolin, all-*trans* RA and their combination in a similar fashion, approximately 2.5-fold each. The shortest fragment of the *CYCLIN C* did not show any inducibility in response to any of the stimulants, but again, it had a much higher basal activity than the longer fragments. The fragment with the two DR2-type RAREs and CREs 1 and 2 was induced 1.4-fold in response to Forskolin and 1.9-fold in response to all-*trans* RA and the combination of both individual stimulants. The fragment covering the first 2,399 bp of the *CYCLIN C* promoter was induced by Forskolin 2.2-fold and 2.8-fold by the combination of all-*trans* RA and Forskolin suggesting the functionality of the promoter in response to Forskolin.

The effects of RAR, RXR and CREB on the *HAS2* and *CYCLIN C* promoter activities were also checked by transfecting the pFN2K -based expression vector for CREB1 and the pSG5-based expression vectors for RARγ and RXRα into HEK293 cells, which were then stimulated with all-*trans* RA, Forskolin and their combination (IV, Fig. 4C). The basal activity of the *HAS2* promoter started to arise from fragment 3 on fragment 12 showing the maximum. Surprisingly, already fragment 2 was induced significantly by Forskolin and combination of Forskolin and all-*trans* RA, 1.4 and 1.3-fold, respectively. Fragment 5 was induced 1.3-fold and 1.5-fold by Forskolin and combination of Forskolin and all-*trans* RA. Interestingly, fragment 6 was reduced by all-*trans* RA 1.7-fold. Fragment 8 that contains CRE1 was induced 1.8-fold by Forskolin, 2-fold by all-*trans* RA and 2.8-fold by the combination indicating interference of retinoids and Forskolin. Fragment 10 was induced significantly by the combination of all-*trans* RA and Forskolin, the induction was 1.7-fold. Fragments 11 and 12 showed induction in response to all treatment conditions. Fragment 11 that contains CRE1 and the RARE cluster was induced by Forskolin 1.8-fold, by all-*trans* RA 1.7-fold and by the combination 1.9-fold. In fragment 12 that contains in addition to elements in fragment 11, also the CREs 2 and 3, the inductions were 1.6-fold, 1.3-fold and 1.8-fold, respectively. The *CYCLIN C* promoter was induced less in response to any stimulant. The shortest fragment did not respond to any of the stimulants. The fragment extending to -1,598 from the TSS was induced by Forskolin only 1.2-fold, though significantly. The all-*trans* RA-mediated response was 1.1-fold and the combination led to a 1.3-fold stimulation. The longest fragment was induced by Forskolin 1.2-fold and by the combination of all-*trans* RA and Forskolin 1.5-fold. Interestingly, all-*trans* RA alone reduced the activity 1.25-fold. Taken together these reporter gene assays indicate an interference of retinoids and CREB signaling on the regulation of both the *HAS2* and *CYCLIN C* gene. These results verify the functionality of all CREs and the RARE cluster on the *HAS2* promoter and also functionality of the *CYCLIN C* promoter in response to both all *trans* RA and Forskolin.

The interference of Forskolin and all-*trans* RA was further investigated with ChIP using Forskolin and all-*trans* RA treatment together (Fig. 9 and 10), (IV, Figs. 5A and 5B). Chromatin was extracted from HEK293 cells that were stimulated for 0 and 60 min with 0.1 μM all-*trans* RA and 10 μM Forskolin. ChIP assays were performed with antibodies against pPol II, RAR, CREB1, SRC-1, MED1, CBP and NCoR1. Only the regions containing the putative REs for CREB and RAR were selected to this study. The ChIP data with the combined treatment demonstrated that Forskolin and all-*trans* RA were interfering with each other. On the *HAS2* promoter, MED1, CBP and NCoR1 mediated their combined action. The effect of the combined treatment on the *HAS2* promoter was best seen in CREs 2 and 3 and in the RARE cluster. The effects of the combination were much the same than the effects of both treatments alone. The most obvious difference was the recruitment of CBP to the region

containing the RARE cluster, where it was not present, when stimulated with Forskolin or all-*trans* RA alone. On the *CYCLIN C* promoter the combined effects were best seen in the region containing the CREs 1 and 2 and two DR2-type RARES, and also at the TSS. The combined treatment showed more differences in case of the *CYCLIN C* gene in the recruitment of transcription regulators compared to individual treatments than in case of the *HAS2* gene. All-*trans* RA treatment alone recruited RAR to the region containing the CREs 1 and 2 and two DR2-type RAREs and the region with CREs 3 and 4, similarly Forskolin recruited RAR to the latter region. When added together, there was no RAR binding to any of the regions. Also CREB1 was recruited to TSS just after the combination, neither of the separate regions recruited it. MED1 binding to the region with CREs 1 and 2 and two DR2-type RAREs was increased just after the combination and surprisingly, the CBP binding that was even increased after either Forskolin or all-*trans* RA to the region with CREs 3 and 4 was gone when the stimulants were used in combination.

