Down-regulation of the *Interleukin 2* gene expression by 1α,25-dihydroxyvitamin D_3

Antti Räsänen
M.Sc thesis
Biochemistry
Department of Biosciences
University of Kuopio
April 2009

ABSTRACT

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UNIVERSITY OF KUOPIO, The Faculty of Natural and Environmental Sciences, Curricula of Biochemistry
RÄSÄNEN Antti Tapani
Thesis for Master of Science degree
Supervisors Dr. Sami Väisänen
M.Sc Juha Matilainen

April 2009

Keywords: Nuclear receptor; Vitamin D receptor; Vitamin D response element; Cytokine; Interleukin-2; IL-2

Interleukin 2 (IL-2) is a signal molecule of the immune system. The protein encoded by *IL-2* gene is a secreted cytokine that is important for the proliferation of T and B lymphocytes. It stimulates T cell proliferation and potentiates the apoptotic cell death of antigen-activated T cells. IL-2 is also required for both induction and self-regulation of T cell-mediated immune responses.

In this study *IL-2* gene was found to be regulated by the nuclear hormone 1α,25$(OH)_2D_3$ in Jurkat cells, which are immortalized T lymphocytes. Combined *in silico* analysis for putative 1α,25$(OH)_2D_3$ response elements (VDREs) and ChIP assays with antibody against vitamin D receptor (VDR) revealed four possible functional VDREs, which were located approximately -8400 bp (ER9), -5400 bp (DR3) and -1500 bp (DR4) upstream and +1500 bp (DR4) downstream from transcription start site (TSS) of the *IL-2* gene. Expression assays in combination with RNAi and cycloheximide confirmed that the *IL-2* gene is repressed by 1α,25$(OH)_2D_3$. Taken together, this study confirmed that the *IL-2* is a primary 1α,25$(OH)_2D_3$ target gene, which is regulated via distal VDREs.

ACKNOWLEDMENTS

I would like to thank my supervisors Dr. Sami Väisänen and M.Sc Juha Matilainen for their guidance and advice during my project. I also would like to thank all my co-workers for pleasant and productive atmosphere in lab. Thanks for Maija and Hanna for providing the cells.

Finally I would like to thank my dear Sanna for all the kicking and pushing through this time.

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ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	$1\alpha,25$-dihydroxyvitamin D_3
AF-1	Activation function 1
AF-2	Activation function 2
AR	Androgen receptor
Blimp-1	B lymphocyte maturation protein-1
CAR	Constitutive androstanone receptor
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CHR	Cytokine-binding homology region
CHX	Cycloheximide
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
CoA	Coactivation protein
CoR	Corepression protein
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DRn	Direct repeat spaced by n nucleotides
ER	Estrogen receptor
ERn	Everted repeat spaced by n nucleotides
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HHV-8	Human herpes virus
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
IL-4	Interleukin 4
IL-7	Interleukin 7
IL-9	Interleukin 9
IL-15	Interleukin 15

IL-21	Interleukin 21
IRn	Inverted repeat spaced by n nucleotides
LIF	Leukemia inhibitory factor
LPD	Ligand binding domain
LPS	Lipopolysaccaride
LXR	Liver x receptor
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NK	Natural killer cell
NR	Nuclear receptor
OSM	Oncostatin-M
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PPAR	Peroxisome proliferator-activated receptors
RAR	Retinoic acid receptor
RE	Response element
RNA	Ribonucleic acid
RNAi	RNA interference
RPLP0	Ribosomal protein, large, P0
RT	Room temperature
RXR	Retinoid X receptor
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TBP	TATA-binding protein
TF	Transcription factor
T_H cell	T helper cell
TNF- α	Tumor necrosis factor-α
TPA	12-O-Tetradecanoyl-phorbol-13-acetate
TSS	Transcription start site
UVB	Ultraviolet radiation B
VDR	Vitamin D receptor

VDIR	Designated VDR interacting repressor
VDRE	$1\alpha,25(OH)_2D_3$ responsive element

1. INTRODUCTION

The expression of several genes involved in growth, differentiation and metabolism of higher organisms is regulated by small compounds, such like steroid hormones or vitamins A or D. These compounds mediate their actions via specific nuclear receptors (NRs). One member of nuclear receptor superfamily is vitamin D receptor (VDR), which mediates the actions of 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$). The vitamin D_3 is synthesized in the human body with the help of sunlight's ultraviolet B (UVB) radiation and hydroxylated to active form, 1α,25$(OH)_2D_3$. 1α,25$(OH)_2D_3$ is involved in calcium and phosphate homeostasis and it plays a role in the regulation of proliferation, differentiation and apoptosis (Dusso et al., 2005). Because of this, 1α,25$(OH)_2D_3$ is considered as a potential agent in prevention of cancer and autoimmune diseases.

In this study the expression of *IL-2* gene in Jurkat cells and the effect of 1α,25$(OH)_2D_3$ treatment was examined. It has been shown that *IL-2* gene is repressed by 1α,25$(OH)_2D_3$ (Alroy et al., 1995), but it is not known how specific is the 1α,25$(OH)_2D_3$ response, what is its mechanism of action and which proteins are involved. This is why *IL-2* was selected as a topic in our study. The protein encoded by *IL-2* gene is a secreted cytokine which is important for the proliferation of T and B lymphocytes. It stimulates T cell proliferation and potentiates the apoptotic cell death of antigen-activated T cells (Taniguchi et al., 1983). *IL-2* is also required for both the induction and self-regulation of T cell-mediated immune responses.

IL-2 has three main activation routes: the Jak-STAT, phosphoinositide 3-kinase (PI3K)/Akt, and RAS-mitogen-activated protein kinase (MAPK) pathways. The contributions of these pathways to T-cell function and cytokine-induced gene expression are essential (Kovanen and Leonard, 2004).

Results of this study help to understand how *IL-2* gene is regulated by 1α,25$(OH)_2D_3$ at molecular level and what is the mechanism of that regulation.

2. LITERATURE REVIEW

2.1 Vitamin D

Vitamin D is a hormone rather than a vitamin. Vitamin D and its active form 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) has many functions in human body. It has many target organs, tissues and cells. The vitamin D target tissues are shown in Table 1 and target cells in Table 2.

The principal role of 1α,25$(OH)_2D_3$ in the immune system is to act as an immunosuppressive agent by down-regulating the activity of T and B cells. The main targets for vitamin D regulation appear to be T helper cells (T_H cells), cytokine profiles of which 1α,25$(OH)_2D_3$ modulates (Casteels et al., 1995;Hewison and O'Riordan, 1997; Lemire, 1995; Manolagas et al., 1994). This immunoregulatory role of 1α,25$(OH)_2D_3$ offers interesting possibilities for development of new medication for several autoimmune diseases including type I diabetes and arthritis as well as for prevention of allograft rejection after transplantation (Lemire, 1997). The 1α,25$(OH)_2D_3$ plays an essential role in calcium homeostasis and bone metabolism, but there are diverse range of biological actions that include induction of cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems (Dusso et al., 2005). Vitamin D analogs have been used in treatment of inflammatory skin conditions such as psoriasis and atopic lesions (Lehmann et al., 2004). It has also been shown that 1α,25$(OH)_2D_3$ suppresses IL-2, interferon-γ and tumor necrosis factor-α (TNF- α) production in Jurkat cells (Lam et al., 1974).

Table 1. Tissues that express the vitamin D receptor for the steroid hormone $1\alpha,25(OH)_2D_3$ (according to Norman, 2008)

Tissue distribution	
Adipose	Muscle, embryonic
Adrenal	Muscle, smooth
Bone	Osteoblast
Bone marrow	Ovary
Brain	Pancreas β cell
Breast	Parathyroid
Cancer cells	Parotid
Cartilage	Pituitary
Colon	Placenta
Eggshell gland	Prostate
Epididymis	Retina
Hair follicle	Skin
Intestine	Stomach
Kidney	Testis
Liver (fetal)	Thymus
Lung	Thyroid
Lymphocytes (B & T)	Uterus
Muscle, cardiac	Yolk sac (bird)

Table 2. $1\alpha,25(OH)_2D_3$ target cells (according to Deluga et al., 2001)

Proven	Putative
1. Intestinal enterocyte	1. Islet cell, pancreas
2. Osteoblast	2. Endocrine cells, stomach
3. Distal renal cells	3. Pituitary cells
4. Parathyroid cells	4. Ovarian cells
5. Keratinocytes of skin	5. Placenta
6. Promyelocytes, monocytes	6. Epididymis
7. Lymphocytes	7. Brain (hypothalamus)
8. Colon enterocytes	8. Myoblasts (developing)
9. Shell gland	10. Aortic endothelial cells
10. Chick chorioallantoic membrane	11. Skin fibroblasts

2.2 Metabolism of vitamin D

The synthesized form of vitamin D in vertebrates is vitamin D_3, known as cholecalciferol, while in plants the form is vitamin D_2, which is known as ergocalciferol (Fig. 1).

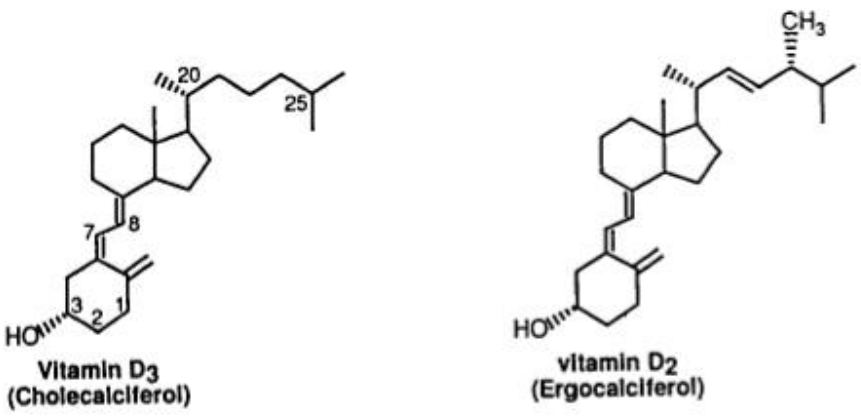

Figure 1. Nutrional forms of Vitamin D. Two different forms of vitamin D.

