Novel Mechanisms in the Regulation of 5-Lipoxygenase Gene Expression by 1,25-Dihydroxyvitamin D_3 and Transforming Growth Factor b

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Abbreviations

$1,25(OH)_2D_3$ 1,25-dihydroxyvitamin D_3 (calcitriol/1,25-dihydroxycholecalciferol)

AA arachidonic acid
aa amino acid
AC 5-azacytidine
ACTR activator of thyroid and retinoic acid receptor
AdC 5-aza-2`-deoxycytidine
AF-2 activation function-2
AKBA 3-acetyl-11-keto boswellic acid
AMH anti-Müllerian hormone
AMP adenosine monophosphate
ANF atrial natriuretic factor
ANT2 adenine nucleotide translocase isoform 2
AP-1/2 activating protein-1/2
AR androgen receptor
ARC activator-recruited co-factor
ASA acetysalicylic acid
ATP adenosine triphosphate
ATRX X-linked, α-thalassemia mental retardation syndrome

BAMBI BMP and activin membrane-bound inhibitor
BG betaglycan
BLT leukotriene B_4 receptor
BMP bone morphogenetic protein
bp base pair(s)
BRG1 brahma related product 1

CAMK Ca^{2+}-calmodulin-dependent kinase
cAMP cyclo AMP
CBP CREB binding protein
CBF-1 Notch-responsive transcription factor

CBFA	core-binding factor A
Cdk	cyclin dependent kinase
cDNA	copy DNA
CHD	chromo-helicase and ATPase-DNA-binding
CHX	cycloheximide
Cip1	cyclin-inhibiting protein
CLP	coactosin-like protein
CMV	cytomegalie virus
CoAA	coactivator activator
Co-Smad	cooperating/common-partner/collaborating/co-mediator Smad
COX	cyclooxygenase
cPLA$_2$	cytosolic phospholipase A_2
CREB	cAMP regulatory element-binding protein/cAMP-regulated enhancer binding protein
CTD	carboxy-terminal domain
CTF	CAT-box binding transcription factor
CYP24	cytochrome P450, subfamily XXIV (vitamin D 24-hydroxylase)
dbcAMP	dibutyryl-cyclo-AMP
DBD	DNA-binding domain
DMAPI	DNMT1-associated protein
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNMT	DNA methyltransferase
DR	direct repeat
DRIP	vitamin D receptor interacting proteins
DTT	dithiothreitol
EGFR	epidermal growth factor receptor
Egr-1	early-growth response factor-1
eIF4E	eukaryotic translation initiation factor 4E

EMSA	electrophoretic mobility shift assays
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
4-ET	eukaryotic translation initiation factor 4E nuclear import factor 1
FCS	fetal calf serum
FKBP12	FK506-binding protein 12
FLAP	5-lipoxygenase activating protein
fig.	figure
Gα	α subunit of guanine nucleotide-binding regulatory (G-) proteins
GGLT	γ-glutamyl leukotrienase
GGT	γ-glutamyl transpeptidase
GM-CSF	granulocyte macrophage colony stimulating factor
GPx	glutathione peroxidase
GR	glucocorticoid receptor
Grb-2	growth factor receptor-bound protein 2
GRIP-1	glucocorticoid receptor-interacting protein-1
GSH	glutathione
GST	glutathione S-transferase
H	histone
HAT	histone acetyl transferase
HC toxin	Helminthsporium carbonium toxin
HDAC	histone deacetylase
HDACi	HDAC inhibitors
H(p)ETE	hydro(pero)xyeicosatetraenoic acid
HeLa cells	epithelial cells derived from a cervix carcinoma
His	histidine
HL-60 cells	human promyeloic leukemic cell line
HL-60TB	HL-60 cells negative for 5-LO
hbrm	human brahma
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A

hnRNP	heterogeneous nuclear RNA particle
HpODE	hydroperoxyoctadecaenoic acid
HUGO	human genome project
ICF	immunodeficiency, centromere instability, facial anomalies
IL	interleukin
Ile	isoleucine
IP	inverted palindrome
ISWI	imitation switch
JNK	c-jun NH_2-terminal kinase
kb	kilobase
kDa	kilo Dalton
KO	knock out
L	leucine
LAP	latency-associated protein
LBD	ligand binding domain
LO	lipoxygenase
LT(R)	leukotriene (receptor)
LX	lipoxin
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MAPKAPK	mitogen-activated protein kinase-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MBD(P)	methylcytosine binding domain (protein)
MeCP	methyl CpG binding protein
MKK	MAPK kinase
MM6 cells	Mono Mac 6 cells
mPit-1	mouse phosphate intestinal transporter
MR	mineralocorticoid receptor
mRNA	messenger RNA