To confirm the roles of MED1, CBP and NCoR1 to all-*trans* RA- and Forskolin-mediated responses of both the *HAS2* and *CYCLIN C* gene, HEK293 cells were transfected with siRNAs against the genes of these co-regulators (IV, Fig. 6). When the *MED1* gene was silenced, the all-*trans* RA-mediated 2.1-fold induction of *HAS2* mRNA was dropped down to 1.1-fold. Silencing the *CBP* gene Also diminished the all-*trans* RA-mediated induction to 1.7-fold, but this reduction was not considered to be significant. Silencing the *NCOR1* gene increased all-*trans* RA-mediated induction of *HAS2* mRNA to 3.8-fold. This indicates that MED1 and NCoR1 may be the main co-regulators in the retinoid-responsiveness of the *HAS2* gene. Forskolin induced *HAS2 mRNA* 3-fold. This induction went down to 2.3-fold, when the *MED1* gene was silenced and to 1.3-fold, when the *CBP* gene was silenced. Only the *CBP* gene silencing was significant. *NCOR1* gene silencing did not have any effect, indicating that CBP is the main co-regulator in the Forskolin-mediated response of the *HAS2* gene. All-*trans* RA induced the *CYCLIN C* gene 1.6-fold. Silencing of the *MED1* gene led to a 1.4-fold reduction and silencing of the *CBP* gene to 1.25-fold reduction. However, these reductions were not significant. Additionally, *NCOR1* gene silencing reduced the activity down to 1.2-fold, but this was not significant either. These results indicate that the all-*trans* RA induction of the *CYCLIN C* gene is not fully requiring MED1, CBP or NCoR1. Forskolin induced the *CYCLIN C* gene 1.6-fold. This induction went significantly down to 0.9-fold when the *MED1* gene was silenced and the silencing of *NCOR1* led to a 2.4-fold induction indicating MED1 and NCoR1 mediated that Forskolin response. Silencing of the *CBP* gene did not have any significant effect.

In summary, both the *HAS2* and the *CYCLIN C* gene were shown to be responsive to all-*trans* RA and Forskolin. Furthermore, regions containing the putative REs for CREB and RAR in their promoter were shown to be functional. All REs recruited proteins involved in the regulation of transcription in response to all-*trans* RA, Forskolin or their combination with a unique pattern. All-*trans* RA and Forskolin signaling were shown to be interfering with each other both on the mRNA and the chromatin level.

6. Discussion

6.1. Regulation of the *HAS2* gene by all-*trans* RA and EGF

Hyaluronan concentration regulates epidermal keratinocyte behavior, their proliferation and differentiation, which should be in a controlled balance for normal function of epidermis. This balance is disturbed in pathologic situations, such as wounding and psoriasis. Malignant transformation of epithelial cells is also frequently accompanied by increased hyaluronan production and accumulation (Toole *et al.*, 2002). Thus, understanding of the regulation of hyaluronan synthesis in epithelial cells becomes extremely important. Various growth factors and hormones regulate hyaluronan concentrations, but before this study, virtually no data has been available on the control of hyaluronan synthesis on the chromatin level (Monslow *et al.*, 2004). However, several studies indicate that the concentration of hyaluronan in different cells and tissues is best correlated with *HAS* gene expression, and in particular with the *HAS2* mRNA levels (Pienimäki *et al.*, 2001; Nishida *et al.*, 1999).

This study demonstrates that *HAS2* is a primary EGF and all-*trans* RA responding gene. Retinoids are widely applied in the therapy of various skin diseases, but only a few primary all-*trans* RA responding genes have been established in human keratinocytes (Xiao *et al.*, 1999). The 8-fold induction of *HAS2* mRNA only after 2 h treatment with all-*trans* RA (I, Fig. 2B) suggests that *HAS2* could be used as a marker for monitoring the efficacy of synthetic RAR ligands in pre-clinical trials (Altucci and Gronemeyer, 2001). Also in rat, in REK cells, the *HAS2* gene was induced my both EGF and all-*trans* RA, but as the effects were more prominent in HaCaT cells, and as there were gaps in the rat sequence, the human *HAS2* gene was selected for further studies. In order to assess the retinoid- and EGF-responsiveness, the first 10 kB of the human *HAS2* gene was screened *in silico* for STAT-REs and DR5-type RAREs. *In vitro* gel shift assays revealed that out of four putative STAT-REs and four putative RAREs found *in silico* only one STAT-RE approximately 380 bp in front of the TSS (STAT-RE1) showed direct binding. This observation was confirmed by ChIP analysis. Without any stimulation only the region spanning the putative STAT-RE1 associated weakly with non-phosphorylated STAT3. Therefore, the inactive form of STAT3 seems to associate its RE weakly even without any stimulus. Still, 60 min of EGF treatment increased the binding and induced also the binding of the active form of STAT3, pSTAT3. This observation strongly supports the idea that this novel STAT-RE is functional and mediates EGFR activation on the *HAS2* promoter. However, it is important to have in mind that signaling via STAT is not the only signaling cascade that EGFR starts. EGFR signaling can also involve RAS and phospholipase Cγ pathways (Carpenter, 2000). The latter can also lead to NF-κB activation. The finding that the STAT-RE1 overlays by a putative NF-κB RE (Monslow *et al.*, 2004, Saavalainen *et al.*, 2007) suggests that both STAT and NF-κB may act co-ordinately in mediating the strong EGF-responsiveness of the receptor via these two overlapping REs.