The synthesis of vitamin D_3 occurs in the skin (Fig. 2), with the aid of sunlight. Vitamin D can be obtained from the diet, either as D_2 from plant or D_3 from animal products (Fig. 1). These compounds can be part of normal diet or from supplements. The importance of vitamin D to normal growth as well as to the overall biology of mammals is stressed by the fact that its sufficient supply is normally ensured by the above independent sources. This feature makes vitamin D unique among other hormones (Jones et al., 1998; Dusso et al., 2005).

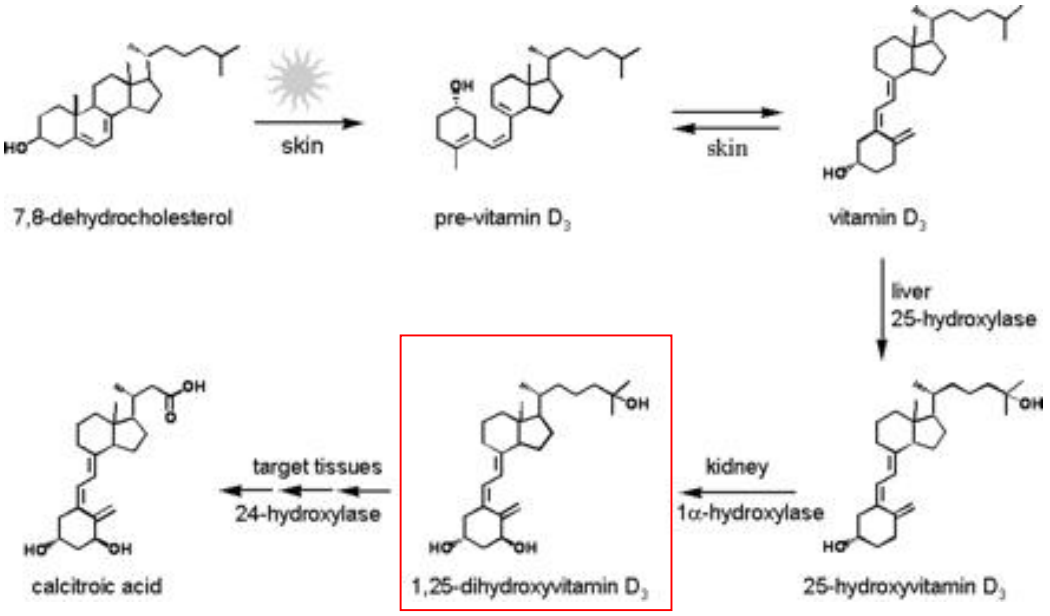

Figure 2. Vitamin D_3 synthesis, activation and catabolism (adapted from Dusso et al., 2005).

Vitamin D_3 is normally inactive and two hydroxylation steps are needed for its activation. First, Vitamin D_3 is hydroxylated in liver by 25-hydroxylase resulting in 25-hydroxyvitamin D_3. Next step takes place in kidney by 1α-hydroxylase resulting in 1α,25-dihydroxyvitamin D_3. The physiologically most active form, 1α,25-dihydroxyvitamin D_3, is inactivated by 24-hydroxylase (Dusso et al., 2005).

2.3. Nuclear receptor superfamily

Gene transcription is controlled by specific transcription factors, which bind directly to DNA. Those proteins bind to specific sequences and recruit cofactors (Kadonaga, 2004). Some of these proteins form a nuclear receptor (NR) superfamily. Human genome encodes 48 members of this superfamily (Maglich et al., 2001).

Nuclear receptors can be divided in three major groups (Fig. 3). These groups are classical endocrine receptors, adopted orphan receptors and orphan receptors. The endocrine receptors include estrogen (ER), androgen (AR), retinoic acid (RAR) and vitamin D

receptor (VDR) (Chawla et al., 2001). Ligands for these receptors are produced mostly in human body e.g. vitamin D_3 is produced in the skin by the photolytic cleavage of 7-dehydrocholesterol followed by thermal isomerization (Dusso et al., 2005).

The adopted orphan receptors include liver X receptors (LXRs), constitutive androstane receptor (CAR), peroxisome proliferator activated receptors (PPARs α, β, γ) and the retinoid X receptor (RXR) subtypes α, β and γ. Many of these receptors are thought to be nutritional sensors for lipids, fatty acids and cholesterol. These orphan receptors are considered adopted, because they can bind physiological ligands and display physiological effects. (Chawla et al., 2001; Wang and Wan, 2008)

Third group is orphan receptors (Chawla et al., 2001). They are called orphan receptors because their ligands are unknown (Mangelsdorf et al., 1995). This subgroup is not only the largest, but also the least characterized of all NRs. Some orphan receptors have genetic association with different human diseases.

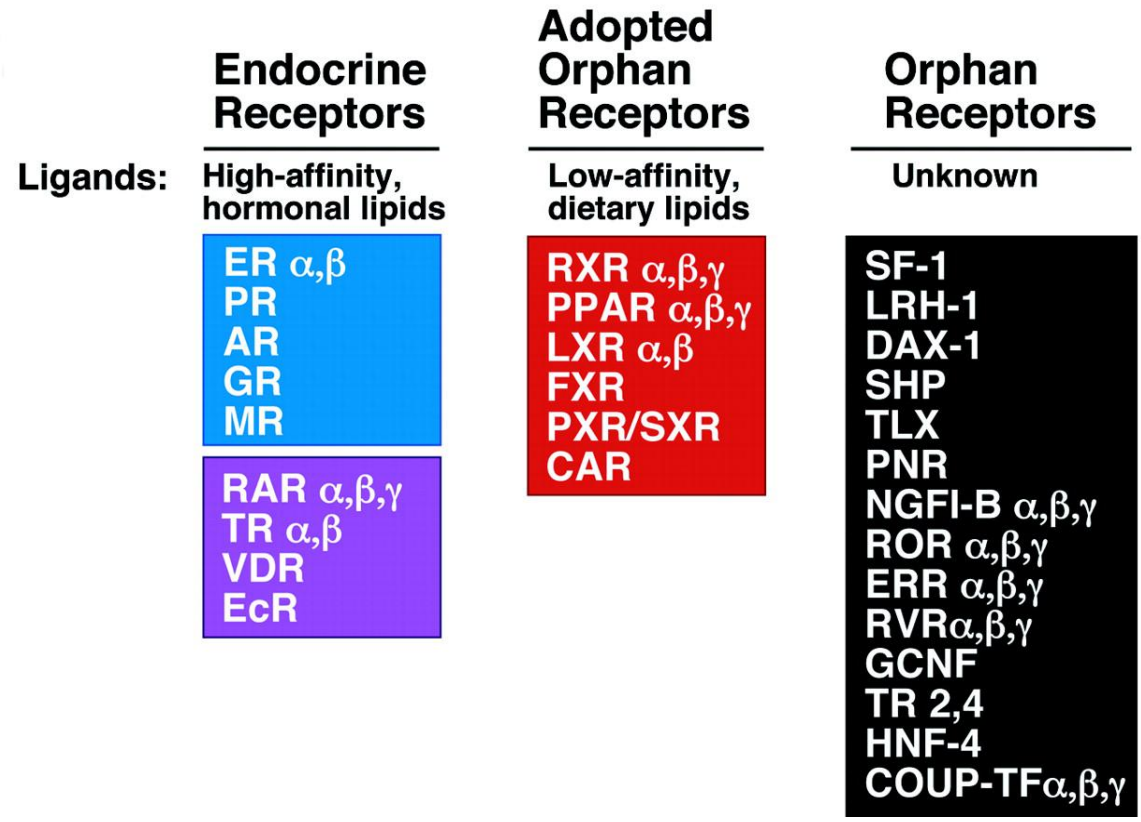

Figure 3. The Nuclear receptor superfamily (according to Chawla et al., 2001). Human nuclear receptors can be sorted into three groups.

Nuclear receptors can also be divided according to their binding to DNA. Steroid hormone receptors can be divided into receptors which form homodimers at response elements (REs) that have inverted repeat structures (class I), and receptors which form heterodimers with RXR at REs that have directly repeated structures (class II). Orphan receptors can be divided into proteins which bind to DNA as monomers (class III), and receptors which form homodimers (class IV) or heterodimers with RXR (class V) at REs formed by direct repeats (Carlberg, 1999).

2.4 Structure of NRs

Structural organization of nuclear receptors is very similar (Fig. 4A). With just few exceptions, these proteins contain an NH_2-terminal region that harbors a ligand-independent transcriptional activation function (AF-1); a core DNA-binding domain (DBD) which contains two highly conserved zinc finger motifs that target the receptor to specific DNA sequences known as hormone response elements (REs); a hinge region which permits protein flexibility to allow for simultaneous receptor dimerization and DNA binding; and a large C-terminal region which encompasses the ligand-binding domain (LBD), dimerization interface, and a ligand-dependent activation functions (AF-2) (Dusso et al., 2005). Without ligand, the NRs can be associated with corepressor (CoR) proteins and this way suppress gene activity. After ligand binding, the LBD of NR undergoes a conformational change which leads to the dissociation of corepressor (CoR) proteins and association of coactivator (CoA) proteins. This ultimately leads to transcriptional activation (Sutton et al., 2003) (Fig. 4B).