MRP1 multidrug resistance-associated protein 1

NAT negative regulator of activated transcription
NCoA62 nuclear coactivator-62 kDa
NCoR nuclear corepressor
NF-1 nuclear factor 1
NFkB nuclear factor of κ-light polypeptide gene enhancer in B cells
NLS nuclear localization sequence
NO nitric oxide
NR nuclear receptor
NuRD nucleosome remodeling histone deacetylase complex

oxoETE oxoeicosatetraenoic acid

PAF platelet-activating factor
PAI plasminogen activator inhibitor
PBS phosphate buffered saline
PCAF p300/CBP-associated factor
PCIP p300/CBP integrator protein
PCNA proliferating cell nuclear antigen
PCR polymerase chain reaction
PG prostaglandin
PGC-1 peroxisome proliferator activated receptor (PPAR) gamma coactivator-1
PI 3-K phosphatidylinositol 3-kinase
PK protein kinase
PL phospholipase
PMA phorbol-12-myristate-13-acetate
PML promyelocytic leukemia
PMNL polymorphonuclear leukocyte
PR progesterone receptor
pRb retinoblastoma protein
pre-mRNA precursor-mRNA
Prp8 pre-mRNA processing factor 8
PTH parathyroid hormone

PTHrP	PTH-related peptide
RA	retinoic acid
RAC	receptor associated coactivator
rANF	rat atrial natriuretic factor
RAR	retinoic acid receptor
RBCE	retinoblastoma control element
RE	response element
RLU	relative light unit
RNA	ribonucleic acid
ROR	retinoid orphan receptor
ROS	reactive oxygen species
R-Smad	receptor-regulated Smad transcription factor
RT-PCR	reverse transcription-polymerase chain reaction
RXRa	retinoid X receptor alpha
RZR	retinoid Z receptor
S	serine
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAP	spliceosome-associated protein
SE	standard error
SBE	Smad binding element
SEAP	secreted alkaline phosphatase
Ser	serine
SF3b2	splicing factor 3b subunit 2
SNF	sucrose non-fermenter
SH3	src-homology
Sin3	SWI independent
SIP	Smad interacting protein
siRNA	small interfering RNA
Ski	Sloan-Kettering Institute oncoprotein
SKIP	Ski-interacting protein
Smad	Sma and Mad related protein

SMC	smooth muscle cell
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SMURF	Smad ubiquitylation regulatory factor
snRNP	small nuclear ribonucleoprotein
Sp1	serum protein1/specific protein1/selective promoter factor1
sPLA$_2$	secretory phospholipase A_2
SRC-1	steroid receptor coactivator-1
STAGA	SPT3-TAF$_{II}$31-GCN5L acetylase
STAT	signal transducers and activators of transcription
SV-40	simian virus-40
SWI	mating type switch
Sxl	Sex lethal
TbRI/II	TGFβ receptor I/II
tab.	table
TAF$_{II}$250	TBP-associated factor II
TAK	TGFβ-activated kinase
TBP	TATA-binding protein
TFIIB	transcription factor IIB
TGFb	transforming growth factor beta
TIF	transcriptional intermediary factor
TNFa	tumor necrosis factor alpha
T$_{(3)}$R	thyroid receptor
tra	transformer
TRAP-1	TGFβ receptor-I-associated protein 1
TRAP	thyroid hormone receptor-associated protein
TbRE	TGFβ responsive element
TsA	Trichostatin A
U2AF	U2 auxiliary factor
U937 cells	human lymphoma cell line
UTR	untranslated region

VD3	1,25-dihydroxyvitamin D_3 (calcitriol/1,25-dihydroxycholecalciferol)
VDR	vitamin D receptor
VDRE	vitamin D response element
Waf	wildtype-p53-activated factor
w/o	without
YY1	Yin-Yan-1

1 Introduction

1.1 Lipoxygenases

Lipoxygenases (LOs) are oxidoreductases which catalyse the conversion of polyunsaturated fatty acids to hydroperoxy fatty acids. In mammals, the main substrate of LOs is arachidonic acid (20:4) (AA), in plants linoleic (18:2) and linoleinic (18:3) acid. To date four human LOs are described, the 5-, 8-, 12-, and 15-LO. The denotation indicates the carbon atom of arachidonic acid where molecular oxygen is inserted. Thus, 5-LO synthesizes 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HpETE) whereas 15-LO produces 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HpETE). Some lipoxygenases are known to convert different substrates or to oxydate at varying positions. Their main substrates determine the terms they are given (Brash, 1999; Ford-Hutchinson et al., 1994).

Lipoxydase, a 15-LO, was the first LO to be characerized and was isolated from soybean (Theorell, 1947). The first mammalian LO was identified in 1974 in human platelets (Hamberg, 1974). Shortly afterwards the first mammalian 5-LO was discovered in rabbit leukocytes (Borgeat et al., 1976). LOs are widespread in animals, including some lower marine organisms, plants and fungi, but are not expressed in bacteria and yeast (Brash, 1999).