Even though none of the four found putative RAREs showed direct binding of RAR *in vitro*, the functionality of the RARE cluster composed of RARE1 and RARE2 was demonstrated in reporter gene assay, in which the RARE cluster could mediate an all-*trans* RA-dependent reporter gene induction that was comparable with that of the idealized DR5-type RARE. Surprisingly, in ChIP assays, all five investigated RE-containing *HAS2* promoter regions even without any obvious RARE candidate associated with RAR. However, this does not mean that all these regions are functionally important in the mediating of the all-*trans* RA –dependence of the *HAS2* promoter. According to the current model of retinoid signaling RARs are only functional in a complex with RXR (Bastien and Rochetty-Egly, 2004). As only promoter regions 2 (the RARE cluster in addition to STAT-RE2) and 3 (STAT-RE3) showed association with RXR, all the other regions could be left out as putative functional sites of retinoid signaling. The direct comparison of both regions concerning the association of other proteins important in retinoid signaling demonstrated that only the RARE cluster containing region showed a significant, all-*trans* RA -induced association of MED protein and activated Pol II, pPol II. Furthermore, a retinoid-induced opening of the chromatin monitored by the levels of histone 4 acetylation could be observed only in region 2. These findings together with the RGA results make it likely that the retinoid response of the human *HAS2* gene is mediated via a larger protein complex containing RARγ, RXRα, CBP, MED proteins and activated Pol II associated with this RARE cluster.

The analysis of the all-*trans* RA-dependent occupation of five RE-containing regions of the *HAS2* promoter with several nuclear proteins suggested that the RARE cluster-containing region is not the only retinoid-responsive region. All-*trans* RA induced the recruitment of both STAT3 and its phosphorylated form to their binding site in region 1 (STAT-RE1). STAT3 and pSTAT3 were also associated in an all-*trans* RA-dependent manner with other regions not containing any putative STAT-RE. As all-*trans* RA is highly specific to retinoids, it is assumed that RAR is the core of these effects. Thus, the *HAS2* promoter is likely to be associated with one or several large protein complexes that may have Pol II or pPol II as its core linked to TFs, CoAs and MED proteins and also to enzymes containing chromatin modulating activity, such as CBP. The chromatin-complexed promoter DNA may loop around this protein complex and is fixed at different REs. This explains, how all-*trans* RA-activated RARs may influence other proteins in the complex without the need of direct contact with DNA or the respective protein partner.

In conclusion, this study demonstrates that the *HAS2* is a primary all-*trans* RA and EGF-responsive gene. The responses are going via a RARE cluster approximately 1200 bp upstream of the TSS and a STAT-RE in the proximal promoter of human *HAS2* gene.

6.2. The 1α,25(OH)$_2$D$_3$-response of the *CYCLIN C* gene

The human *CYCLIN C* is known to be a primary 1α,25(OH)$_2$D$_3$-responsive gene (Polly *et al.*, 2000). This study provides a deeper understanding of the chromatin level events in the regulation of the *CYCLIN C* gene by 1α,25(OH)$_2$D$_3$. The *CYCLIN C* gene is an interesting 1α,25(OH)$_2$D$_3$ responsive gene as its function is not linked to the classical endocrine functions of 1α,25(OH)$_2$D$_3$, such as the regulation of calcium homeostasis and bone

mineralization. Therefore, the regulation of the *CYCLIN C* gene should provide a more detailed understanding of the mechanisms how $1\alpha,25(OH)_2D_3$ regulates cellular functions, such as proliferation, differentiation and apoptosis. Still, despite some function in the G_0-G_1 transition in the cell cycle (Ren and Rollins, 2004), the main function of the *CYCLIN C* gene is considered to being a part of MED complexes suggesting that Cyclin C protein regulates the general transcription rate rather than the cell cycle. Therefore, it is possible that the effects of Cyclin C on the cell cycle are secondary in nature and derive from an effect on the build up of other products involved more intimately in cell cycle regulation. Interestingly, Cyclin C has been reported to be contained only in those MED complexes that have a transcriptionally repressive function (Wang *et al.*, 2001). The Cyclin C-CDK8 complex phosphorylates the C-terminal domain of Pol II (Rickert *et al.*, 1996) and the basal TF TFIIH (Akoulitchev, 2000) both phosphorylations terminating the transcription. These two functions of Cyclin C make it important to understand its regulation in detail.