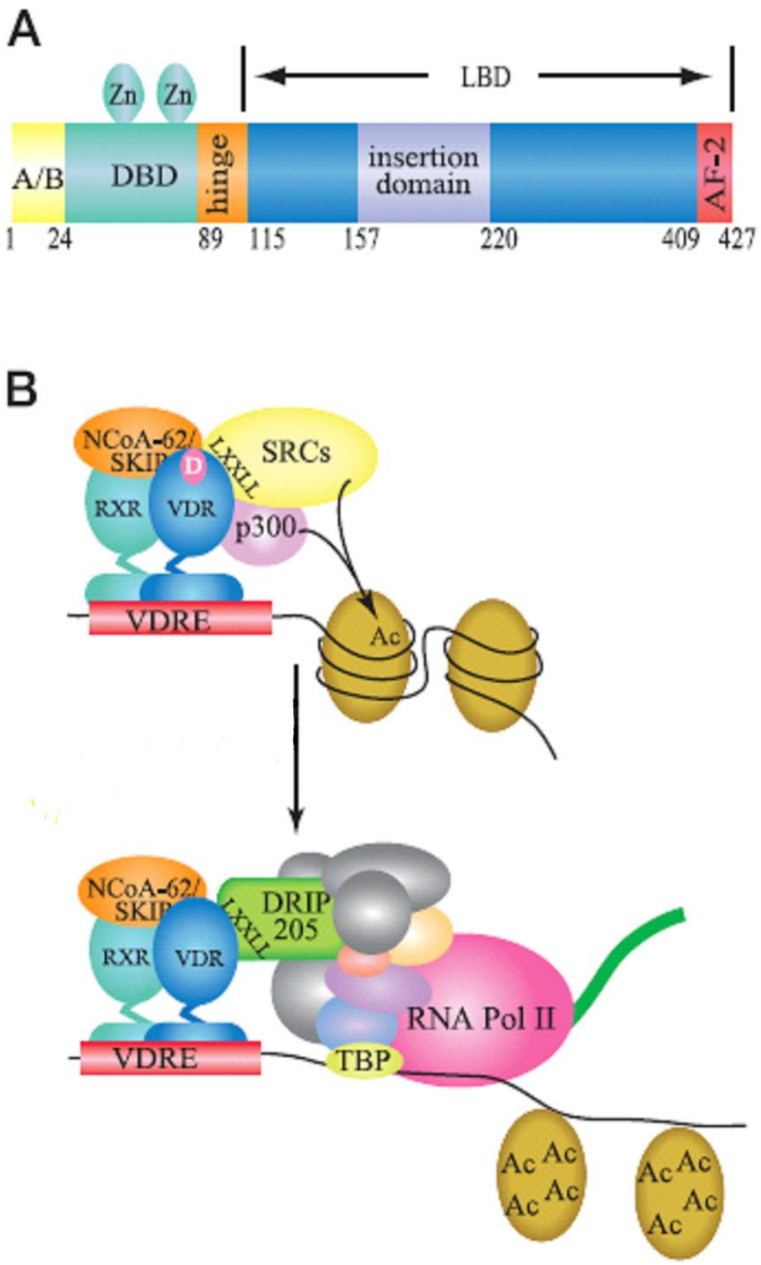

Figure 4. Domain structure of NRs and two step model of VDR-mediated transcription (adapted from Sutton et al., 2003). (**A**) NRs consist of six domains. The N-terminus (A/B) is variable, the DNA binding domain DBD is the most conserved region and contains two zinc fingers. The role of the hinge region is to be the connection between the DBD and the ligand-binding domain (LBD). The LBD is mainly responsible for ligand binding and dimerization and contains the activation function 2 (AF-2). **(B)** Temporal association of coactivators during VDR-mediated transcription. The liganded VDR-RXR complex recruits SRCs and CBP/p300, resulting in the acetylation of histones. The open chromatin template allows binding of the DRIP complex and entry of the core transcription machinery.

2.5 Function of NRs

NRs act as transcription factors (TFs) in the cell. They can be located either in the cytoplasm or in the nucleus and can be activated in many ways. An activated ligand or hormone, e.g. vitamin D_3, is generated from a pre-cursor or pro-hormone. The ligand for NRs can be generated either inside or outside of the cell. After synthesis, a hormone or ligand enters the cytoplasm or the nucleus where it binds to a specific receptor. The ligand can be also a metabolite which is formed in the cell. There are also alternative ligand independent pathways for activation of NRs (Aranda and Pascual, 2001). NRs can either activate or repress target genes by binding directly to response elements (RE) or other classes of DNA-bound TFs (Glass and Rosenfeld, 2000).

NRs regulate transcription mostly via binding to specific DNA sequences within target genes. These sequences are REs which contain the consensus sequence RGKTCA (R = A or G, K = G or T, Y = T or C, M = A or C, N = A, G, C or T) (Fig. 5). NRs can bind to REs as monomers, homodimers or heterodimers. The retinoid X receptor (RXR) subtype acts as a common partner for other NRs, such as VDR. Heterodimeric RXR-VDR complex binds to direct repeat (DR) or everted repeat (ER) type REs (Calberg et al., 2007).

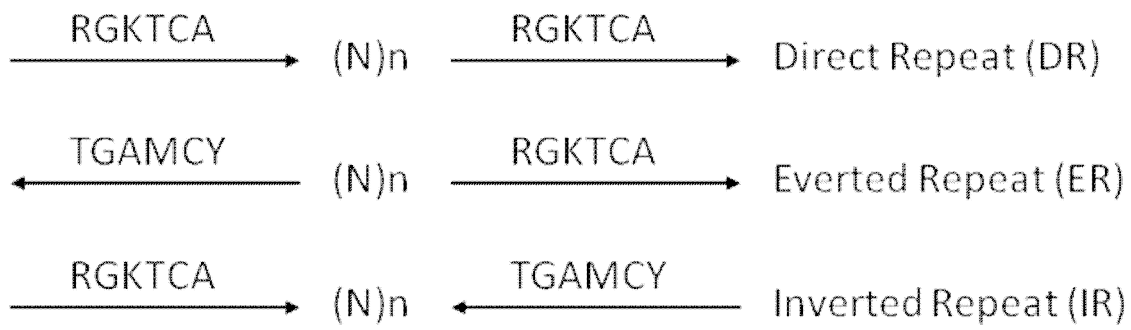

Figure 5. Different types of NR REs. (R = A or G, K = G or T, Y = T or C, M = A or C, N = A, G, C or T). Depending on NR, n can be 1, 2, 3….etc

2.6 VDRE and E-box elements

The VDR mediates biological actions of 1α,25$(OH)_2D_3$ and its analogues. VDR forms heterodimer with RXR. Activation of VDR-RXR complex by ligand allows its binding to the DNA (Cheskis and Freedman, 1994). There are suggestions that RXR could have an active role in 1α,25$(OH)_2D_3$-mediated regulation (Bettoun et al., 2003).

The VDR-RXR heterodimer binds to Vitamin D_3 Response element (VDRE) which consists of two half-sites separated by three to four nucleotides (Fig. 5). VDR-RXR heterodimer preferably binds to direct repeats (DR) with three spacing nucleotides (DR3), but also DR4 type response elements are common (Wang et al., 2005). It can also bind everted repeat with 6, 7, 8, or 9 spacing nucleotides (Tavera-mendoza et al., 2006). The non-liganded form of VDR-RXR complex can also bind DNA, but liganded form gives stronger DNA-protein interaction (Ross et al., 1993).

E-box-like motif (CANNTG) is an another class of nVDRE in the human 1α(OH)ase promoter. The VDR, activated by 1α,25$(OH)_2D_3$, does not directly bind to the negative VDRE, but instead associates with designated VDR interacting repressor (VDIR). VDIR transactivates through direct binding to this E-box-type element (1αnVDRE). However, the VDIR transactivation function is transrepressed through ligand-induced protein-protein interaction of VDIR with VDR/RXR (Kim et al., 2007).

2.7 Cytokine superfamily

Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells (Fantini et al., 2007).

Cytokines are polypeptides produced in response to microbes and other antigens, and they mediate and regulate immune and inflammatory reactions. Although cytokines are structurally diverse, they share several properties. For example, cytokines are characterized by considerable "redundancy" in that many cytokines appear to share similar functions (Ozaki and Leonard, 2002).

Cytokines are signaling- and glycoproteins which main function is cell-cell signaling. While hormones are secreted from specific organs to the blood, and neurotransmitters are related to neural activity, the cytokines are a more diverse class of compounds in terms of origin and purpose. They are produced by a wide variety of hematopoietic and non-hematopoietic cells and can have autocrine, paracrine and endocrine effects, sometimes strongly dependent on the presence of other chemicals (Wang et al., 2008).