This study identified up to four VDREs within the first 8.4 kB of the *CYCLIN C* promoter. The ChIP assays with anti-VDR and anti-RXR antibodies suggested that the *CYCLIN C* promoter may contain up to five VDREs. Combined *in silico/in vitro* scanning of the first 8.4 kB identified in total four putative VDREs, two of which were DR3-type and two DR4-type REs. In order to further check the functionality and the chromatin composition of these putative VDREs, Re-ChIP assays were performed in order to find out the VDR-complexes on these elements. In this assay, first the anti-VDR antibody and then antibodies against RXR, NCoA3, MED1 and phosphorylated Pol II were used for immuno-precipitation. All the regions showed unique patterns of VDR and its partner proteins in their relative strength and time scale. All regions showed simultaneous associated of VDR with all the partner proteins, and most of them showed ligand-responsiveness. The most regions showed their maximum binding at the time point 60 min after $1\alpha,25(OH)_2D_3$ treatment. These findings support the functionality of the regions further suggesting that these regions are the VDREs via which the $1\alpha,25(OH)_2D_3$ responsiveness of the *CYCLIN C* gene is mediated.

In conclusion, this study provides insight into the regulation of the *CYCLIN C* gene by $1\alpha,25(OH)_2D_3$. One strong and three weaker VDREs were found to bind VDR-RXR heterodimers and complexes of VDR and co-regulators. In addition, each RE showed an individual pattern of these complexes suggesting that each of the VDREs is unique in its response to $1\alpha,25(OH)_2D_3$. Because of the role of the *CYCLIN C* gene in repression, this detailed analysis may help to understand the downregulation of a number of secondary $1\alpha,25(OH)_2D_3$-responding genes with an impact on cellular growth, differentiation and apoptosis.

6.3. Chromatin level mechanisms of RAR- and NF-κB-mediated regulation of the *HAS2* gene

In addition to RAREs and STAT-REs identified in the first paper (I), the human *Has2* was found to contain also binding sites for Sp1 and NF-κB. *In silico* screening of the first 2,250 bp of the promoter identified three NF-κB-REs, the functionality of which was demonstrated in HaCaT cells. In addition, in total 12 Sp1 binding sites were found within the 2,250 bp upstream of the TSS. NF-κB RE1 at position -380 partially overlaid the STAT3

binding site reported in the first paper and may contribute to the known response of the *HAS2* gene to EGF. Two other NF-κB REs at positions -1,420 and -1,890, also associated with the NF-κB component p65 in living cells. However, the NF-κB RE1 showed the strongest response to TNFα stimulation. The TSS showed the most prominent recruitment of pPol II, MED and CoA proteins in response to TNFα most likely integrating the activity of the three NF-κB REs by DNA looping. The activity of all three NF-κB REs was confirmed by reporter gene assay. Interestingly, promoter region between -640 and -512 bp showed an inhibitory effect of NF-κB activity on RE1 according to reporter gene assays. In addition, ChIP assays demonstrated strong NCoR1 association that was responsive to TNFα. As TNFα has pleiotropic actions, it may affect the *HAS2* gene both positively and negatively. This may explain why the *HAS2* gene is induced by TNFα only 1.8-fold even though its promoter contains three functional NF-κB REs.

The RARE cluster at position -1208 to -1226 was found to be the main region responsible for the all-*trans* RA – induction of the *HAS2* gene. In addition to showing the binding of RAR to the respective promoter region by ChIP assay, all-*trans* RA-dependent recruitment of CBP and Sp1 proteins and dissociation of NCoR1 to the same promoter region could be seen. As the ChIP method has its limitations concerning the resolution, some retinoid-responsive effects were seen also in regions flanking the RARE cluster. RAR can associate with sites in the vicinity of the RARE cluster, maybe without direct DNA contact in conjunction with other TFs. This idea is further supported by reporter gene assay that showed all-*trans* RA-dependent activation in the region between positions -1,129 and -930.