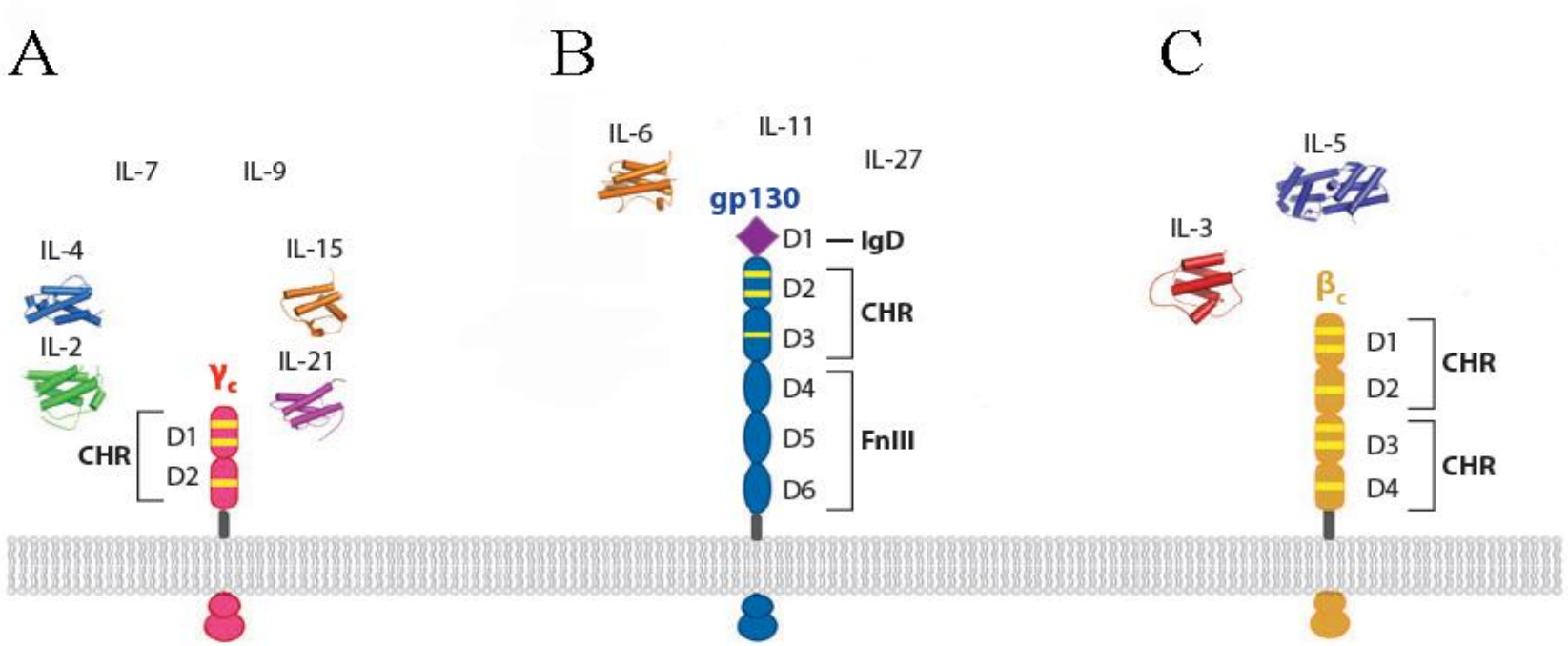

Figure 6. Diversity of shared cytokine-receptor interactions. Shared cytokine receptors γc (A), gp130 (B), and βc (C) are represented schematically on a cell membrane. The respective interacting cytokines with known three-dimensional structures are shown with cylinder representations of the four-helix bundles. (Wang et al., 2008)

The cytokine family consists mainly of small water-soluble proteins and glycoproteins with a molecular mass between 8 and 30 kDa. Each cytokine binds to a specific cell-surface receptor (Fig. 6). Subsequent cascades of intracellular signalling then alter cellular functions. This may include upregulation and/or downregulation of several genes, resulting in production of other cytokines, an increase in the number of surface receptors for other molecules or suppression of their own effect by feedback inhibition (Wang et al., 2008).

The effect of a particular cytokine in a given cell depends on the cytokine, its extracellular abundance, the presence and abundance of the complementary receptor on the cell surface, and downstream signals activated by receptor binding; these last two factors can vary by cell type (Ozaki and Leonard, 2002).

Cytokines are critical to the development and function of both innate and adaptive immune responses. They are often secreted by immune cells that have encountered a pathogen or chemical compound, such as lipopolysaccaride (LPS), thereby activating and recruiting further immune cells to increase the system's response to the pathogen (Schmelzer et al., 2009). Interleukins are one group of cytokines. Other groups are lymphocines and chemokines.

2.7.1 Interleukin 2

Interleukin 2 (*IL-2*) is a signaling molecule of the immune system. It is a 15-kDa α-helical cytokine produced predominately by activated $CD4^+$ and $CD8^+$ T cells (Taniguchi et al., 1983). The protein encoded by this gene is a secreted cytokine that is important for the proliferation of T and B lymphocytes and it stimulates T cell proliferation and potentiates the apoptotic cell death of antigen-activated T cells (Taniguchi et al., 1983). IL-2 is also required for both the induction and self-regulation of T cell-mediated immune responses. Activation of T cells through the T cell receptor (TCR) and costimulatory molecules such as CD28 lead to the production of *IL-2* and the expression of the IL-2 receptor (IL-2R) (Willerford et al., 1995).

The IL-2R is a heterotrimeric protein complex, gamma chain of which is common for all hematopoietic cells and is also shared by the receptors for *IL-4, IL-7, IL-9, IL-15,* and *IL-21* (Leonard, 2001; Nelson et al., 1998). The expression of this gene in mature thymocytes is monoallelic, which represents an unusual regulatory mode for controlling the precise expression of a single gene. The transient nature of *IL-2* secretion depends on transcriptional induction by TCR signals and stabilization of *IL-2* mRNA by costimulatory signals, followed by transcriptional silencing of the *IL-2* gene and rapid degradation of the IL-2 mRNA (Fraser et al., 1991).

The IL-2R heterotrimeric protein complex induces *IL-2* signaling that depends on the cytoplasmic tails of CD122 and γc (Fig. 7). When in close proximity, Jak-3 via γc and Jak-1 via CD122 phosphorylate key tyrosine residues on CD122, leading to the association of the adapter Shc and either Stat5 or, to a lesser extent, Stat3. Shc provides a platform to activate the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways, important for cell growth and survival (Nelson et al., 1998; Gaffen et al., 2001; Kovanen and Leonard, 2004). The associated Stats are further phosphorylated, allowing their dimerization and translocation into the nucleus. In activated T cells Stat5 is the main IL-2-induced Stat which regulates genes important for the effector function and T cell growth. Although activation of the MAPK, PI3K, and Stat5 pathways are important for conventional activated T cells, Stat5 is the main pathway by which IL-2R contributes to the regulatory T cell (Treg) production and maintenance (Fig. 7) (Kovanen and Leonard, 2004).

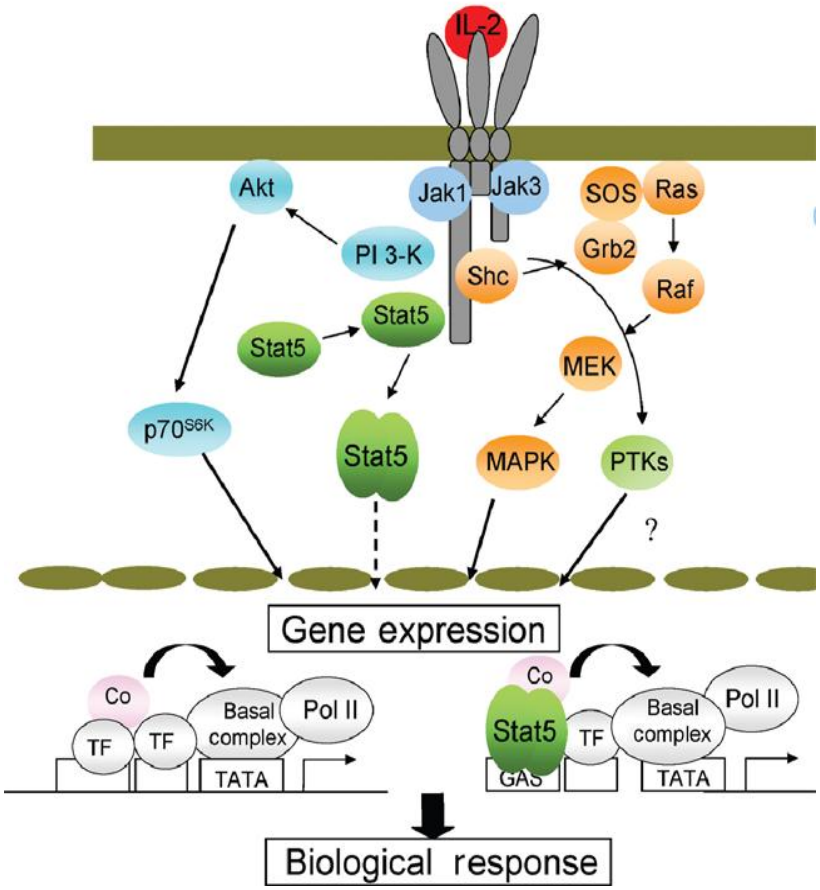

Figure 7: A schematic representation of the major signaling pathways activated by IL-2 (adapted from Kovanen and Leonard, 2004). Main routes in *IL-2* activation are Stat5, PI3-K, MAPK and PTK pathways. 'PTK' stands for protein tyrosine kinases activated by *IL-2*. Some of the PTKs reported to be activated by *IL-2* include Syk, Pyk2, p56lck, p53/p56lyn, and p59fyn.

IL-2 is an autocrine growth factor for T-cells (Bemiss et al., 2002). It has been reported that proliferation of lymphocytes activated with mitogen is inhibited by $1\alpha,25(OH)_2D_3$ and that the lymphocytes exhibit reduced *IL-2* activity (Lam et al., 1974, Tsoukas et al., 1984). In addition, the $1\alpha,25(OH)_2D_3$ inhibites *IL-2* transcription (Alroy et al., 1995).

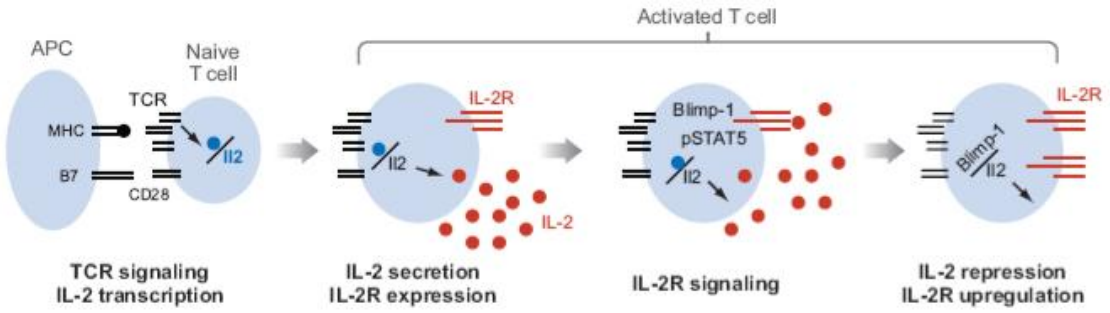

Figure 8. The autocrine *IL-2* auto-inhibition loop (according to Malek, 2008).

This autoregulatory loop depends on activation of Stat5 and IL-2-dependent induction of the transcriptional repressor B lymphocyte maturation protein-1 (Blimp-1) (Fig. 8). Thus, after antigen-activation of a naive T cell, *IL-2* is produced and the high-affinity IL-2R is expressed. Then the secreted IL-2 binds to the IL-2R leading to Stat5 activation and Blimp-1 induction and ultimately to the repression of the *IL-2* gene (Villarino et al., 2007; Gong et al., 2007). Blimp-1 is a key downstream mediator of *IL-2* repression because ectopic expression of Blimp-1 in activated T cells inhibits *IL-2* production and the 8.4 kb IL-2/GFP reporter (Martins et al., 2006), and Blimp-1-deficient T cells produce increased *IL-2* (Gong et al., 2007; Kallies et al., 2006). For example, mice with T cell specific knockout of Blimp-1 exhibit severe inflammatory bowel disease leading to early death (Gong et al., 2007; Kallies et al., 2006).

The activation of naive T cells leads to very high expression levels of CD25, which is a part of IL-2R (Dendrou and Wicker, 2008), through a two-step process (Fig. 8). First, moderate levels of CD25 are rapidly induced by TCR and costimulatory signals, in part by activation of NF-κB, NFAT, AP-1, and CREB/AFT. Subsequently, *IL-2* binds to the IL-2R and increases the initial level of CD25 through a Stat5-dependent positive feedback loop. Such a mechanism increases *IL-2* binding and hence signaling by activated T cells through enhanced capture of *IL-2* by CD25 (Kim et al., 2006).

3. AIMS OF THE STUDY

This study aims to investigate the mechanism behind the $1\alpha,25(OH)_2D_3$ dependent down-regulation of the *IL-2* gene. More specific aims are as follows:

1. To use actinomycin-D, cycloheximide and RNAi in combination with RT-PCR in order to study the effect of $1\alpha,25(OH)_2D_3$ to the expression of the *IL-2* gene.
2. To identify possible, previously unknown $1\alpha,25(OH)_2D_3$ responding regions within the distal regions of the *IL-2* gene by using *in silico* and ChIP methods.
3. To monitor the importance of these specific regions to the overall regulation of the *IL-2* gene.

4. MATERIALS AND METHODS

4.1 Cell culture

Human immortalized T lymphocytes (Jurkat) were cultured in RPMI-1640 medium containing 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin in a humified 95 % air / 5 % CO_2 incubator at 37°C. In the experiments, FBS was stripped by stirring it with 5 % activated charcoal for 3 h at RT. Charcoal were removed by centrifugation and sterile filtration. Prior to total RNA, chromatin extraction and RNAi, the cells were grown overnight in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5 % charcoal-stripped FBS, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin in a humidified 95 % air / 5 % CO_2 incubator at 37°C. For expression assays the cells were treated with phytohemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA) and 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, followed by an exposure to the ligand. Next the cells were treated either with solvent (EtOH 0.1 % final concentration) or 10 nM 1α,25$(OH)_2D_3$ (diluted in ethanol).

4.2 PCR-primers

PCR-primers for ChIP analysis (Table 3) were designed with Oligo software (Molecular Biology Insights, Inc., Cascade, USA) and ordered from Oligomer (Oligomer, Helsinki, Finland). The PCR conditions were optimized with My-IQ-cycler (BioRad, California, USA). The PCR cycling conditions used were 5 min at 95°C, 45 cycles for 30 s at 95°C, for 30 s at 50-60°C and for 30 s at 72°C.

Table 3: IL-2 PCR-primers used in ChIP scanning

Sequence	Name	Location from TSS	Size of amplicon (bp)
5'-TTCTACCACCCCCTAAAT-3'	IL-2 prom1	1898	
5'-TGCCAGGGTGAATCCAA-3'	IL-2 prom2	1506	391
5'-TTGGATTCACCCTGGCA-3'	IL-2 prom3	1523	
5'-GAGGCAGCATAACACTAA-3'	IL-2 prom4	999	523
5'-TTAGTGTTATGCTGCCTC-3'	IL-2 prom5	1017	
5'-GATGGGACTAATAGCAGC-3'	IL-2 prom6	662	354
5'-GCTGCTATTAGTCCCATC-3'	IL-2 prom7	680	
5'-TCAACTCCTGCCACAATG-3'	IL-2 prom8	272	408
5'-CATTGTGGCAGGAGTTGA-3'	IL-2 prom9	290	
5'-TCTTGCTCTTGTCCACCA-3'	IL-2 prom10	-138	428
5'-TGGTGGACAAGAGCAAGA-3'	IL-2 prom11	-574	
5'-CCAGGTGATTTAGAGGAT-3'	IL-2 prom12	-120	450
5'-ATCCTCTAAATCACCTGG-3'	IL-2 prom13	-957	
5'-TAGACTAAGTGCCTGCCT-3'	IL-2 prom14	-552	405
5'-AGGCAGGCACTTAGTCTA-3'	IL-2 prom15	-1285	
5'-CACCCTCCTCAAAATCCA-3'	IL-2 prom16	-939	346
5'-TGGATTTTGAGGAGGGTG-3'	IL-2 prom17	-1676	
5'-GCCTGAGTGATGATGCTG-3'	IL-2 prom18	-1267	409
5'-CAGCATCATCACTCAGGC-3'	IL-2 prom19	-2058	
5'-CAGGGGGTATGACACAC-3'	IL-2 prom20	-1658	400
5'-GTGTGTCATACCCCCTG-3'	IL-2 prom21	-2452	
5'-TCTCTGGATGGTGTGGAA-3'	IL-2 prom22	-2041	411
5'-TTCCACACCATCCAGAGA-3'	IL-2 prom23	-2899	
5'-ATGTAGCCTAATGGGTCC-3'	IL-2 prom24	-2434	465
5'-GGACCCATTAGGCTACAT-3'	IL-2 prom25	-3622	
5'-GAAGTCAGTATGGCGATT-3'	IL-2 prom26	-2899	740
5'- AATCGCCATACTGACTTC-3'	IL-2 prom27	-4035	
5'-AATCTGACAAAAGGGCTA-3'	IL-2 prom28	-3604	430
5'- TAGCCCTTTTGTCAGATT-3'	IL-2 prom29	-4461	
5'-GGAACAAAACAGTGCCC-3'	IL-2 prom30	-4017	443
5'-GGGCACTGTTTTGTTCC-3'	IL-2 prom31	-4846	
5'-GAATCCAACTCACAAGGG-3'	IL-2 prom32	-4444	402

5’-CCCTTGTGAGTTGGATTC-3’	IL-2 prom33	-5251	
5’-GACCAACCCACAGCCAA-3’	IL-2 prom34	-4828	423
5’-TTGGCTGTGGGTTGGTC-3’	IL-2 prom35	-5682	
5’-CTGGTACGATTCCTTCTG-3’	IL-2 prom36	-5234	458
5’-CAGAAGGAATCGTACCAG-3’	IL-2 prom37	-6520	
5’-AAAGCACTCCTCAGCAAA-3’	IL-2 prom38	-5674	846
5’-TTTGCTGAGGAGTGCTTT-3’	IL-2 prom39	-7075	
5’-AGACTGGCAAACTGGATA-3’	IL-2 prom40	-6502	563
5’-TATCCAGTTTGCCAGTCT-3’	IL-2 prom41	-4792	
5’-AGCCCATCAGATTAACAG-3’	IL-2 prom42	-7057	435
5’-CTGTTAATCTGATGGGCT-3’	IL-2 prom43	-8046	
5’-GAACCCACGGCAAAGAA-3’	IL-2 prom44	-7475	571
5’-TTCTTTGCCGTGGGTTC-3’	IL-2 prom45	-8493	
5’-ATGGCTGGGTACTCCTC-3’	IL-2 prom46	-8029	464
5’-GAGGAGTACCCAGCCAT-3’	IL-2 prom47	-8935	
5’-GCACACCACCAAGAGATT-3’	IL-2 prom48	-8476	459
5’-AATCTCTTGGTGGTGTGC-3’	IL-2 prom49	-9380	
5’-CATCAAGTGTGCTGGTGT-3’	IL-2 prom50	-8917	463
5’-ACACCAGCACACTTGATG-3’	IL-2 prom51	-9988	
5’-AAAGCCACTACAGGAGAA-3’	IL-2 prom52	-9362	626

4.3 Total RNA extraction and cDNA synthesis

Total RNA was extracted using mini RNA isolation II kit (Zymo Research, Hiss Diagnostics, Freiburg, Germany) according to the instructions of the manufacturer. Before collection, the cells were treated with phytohemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, followed by exposure to the ligand. Next, the cells were treated either with solvent (EtOH 0.1 % final concentration), 10 nM 1α,25$(OH)_2D_3$ (diluted in ethanol) or with the actinomycin D (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 3, 6 and 24 h. To ensure that IL-2 is a primary 1α,25$(OH)_2D_3$ target gene the cells were treated simultaneously with 10 mM cycloheximide (CHX) (Sigma-Aldrich, St. Louis, MO,

USA) and 10 nM 1α,25$(OH)_2D_3$. Purity and concentration of RNA were measured by a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA).

Complementary DNA synthesis was performed with Roche Transcriptor First strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions of the manufacturer using 1 µg of total RNA.