The constitutive nuclear factor Sp1 appeared to act not only as a constitutive TF but also as a co-factor of both retinoid and NF-κB signaling. Therefore, Sp1 is thought to contribute both to the basal mRNA expression of the *HAS2* gene as well as to its induction by all-*trans* RA and TNFα. The latter is supported by the observation that Sp1 is known to interact with CBP (Song *et al.*, 2002; Zhang *et al.*, 2001). As CBP interacts with many TFs, including NRs, such as RARs, and NF-κB (Chen and Greene, 2003), it is well suited to integrate the actions of these regulatory proteins. A similar role could be also played by CoA, CoR and MED proteins, as their interaction domain do not require very strict target recognition.

A co-stimulation of TNFα and all-*trans* RA did not have any additional effect on *HAS2* mRNA expression. However, all-*trans* RA stimulation was reduced by the application of a NF-κB inhibitor indicating that RAR and NF-κB signaling do have interactions. This interference might happen in several levels. Firstly, it can be due to the co-operative action of both TFs on the chromatin organization of the same promoter region. Secondly, RAR and NF-κB can share common co-factors as suggested in this study. Finally, the interference might be on the level of modulating of target gene mRNA stability and the communication of RAR and NF-κB with other signal transduction pathways.

Taken together, this study describes the human *HAS2* gene promoter being under the control of the inducible TFs NF-κB and RAR and constitutively active factor Sp1. These regulatory proteins are thought to share common co-factors providing numerous possibilities for functional interactions between the different signaling pathways.

6.4. Comparisons of the regulation of the genes *HAS2* and *CYCLIN C* by Forskolin and all-*trans* RA

The regulation of the two genes, human *HAS2* and *CYCLIN C* have not been studied in detailed so far (Monslow *et al.*, 2004; Saavalainen *et al.*, 2005 and 2007; Sinkkonen *et al.*, 2005). Also the complete picture of the regulation of gene expression on chromatin level in general is still unclear becoming an important aspect of the research in molecular biology. The altered amounts of hyaluronan are associated with many pathological situations including wounding and psoriasis in addition to several inflammatory conditions and malignant tumours. As it is also known that the hyaluronan concentration is best correlated with *HAS2* gene transcription, (Tammi *et al.*, 2005; Pienimäki *et al*., 2001; Nishida *et al.*, 1999; Karvinen *et al.*, 2003) a detailed understanding of the regulation of the *HAS2* gene is extremely important. *CYCLIN C* has also important roles both in normal physiology and in disease being a regulator of cell cycle (Ren and Rollins, 2004) and a component of the MED complexes.

This study extends our previous studies (I, II and III) concerning *HAS2* and *CYCLIN C* gene regulation (Saavalainen *et al.*, 2005 and 2007, Sinkkonen *et al.*, 2005) and provides the regulation by Forskolin and combination of all-*trans* RA and Forskolin as a new aspect.

In silico screening within the first 2,250 bp yielded three and four putative CREs on the *HAS2* and *CYCLIN C* promoters, respectively, and a cluster of two RAREs on the *HAS2* promoter and two DR2-type RAREs that are responsible for the all-*trans* RA-mediated response of the *CYCLIN C* promoter. The *HAS2* promoter was shown to bind many transcriptional regulators in an all-*trans* RA or Forskolin–dependent manner. Both responses showed some independent features but also interference in their signaling. The interference of all-*trans* RA with Forskolin signaling has been demonstrated earlier and might be due to the non-classical action of RA by activating CREB1 via PKC, ERK or RSK without RAR (Aggarwal *et al.*, 2006). This study indicates that the interference might be, at least in partly, due to common co-regulator recruitment. Many co-factors are rather promiscuous in their interaction with TFs. MED1, CBP, SRC-1and NCoR all interact both with CREB1 and RARγ. Their short interaction domains do not require very specific target recognition making these co-factors suitable for integrating the actions of basically all TFs that they interact with. This general mechanism is supposed to apply to all genes that contain functional CREs and RAREs in their regulatory regions making *HAS2* and *CYCLIN C* only as representative examples.

All putative REs showed a unique pattern of TF and co-regulator binding in response to different treatment conditions. There was also quite a lot of ligand-independent association of TFs and co-regulators to the *HAS2* promoter making the chromatin level mechanisms of the regulation of the *HAS2* gene complex (Fig. 9). In the absence of any stimulus the RARE cluster associated with NCoR1. Also CBP and pPol II to some extent associated with this region. The surprising finding that both a CoA and a CoR associated to the same region was probably due to looping of the DNA that cannot be avoided in a ChIP protocol using sonicated DNA fragments.

In this protocol the average size of the chromatin fragments ranges from 300 to 1000 bp limiting the resolution of the promoter scanning. All-*trans* RA induced RAR association to the RARE cluster, as well as NCoR1 dissociation from the same region. Also pPol II associated more strongly than without any treatment. Interestingly, CBP alone was not immunoprecipitated to this region, but the combination of CBP with pPol II was found in the RARE cluster. This indicates that there might be some factor "X" masking CBP and the interaction with RAR overcomes this masking. Forskolin treatment recruited RAR to the RARE cluster. It did not induce NCoR1 dissociation but increased pPol II binding. Also CBP was bound to this region. The association of both NCoR1 and CBP suggests that there is a bigger complex of regulators involved in this region. The combination of all-*trans* RA and Forskolin treatments did not induce significant RAR binding to its RE even though it caused NCoR1 to dissociate from this region. Surprisingly, the combination recruited CREB to this region although either of the separate treatments did not cause any CREB binding. This might also be due to the chromatin looping and association with CBP that still binds the RARE.

CRE1 on the *HAS2* promoter harvested a wide range of factors in the absence of any stimulus. It bound NCoR1 and CREB1 and also showed some association with RAR, CBP, MED1 and pPol II. The association of CREB1 without ligand stimulation has been proved earlier (Hagiwara *et al.*, 1993; Richards *et al.*, 1996; Gonzalez and Montminy, 1989). It is assumed that the regulation of CREB activity happens mainly by regulating CBP recruitment rather than CREB binding to CRE. All-*trans* RA increased RAR binding and also MED1 binding and caused NCoR1 to dissociate from the promoter. The increase in the binding of RAR was again probably due to looping of chromatin. Forskolin induced MED1 and CBP binding, which was logic, as CBP should be the main regulator of CREB binding. Forskolin caused also NCoR1 dissociation from CRE1. The combined treatment of all-*trans* RA and Forskolin caused a similar pattern to CRE1 than Forskolin alone highlighting the role of Forskolin as the regulator via this RE.

As CREs 2 and 3 were relatively close to each other (-1,731 to -1,751 and -1,878 to -1,889) and as there are limitations in the resolution of ChIP method CREs 2 and 3 were combined in the model (Fig. 9). In the absence of ligand there were NCoR1, CBP, MED1 and again CREB binding. All-*trans* RA recruited pPol II and RAR there but did not affect the binding of NCoR1. Forskolin, on the other hand, caused NCoR1 dissociation and recruited in addition to MED1 also RAR there. Surprisingly, the combined treatment did not cause RAR association even though both separate treatments did.

The *CYCLIN C* promoter also bound many transcriptional regulators in an all-*trans* RA- or Forskolin-dependent manner. The conclusions concerning the chromatin level events on the *CYCLIN C* promoter are quite complex because of the fact that all other putative REs are at least partly in repetitive sequence except for CRE1. This raises CRE1 as the most potent RE concerning the Forskolin response of the *CYCLIN C* gene. The responsiveness to retinoids is even far more complex. The first 2,250 bp of the promoter did not contain any putative DR5-type REs or even DR1-type REs that could bind RAR. Even scanning a few thousand bp further upstream did not provide any putative RAREs. Anyway, two DR2-type RAREs that were in the repetitive sequence were found on the *CYCLIN C* promoter. Laperriere and co-workers (Laperriere *et al.*, 2007) noticed that DR2-type REs elements in Alu sequences were functional RAREs. Without any stimulant, the region

containing both DR2-type RAREs in addition to CREs 1 and 2 showed association with RAR and CREB1 (Fig. 10). Also CBP and pPol II were found in this region. All-*trans* RA increased RAR binding supporting the idea that something within this fragment serves as a RARE (most likely either or both of the DR2s). Otherwise the chromatin composition remained the same as without any stimulants. The combination of all-*trans* RA and Forskolin increased CREB1 and pPol II binding. The fact that CBP, CREB1 and also pPol II were found in this region suggests that this fragment is also functional concerning Forskolin signaling.

The putative CREs 3 and 4 containing region of the *CYCLIN C* promoter was found out to be even more complicated. In the absence of any stimulus NCoR1, CBP and RAR where associated with this region indicating the presence of a bigger complex with also some other proteins involved. Significant CREB1 binding was not seen. All-*trans* RA diminished NCoR1 binding and recruited pPol II indicating that this region although not having any RAREs is also functional concerning retinoid-response. Forskolin also recruited pPol II and caused the dissociation of NCoR1 in addition to recruiting CREB1 indicating that both or either of the CREs is functional. Surprisingly, the combined treatment did not recruit CREB1 or pPol II indicating some kind of interference of all-*trans* RA and Forskolin with each other.