4.4 IL-2 expression assays

Real time PCR was performed using a Roche Lightcycler 480 (Roche Diagnostics GmbH, Mannheim, Germany), TaqMan® probes (Applied Biosystems Inc, USA) and TaqMan® Gene Expression Master Mix (Applied biosystems Inc., USA). Each reaction was performed using specific assay (for IL-2: #4331182; for ribosomal protein, large, P0 (RPLP0): #4333761F), cDNA template and Master mix. PCR cycling conditions were: pre-incubation for 10 min at 95°C, 45 cycles of 30 s at 95°C and 1 min at 60°C.

Fold inductions were calculated using the formula $2^{(\Delta\Delta Ct)}$, where ΔΔCt is the ΔCt (stimulus) - ΔCt(solvent), and ΔCt is Ct(IL-2) - Ct(RPLP0). Ct is the cycle were the signal crosses the threshold value and RPLP0 is a housekeeping gene.

4.5 In silico screening for putative VDREs

The *in silico* screening of the VDR binding sites to the 5'-flanking sequence of the IL-2 gene was done by using the RESearch program. Screening was based on the hexameric core sequence RGKTCA (R = A or G, K = G or T, Y = T or C, M = A or C, N = A, G, C or T) for VDREs (Fig. 5) and CANNTG for E-box elements (Kim et al., 2007). Only DR3, DR4, ER6, ER7, ER8 and ER9 were considered for VDREs.

4.6 Chromatin immunoprecipitation (ChIP assays)

ChIP assays were performed as previously described (Väisänen et al., 2005). The antibodies against VDR (sc-1008), NCoR (sc-8994) and pPol II (sc-13583) were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). The unspecific IgG was from Upstate Biotechnology (Upstate Biotechnology Inc, Lake Placid, NY, USA). The DNA yield and purity were determined by NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The ChIP templates were analyzed by semi-quantitative real-time PCR. For each of the 26 regions of the human *IL-2* promoter, primer pairs were designed (Table 3), optimized and controlled by running PCR with 25 ng genomic DNA (input) as a template. When running immunoprecipitated DNA (output) as a template, the following PCR profile was used: pre-incubation for 5 min at 95°C, 38 cycles of 30 s at 95°C, 30 s at a primer-specific annealing temperature and 30 s at 72°C, and one final incubation for 10 min at 72°C. The PCR products were separated by electrophoresis through 2 % agarose gels. Gel images were scanned on a FLA-3000 reader (Fuji, Tokyo, Japan) and analyzed using Image Gauge software (Fuji, Tokyo, Japan).

4.7 RNA interference

Before siRNA transfections the Jurkat cells were split to 500 000 cells/ml/well in 6-well plates. The cells were transfected with Stealth™ siRNAs targeting the VDR mRNA (Invitrogen, Carlsbad, California, USA) or non-specific siRNA oligomers (Invitrogen, Carlsbad, California, USA) with Interferin reagent (Poly-Plus-transfection, Illkirch, France) according to the instructions of the manufacturer. The transfection reagent and oligos were diluted within GIBCO™ Opti-MEM (Invitrogen, Carlsbad, CA, USA) and pipetted onto the cells. Total protein extraction for Western blot was performed 72 h after the onset of the transfection.

4.8 Western Blot

The cells were collected and suspended to a lysis buffer. Total protein concentration was measured with Victor^3 (Perkin Elmer, Massachusetts, USA) at wave length 595 nm using Bio-Rad protein assay (BioRad, California, USA). Bovine serum albumin (BSA) (2 mg/ml) was used as a standard.

Silencing of VDR at the protein level was verified using 25 µg of whole cell extract from Jurkat cells and anti VDR antibody (sc-1008, Santa Cruz Biotechnologies, Heidelberg, Germany). Anti β-actin antibody (Sigma Aldrich, St. Louis, MO, USA) was used to control for equal protein loading. Cellular proteins were separated using 9% SDS polyacrylamide gel electrophoresis. The blotted proteins were blocked and incubated with antibodies by using SNAP i.d. Protein Detection System (Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions. DyLight™ 800 conjugated goat anti-rabbit IgG (Thermo Fisher Scientific Inc, Rockford, IL, USA) was used for detection. Detected proteins were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Nebraska, USA).

5. RESULTS

5.1 Cell line selection

At the beginning of the study, several cell lines tested for *IL-2* expression. Reverse transcriptase PCR was performed to measure basal expression of *IL-2*. Best cell line was Jurkat (Fig. 9). Primers used for PCR were 5'-CAAGAATCCCAAACTCACCAG-3' and 5'-GTTTCAGATCCCTTTAGTTCCA-3' for *IL-2* and 5'-GTGGTGATACCTAAAGCCTG-3' and 5'-AGATGCAGCAGATCCGCA-3' for control 36B4. The PCR cycling conditions used were 5 min at 95 ºC, 45 cycles for 30 s at 95 ºC, for 30 s at 60 ºC and for 30 s at 72 ºC.

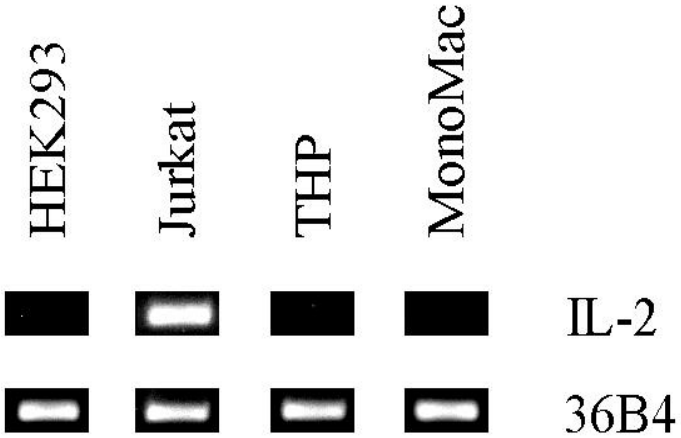

Figure 9. IL-2 basal expression in various cell lines.

5.2 RT-PCR

The expression levels of *IL-2* gene and its response to 1α,25$(OH)_2D_3$ in Jurkat cells were monitored by real-time quantative PCR in relation to the control gene RPLP0 (Fig. 10). The expression of *IL-2* gene decreased significantly after 3 and 6 h treatment and returned back to the basal level after 24 h treatment. The lowest fold change (0.5-fold) could be observed 6 h after onset of 1α,25$(OH)_2D_3$ treatment.

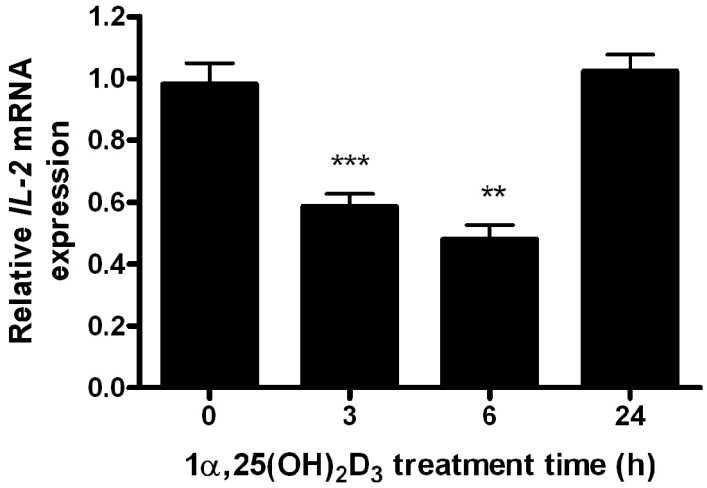

Figure 10. The effect of 1α,25$(OH)_2D_3$ to the expression of the *IL-2* gene in Jurkat cells. RT-PCR was used to determine the ratio of the 1α,25$(OH)_2D_3$ induced mRNA expression of the *IL-2* gene relative to the control gene RPLP0. Fold changes relative to basal level (0 h) were determined after 3, 6 and 24 h treatments with 1α,25$(OH)_2D_3$. Statistical significance was calculated with the 2-tailed unpaired Student's t-test (*$p<0.05$, **$p<0.01$) using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

5.3 RNAi

The effect of silencing of the *VDR* mRNA to the *IL-2* gene expression was studied using the siRNA technique. The cells were transfected with unspecific control siRNA oligomers or with specific siRNAs against the VDR mRNA. The siRNA treatment time was 72 h. After siRNA treatment, the cells were further treated with 10 nM 1α,25$(OH)_2D_3$ for 6 h. Western blot confirmed that the VDR protein was down-regulated by VDR siRNA treatment (Fig. 11A). Quantitative real-time PCR analysis showed that siVDR treatment increased *IL-2* mRNA expression in Jurkat cells. When unspecific siRNA was used, 1α,25$(OH)_2D_3$ was able to down-regulate IL-2 mRNA expression 0.5-fold (Fig. 11B).