Even though each RE seemed to recruit to some extent an individual pattern of co-regulators, some patterns of the recruitment in response to certain stimulus was seen. RNA silencing results confirmed that mainly MED1 and NCoR1 mediated the retinoid-responsiveness of the *HAS2* gene. The Forskolin-responsiveness, on the other hand, was mediated mostly by CBP. MED1 and NCoR1 were shown to be the main co-regulators involved in the Forskolin-responsiveness of the *CYCLIN C* gene, while none of the co-regulators investigated was shown to affect significantly the retinoid-responsiveness of the *CYCLIN C* gene. As there are clear differences in the use of co-regulators between different genes, the responses of each individual gene are most likely a mixture of general and gene-specific mechanisms, how co-factors interfere signalling pathways.

Taken together, this study demonstrated that both human *HAS2* and *CYCLIN C* genes are regulated by all-*trans* RA and Forskolin. On the *HAS2* promoter the RARE cluster could be confirmed as the mediator of retinoid-response. In addition, three CREs were shown to be functional concerning the CREB-response. On the human *CYCLIN C* promoter it can be strongly assumed that the retinoid-response goes via both or either of the two DR2-type REs and the CREB-response via the CRE1 in unique region, although the existence of RAREs more distant from the TSS cannot be ruled out. All REs showed different patterns of co-regulators in response to different stimulus indicating that RAR and CREB signaling pathways differ from each other by the use of co-regulators. On the other hand, the interference of the two signaling pathways is also likely to happen via the use of common co-regulators.

7. Summary and conclusions

In conclusion, the four publications, on which this thesis is based, represent a detailed analysis of the chromatin level mechanisms of the regulation of human genes *HAS2* and *CYCLIN C*. RAR-, STAT-, NF-κB- and CREB-mediated regulation of *HAS2* gene was investigated. In addition, the chromatin composition of the VDREs on the *CYCLIN C* promoter, as well as its regulation by RAR and CREB was studied.

Regulation of HAS2 gene by all-trans RA and EGF (I)

This study indicates that both the rat and human *HAS2* genes are primary EGF and all-*trans* RA responding genes. However, the details of the mechanisms differ between the two species. The human *HAS2* gene contains a functional STAT-RE in its proximal promoter. This STAT-RE binds both STAT and the active phosphorylated form of STAT in an EGF-responsive way, indicating strongly that it mediates the effects of EGF on the regulation of *HAS2* gene. A cluster of two RAREs approximately 1,200 bp upstream of the TSS mediates the retinoid response of the human *HAS2* gene.

Chromatin composition of the VDREs on human CYCLIN C gene in response to 1α,25(OH)$_2$D$_3$(II)

In this study, the details of the 1α,25(OH)$_2$D$_3$-response of human *CYCLIN C* gene were studied on chromatin level. The first 10 kB of the *CYCLIN C* promoter contains in total four functional VDREs. The association pattern of VDR and its partner proteins, the heterodimerization partner RXR, the CoA NCoA3, MED and activated Pol II is different in each VDRE. All four VDREs bind a complex containing VDR and the partner proteins in a 1α,25(OH)$_2$D$_3$-dependent way. The intensity and the timing of binding differ between the elements making each VDRE unique in the recruitment of VDR and other proteins involved in the regulation of transcription.

Integration of the activation of human HAS2 gene by RAR and NF-κB (III)

The chromatin level mechanisms of the activation of human *HAS2* gene by both RAR and NF-κB and the integration of the two signaling pathways were investigated in this study. The *HAS2* gene promoter was found to be under the control of the inducible TFs RAR and NF-κB and the constitutive active factor Sp1. The *HAS2* promoter contains three functional NF-κB REs within the first 2250 bp of the promoter. All three NF-κB REs associate with NF-κB and recruit pPol II and transcriptional co-regulators in response to TNFα and also constitutively each RE having still a unique pattern of proteins involved in transcription. The RARE cluster on the *HAS2* promoter associates with RAR, CBP and Sp1 in a retinoid-dependent way. Also NCoR1 is dissociated from that region in response to all-*trans* RA. Sp1 affects both the retinoid and NF-κB signaling in addition to being a constitutive TF also by acting as co-factor for both signaling pathways probably via interaction with

CoAs, CoRs or MED proteins. As all three regulatory proteins affect the activation of the *HAS2* gene and its chromatin composition, they are likely to act by sharing common co-factors, which provide numerous possibilities for functional interactions between the signaling pathways, of which they are the end point.

Comparison of RAR and CREB-mediated regulation of the HAS2 and CYCLIN C genes (IV)

This study investigated the chromatin level mechanisms of the *HAS2* and *CYCLIN C* genes by RAR, and CREB1. Additionally, the interference of the two signaling pathways was studied. It was demonstrated here that both the *HAS2* and *CYCLIN C* genes are regulated by all-*trans* RA and Forskolin. On the *HAS2* the retinoid-responsiveness goes via the RARE cluster. In addition, three CREs were shown to be functional concerning the CREB-response. On the *CYCLIN C* promoter the retinoid-response is supposed to go via both or either of the two DR2-type RAREs and the CREB-response via the CRE1 in unique region. On both promoters both signaling pathways have some independent features but they also show some interference. Each RE recruits a different pattern of co-regulators making them unique.

8. Future aspects

Each cell of an organism contains the same genetic material and the differences between cells arise from differences in gene expression. The genome sequences of many organisms have now been completed, which raises the mechanisms of gene expression to be the next important aspect in genomics. Recent fast improving technologies in the field of bioinformatics and molecular biology in general have provided enormous amounts of data that are useful in clarifying the regulatory mechanisms of genes. However, there is not a complete understanding of either the regulatory regions or the regulatory proteins involved in the process. Furthermore, the dynamics of the process are still poorly characterized.

The genomic data has revealed a huge number of genes with clinical phenotypes. In case of monogenic disorders, the disease is due to mutations, mostly in the protein coding sequence of the gene. However, it is now widely accepted that the defects in the highly regulated events of gene regulation can disrupt normal gene function and lead to pathological states. Knowing the susceptible genes for common diseases, such as cancer, type 2 diabetes and Alzheimer's, is the first step in the fight against the disease. The challenge in the future is not only to understand that certain gene is regulated by certain compound but also have a chromatin level understanding of the mechanisms by which the process of that gene's transcription occurs. This provides us a multitude of potential target molecules on which to attack, if we want to affect the expression of the gene. We do not have to target necessarily always the TF that is known to regulate the gene but the targets could also be the other proteins involved in the processes leading to gene expression. A complete picture of the whole process with the regulatory regions and the regulators as such enable us to specify the treatments for each patient. In addition the increasing number of potential targets makes it possible to overcome the putative side effects of one compound.

It will be very challenging to find a general model on chromatin level events leading to gene expression that could be applied to all or at least to most of the signaling pathways. Due to the high diversity of the signaling routes, it is easy to speculate that those events do not fit into one model. However, understanding the principles of the chromatin level regulation serves as a building block, on which to develop the understanding of physiological states.

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Appendix: Original publications

Kuopio University Publications C. Natural and Environmental Sciences

C 199. Tarvainen, Tanja. Computational Methods for Light Transport in Optical Tomography.
2006. 123 p. Acad. Diss.

C 200. Heikkinen, Päivi. Studies on Cancer-related Effects of Radiofrequency Electromagnetic Fields. 2006. 165 p. Acad. Diss.

C 201. Laatikainen, Tarja. Pesticide induced responses in ectomycorrhizal fungi and symbiont Scots pine seedlings.
2006. 180 p. Acad. Diss.

C 202. Tiitta, Markku. Non-destructive methods for characterisation of wood material.
2006. 70 p. Acad. Diss.

C 203. Lehesranta, Satu. Proteomics in the Detection of Unintended Effects in Genetically Modified Crop Plants.
2006. 71 p. Acad. Diss.

C 204. Boman, Eeva. Radiotherapy forward and inverse problem applying Boltzmann transport equation.
2007. 138 p. Acad. Diss.

C 205. Saarakkala, Simo. Pre-Clinical Ultrasound Diagnostics of Articular Cartilage and Subchondral Bone.
2007. 96 p. Acad. Diss.

C 206. Korhonen, Samuli-Petrus. FLUFF-BALL, a Fuzzy Superposition and QSAR Technique - Towards an Automated Computational Detection of Biologically Active Compounds Using Multivariate Methods.
2007. 154 p. Acad. Diss.

C 207. Matilainen, Merja. Identification and characterization of target genes of the nuclear reseptors VDR and PPARs: implementing in silico methods into the analysis of nuclear receptor regulomes.
2007. 112 p. Acad. Diss.

C 208. Anttonen, Mikko J. Evaluation of Means to Increase the Content of Bioactive Phenolic Compounds in Soft Fruits.
2007. 93 p. Acad. Diss.

C 209. Pirkanniemi, Kari. Complexing agents: a study of short term toxicity, catalytic oxidative degradation and concentrations in industrial waste waters.
2007. 83 p. Acad. Diss.

C 210. Leppänen, Teemu. Effect of fiber orientation on cockling of paper.
2007. 96 p. Acad. Diss.

C 211. Nieminen, Heikki. Acoustic Properties of Articular Cartilage: Effect of Structure, Composition and Mechanical Loading.
2007. 80 p. Acad. Diss.

C 212. Tossavainen, Olli-Pekka. Shape estimation in electrical impedance tomography.
2007. 64 p. Acad. Diss.

C 213. Georgiadis, Stefanos. State-Space Modeling and Bayesian Methods for Evoked Potential Estimation.
2007. 179 p. Acad. Diss.