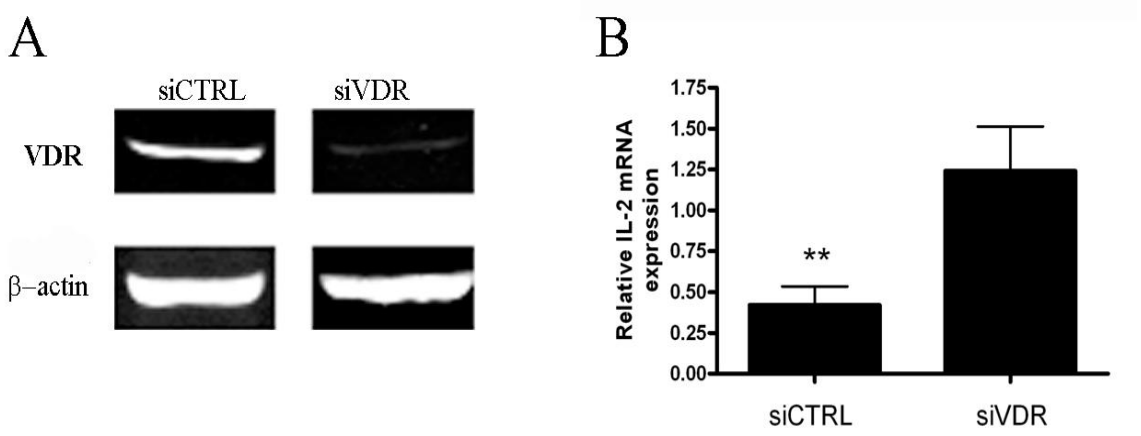

Figure 11. The effect of silencing of the VDR to the ligand dependent IL-2 expression. RT-qPCR and western blot were used to determine effect of VDR-specific siRNA on the mRNA expression of *IL-2* gene in Jurkat cells. (A) Silencing of VDR at protein level. Representative blots are shown. (B) Relative IL-2 mRNA expression after silencing of the VDR. Columns represent the means of at least three independent treatments and the bars represent standard deviations. Statistical significance was calculated with the 2-tailed unpaired Student's t-test (*$p<0.05$, **$p<0.01$) using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

5.4 Effect of actinomycin-D and cycloheximide to the IL-2 mRNA expression

The stability of IL-2 mRNA was determined with actinomycin-D treatment. The expression of *IL-2* mRNA decreased significantly after 1 h treatment with actinomycin-D (Fig. 12A). The primary 1α,25(OH)$_2$D$_3$ target gene *IL-2* mRNA levels decreased significantly after 3 h of 10 mM CHX and 1α,25(OH)$_2$D$_3$ treatment. The lowest fold change (0.6-fold) was observed 3 h after CHX and 1α,25(OH)$_2$D$_3$ treatment (Fig. 12B).

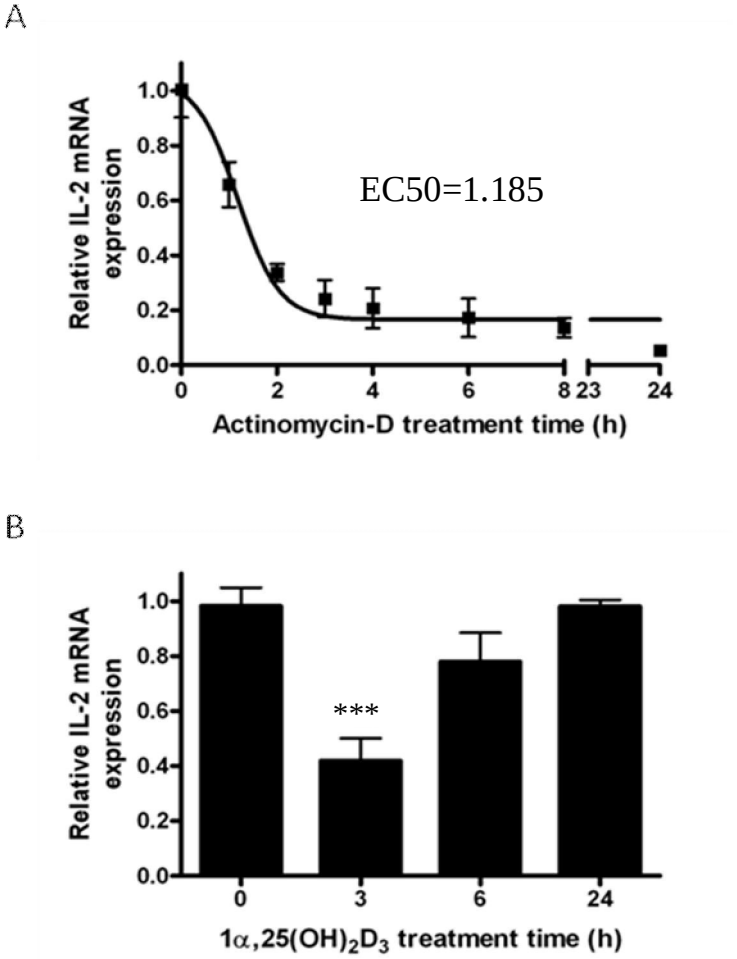

Figure 12. Expression profiles of the human *IL-2* gene. (A) PHA and TPA induced Jurkat cells were treated with 20 nM actinomycin-D for indicated times. EC50=1.185 (B) The *IL-2* mRNA expression after 3 h, 6 h and 24 h treatments with 10 nM 1α,25(OH)$_2$D$_3$ in the presence of 10 mM CHX. Columns represent the means of at least three independent treatments and the bars represent standard deviations. Statistical significance was calculated with the 2-tailed unpaired Student's t-test (*p<0.05, **p<0.01, ***p<0.001) using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

5.5 *In silico* and ChIP scanning results for putative VDREs

In silico screening of the *IL-2* gene revealed four putative VDREs when the consensus hexameric sequence RGKTCA was used and four possible E-box elements when the consensus sequence CANNTG was used (Fig. 13A). Three VDREs located upstream and one downstream of the *IL-2* TSS. All E-box elements located upstream of the *IL-2* TSS. All of the putative VDREs contain one motif with one nucleotide mismatch compared to VDRE consensus sequence RGKTCA. Two of the REs were DR4-types, one DR3-type and one ER9-type (Fig. 13 A).

In order to find out whether VDR is associated with the found putative VDREs, ChIP assays were performed using chromatin that was extracted from PHA and TPA treated Jurkat cells which were stimulated for 60, 120 and 180 min with $1\alpha,25(OH)_2D_3$ (Fig 13B). Results suggest that the VDR associates with regions 23 (-8493 to -8029), 21 (-7492 to -7057), 20 (-7075 to -6502), 17 (-5251 to -4828), 14 (-4035 to -3604), 9 (-1676 to -1267), 8 (-1285 to -939) and 1 (1506 to 1898) (Fig. 13B). Strongest binding of VDR was observed after 60 min, 120 min and 180 min treatments.

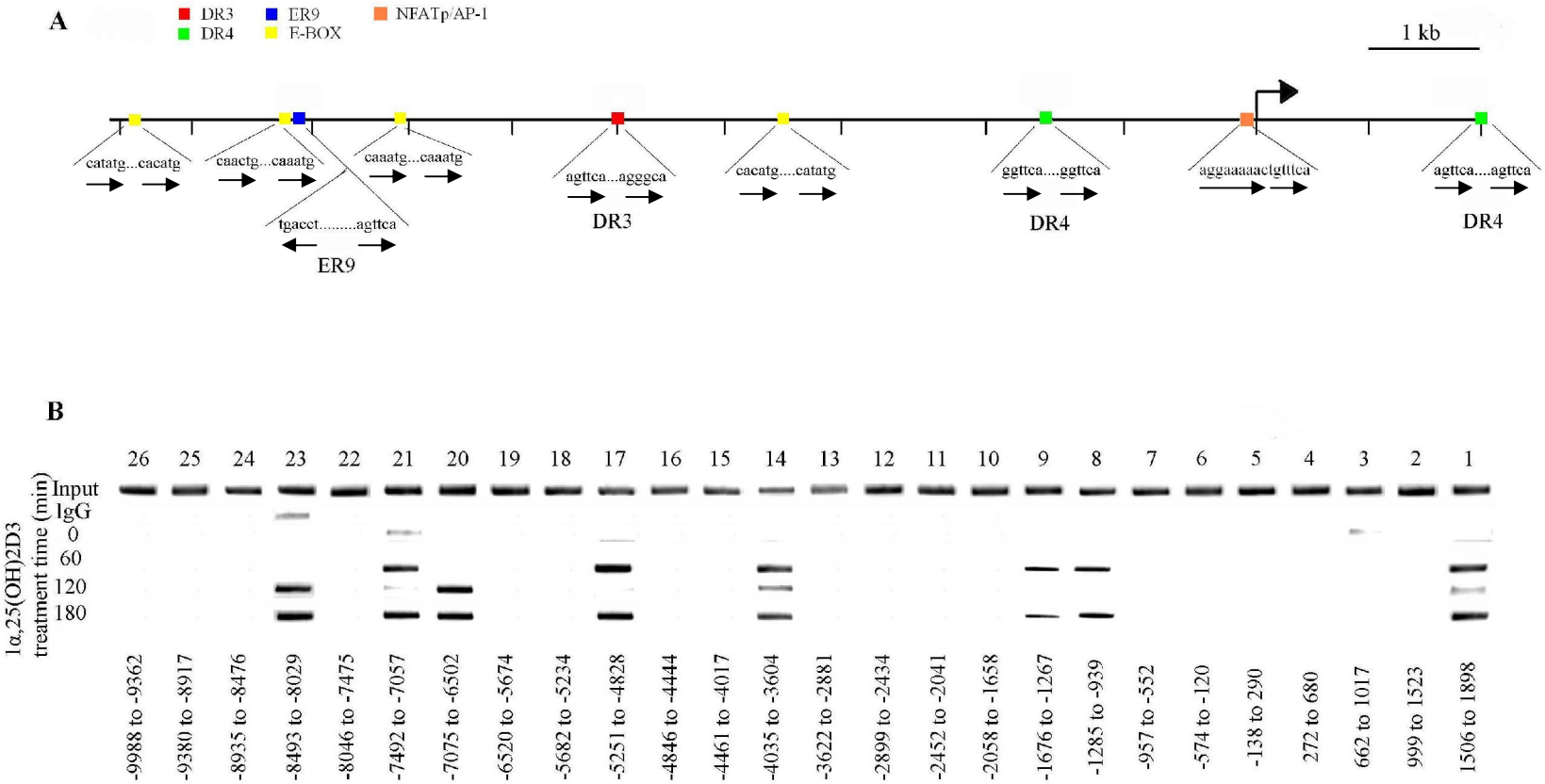

Figure 13. Putative VDREs and ligand dependent recruitment of VDR within the *IL-2* gene. (A) Four putative VDREs were revealed by *in silico* screening. Three of the VDRE candidates are located upstream and one downstream, and four E-box elements are located upstream of the *IL-2* TSS. (B) Chromatin was extracted from Jurkat cells, which were treated with 10 nM 1α,25-dihydroxyvitamin D_3 for indicated times. ChIP assays were performed using anti-VDR antibody or nonspecific IgG.

5.6 ChIP results for selected VDREs

According to the ChIP scanning results, regions 23, 21, 20, 17, 14, 9, 8 and 1 (Fig. 13B) recruited VDR. These were thus studied further to clarify if NCoR and pPol II were also present. The results suggest that NCoR associates with regions 23, 21, 20, 17, 14 and 8 after 30 min and disappears after 120 min of treatment (Fig. 14). pPol II associates with regions 21, 14 and 8 at 60 min treatment. The strongest recruitment of NCoR was observed with 30 min and 120 min ligand treatments while the strongest recruitment of pPol II was observed without ligand (Fig 14).

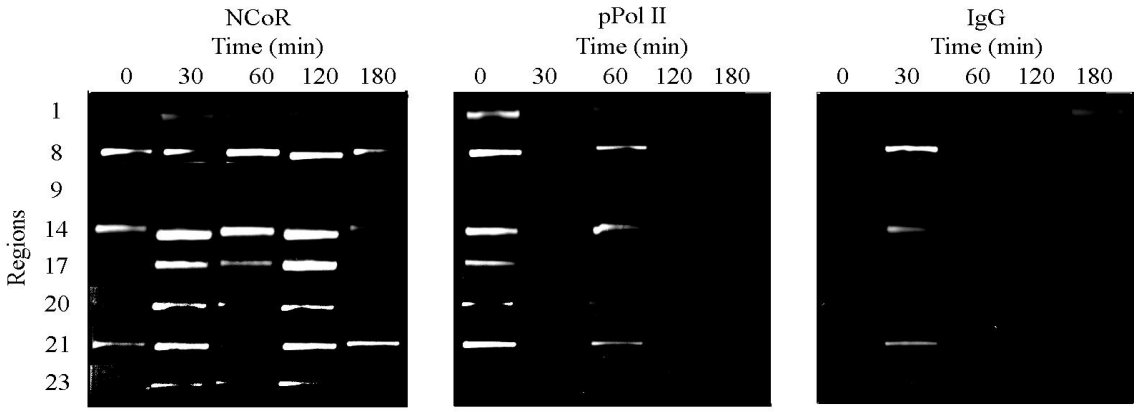

Figure 14. Association of VDR containing regions with NCoR and pPol II. Chromatin was extracted from Jurkat cells which were treated with 10 nM $1\alpha,25(OH)_2D_3$ for indicated times. ChIP assays were performed using anti-NCoR or anti-pPol II antibodies.

6. DISCUSSION

This study confirms previous findings that the expression of the *IL-2* gene is down-regulated by the nuclear hormone $1\alpha,25(OH)_2D_3$ in Jurkat cells (Lam et al., 1974; Tsoukas et al., 1984; Alroy et al., 1995). Different repression mechanisms have been proposed for steroid/nuclear receptor family members. In some cases, DNA binding is required (Diamond et al., 1990), while in other examples protein-protein interactions appear to be sufficient in conferring repression (Kerppola et al., 1993, Jonat et al., 1990). Therefore, it was interesting to try to clarify the repression mechanism of *IL-2* by VDR.

In this study, $1\alpha,25(OH)_2D_3$-dependent responses of *IL-2* were studied in Jurkat cells. The observed 0.6-fold repression by $1\alpha,25(OH)_2D_3$ is not a very strong downregulation. We tested the basal expression levels of *IL-2* also in other cell lines (MonoMac, THP and HEK293), but detected hardly any (Fig. 9). In addition, the Jurkat cells had to be activated by PHA and TPA before *IL-2* expression could be observed. Our data confirms the previous findings of Alroy and co-workers that Jurkat is the only cell line which expresses *IL-2* gene in high levels (Alroy et al., 1995).

When the VDR was silenced in Jurkat cells by using siRNA, the $1\alpha,25(OH)_2D_3$ treatment had no effect on the IL-2 expression. When unspecific siRNA was used, $1\alpha,25(OH)_2D_3$ was able to down-regulate *IL-2* mRNA expression 0.5-fold (Fig. 11). This confirms that *IL-2* gene is regulated by $1\alpha,25(OH)_2D_3$.

Actinomycin D is a cyclic polypeptide-containing antibiotic that inhibits RNA synthesis. It binds to β-DNA found within the boundaries where double-stranded DNA connects with single-stranded DNA in the transcriptional complex. This immobilizes the complex, interfering with the elongation of growing RNA chains. Nucleolar (ribosomal) RNA synthesis is particularly sensitive to the presence of actinomycin, and this probably accounts for its pharmacological activity as well as its extreme toxicity to mammalian cells (Sobell, 1985). In this study actinomycin D was used to determine the stability of IL-2

mRNA. The expression of *IL-2* mRNA decreased significantly after 1 h treatment with actinomycin-D and *IL-2* mRNA half-life was determined to be 1.185 hours (Fig. 12A).

Cycloheximide is an inhibitor of protein biosynthesis. Cycloheximide exerts its effect by interfering with the translocation step in protein synthesis and blocking translational elongation. It works rapidly and its effects are rapidly reversed by simply removing it from the culture medium. It may be used to distinguish between genes expressed in organelles and genes expressed in the nucleus. Genes expressed in the eukaryotic nucleus will not be expressed in the presence of cycloheximide (Kay and Korner, 1966) and thus cycloheximide can be used to determine if the gene of interest is a primary target gene for a given treatment. In this study *IL-2* was confirmed to be a primary $1\alpha,25(OH)_2D_3$ target gene (Fig 13B).

In this study, we have shown that the expression of IL-2 was increased by PHA and TPA treatments and repressed by $1\alpha,25(OH)_2D_3$ treatment. Our data is in agreement with previously published data (Alroy et al., 1995). *IL-2* repression induced by $1\alpha,25(OH)_2D_3$ seems to be transient, because 24 h after the onset of $1\alpha,25(OH)_2D_3$ treatment the mRNA levels of *IL-2* returned to basal level (Fig 10).

In silico scanning revealed four putative VDREs and four candidate E-box binding sites in our standard 10 000 bp upstream and 2 000 bp downstream promoter search. Three VDREs and four E-box binding elements located upstream from TSS and one downstream from TSS. We also performed ChIP scanning for the whole region and found three more possible VDR binding sites. According to Carlberg and co-workers (2007) the expression of $1\alpha,25(OH)_2D_3$-induced genes relies on multiple REs. Therefore it is possible that the REs can work together. However this possibility cannot be ruled out until ChIP experiments are performed. Finally it is possible that in isolation the regions containing the REs are suppressive in isolation, and rely on other elements to drive positive transcriptional response. It was shown by Murayama et al. (2004) that the bHLH-type transcriptional activator (VDIR) binds the suppressing element in the *CYP27B1* gene. In the presence of ligand VDR binds to VDIR and causes the recruitment of CoRs. Subsequently it has been

shown by Turunen and colleagues (2007) that additional classical VDREs participate in this process as well. These elements contain traditional structures that bind VDR-RXR heterodimers. Therefore in their model these additional VDREs help in the execution of the response and there is a master regulator that does not necessary require direct DNA binding of VDR.

A more detailed ChIP analysis of the seven pre-scanned VDREs showed the strongest recruitment of VDR at regions 23, 21, 20, 17, 9 and 8 at 60 min after the onset of 1α,25$(OH)_2D_3$ treatment, and 21 and 20 at 120 min after the onset of 1α,25$(OH)_2D_3$ treatment. It is possible that there are some binding sites that are not found. There are some E-box binding sites or other binding sites which associate with VDR (Kim et al., 2007). It is also possible that *IL-2* gene is regulated via multiple VDREs (Saramäki et al., 2006, Kim et al., 2007).

The ChIP data is in agreement with the mRNA expression profile of the *IL-2* where significant fold change can be seen after 3 h treatment. We also used antibodies against other transcription factors, such as NCoR and pPol II, to identify true transcriptional activity. Rising intensity of the binding of NCoR indicates that the *IL-2* gene was repressed after 1α,25$(OH)_2D_3$ treatment. The disappearance of pPol II binding, thus indicating reduced polymerase activity, corroborates the repression of *IL-2* gene upon 1α,25$(OH)_2D_3$ treatment.

In conclusion, this study demonstrates that the *IL-2* is a primary 1α,25$(OH)_2D_3$ target gene, containing seven possible VDR associated regions. Four of these regions contained classical VDREs and the other three E-box elements. Real time PCR demonstrated that *IL-2* is negatively regulated by 1α,25$(OH)_2D$,. This was confirmed by ChIP which showed arrested transcriptional activation of *IL-2* upon 1α,25$(OH)_2D_3$ treatment. siRNA results confirmed that *IL-2* is indeed regulated via VDR. This study provided insight into the mechanism of transcriptional downregulation by 1α,25$(OH)_2D_3$, which is at the moment poorly understood, although according to recent micro array data approximately 50 % of all 1α,25$(OH)_2D_3$ responding genes are downregulated this VDR ligand (Swami et al., 2003).

Further studies are thus needed to understand the mechanisms of how VDR-RXR regulates *IL-2*. For example, a 3C analysis would be required to see whether these relatively distal elements loop to the TSSs and what are the kinetics of this possible looping. In the future, discovery of TF binding sites over the whole genome *in vivo* by ChIP-seq analysis would help in the identification of response element networks that regulate genes upon a particular stimulus. This would narrow down the number of sequences in which to search for REs by computer methods and thus improve this method considerably. There are lots to be done before we can fully understand the whole mechanism behind the transcriptional regulation of *IL-2*.

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