1.2 5-LO protein structure

The molecular mass of the monomeric LOs in mammals and plants was determined as 75-80 kDa and 94-104 kDa, respectively. 5-LO copy desoxyribonucleic acids (cDNAs) have been cloned from human, mouse, rat and hamster and the human cDNA codes for a protein with 673 amino acids (aa) and a molecular weight of 77,839 kD (Matsumoto et al., 1988). Since no crystal structure for 5-LO is available so far, a model of 5-LO based on experimental data and the structure of reticulocyte 15-LO has been proposed (Hemak, 2002). 5-LO like other LOs consists of a C-terminal catalytic domain (aa 115-673) and an N-terminal β-barrel domain (aa 1-114) (Hammarberg et al., 2000; Hemak, 2002). Ca^{2+}-binding to the 5-LO β-barrel domain mediates nuclear membrane

translocation (Chen and Funk, 2001). The human LOs (5-, 12-, 15-LO) share a 60 % sequence similarity (plant LOs: 25 %) (Ford-Hutchinson et al., 1994). All LOs contain a characteristical iron centre in which a non-heme, non-sulfur iron is liganded by conserved histidine (His) residues and a C-terminal isoleucine (Ile) (Hammarberg et al., 1995). Two or three possible adenosine triphosphate (ATP) binding sites have been proposed (Hemak, 2002; Zhang et al., 2000), that deviate from typical nucleotide binding sites, though. In 5-LO, an src homology SH3 binding motif that interacts with the src homology domain of growth factor receptor-bound protein 2 (Grb2) and cytoskeletal proteins has been identified (Lepley and Fitzpatrick, 1994). A characteristic feature of 5-LO is its temporary nuclear localization. Some putative nuclear localization signal (NLS) sequences as determinants for nuclear import have been supposed so far (Chen, 1998; Healy, 1999; Jones, 2003; Jones et al., 2002).

1.3 The 5-LO enzyme

5-LO catalyzes the two initial steps in the biosynthesis of leukotrienes, the oxygenation of arachidonic acid to 5-HpETE and the further reaction to the allylic epoxide leukotriene A_4 (5(S)-6-oxido-7,9,11-trans-14-cis-eicosatetraenoic acid, LTA_4) (Ford-Hutchinson et al., 1994; Samuelsson, 1983). LTA_4 then can be converted into either LTB_4 or the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 (fig. 2, chapter 1.6).

Isolated LOs are usually in the inactive form, in which the iron in the active site is in the ferrous state (Fe^{2+}). For catalysis, it needs to be restored into the active, ferric form (Fe^{3+}) by redox reaction with fatty acid hydroperoxides (Hammarberg et al., 2001; Riendeau et al., 1989; Rouzer and Samuelsson, 1986). The exact chemical mechanism of the lipoxygenase reaction is not yet clarified but a radical mechanism is supposed (de Groot, 1975).

Several factors stimulating 5-LO enzyme activity have been identified. Ca^{2+} binding to the β-barrel C2 domain has been shown (Hammarberg et al., 2000) with a stoichiometry of binding of two Ca^{2+}/5-LO (Hammarberg and Rådmark, 1999). Ca^{2+} increases the hydrophobicity of 5-LO (Hammarberg and Rådmark, 1999) and promotes membrane association. Also, translocation to the nuclear envelope is stimulated (Rouzer and Kargman, 1988). Ca^{2+} can significantly increase 5-LO product formation *in vitro*

(Hammarberg and Rådmark, 1999). Similar to Ca^{2+}, but less efficient, Mg^{2+} binding to 5-LO and activation of the enzyme has been demonstrated (Reddy et al., 2000). Another well known factor for 5-LO catalytic activity is ATP, for which a binding site was identified in 5-LO (Noguchi et al., 1996; Rouzer and Samuelsson, 1985). This binding ability is used for 5-LO purification by affinity chromatography using an ATP-agarose column (for review, see (Werz, 2002a)). Cellular membrane fractions stabilize purified 5-LO and upregulate enzyme activity. Synthetic phosphatidylcholine vesicles alone could also stimulate 5-LO activity (Rouzer and Samuelsson, 1985). As mentioned above, activation of resting 5-LO requires oxidation of the ferrous iron in the active site to its ferric form. 5-HpETE, 12-HpETE and 13-hydroperoxyoctadecaenoic acid (13-HpODE) have been shown to act as redox partners in this reaction (Rouzer and Samuelsson, 1986). Reducing agents, such as glutathione, can inhibit 5-LO activity by acting as a substrate and blocking the oxidation of its iron. In intact cells, glutathione peroxidases (GPxs) regulate 5-LO activity by reducing lipid hydroperoxides (Hatzelmann and Ullrich, 1987; Weitzel and Wendel, 1993).

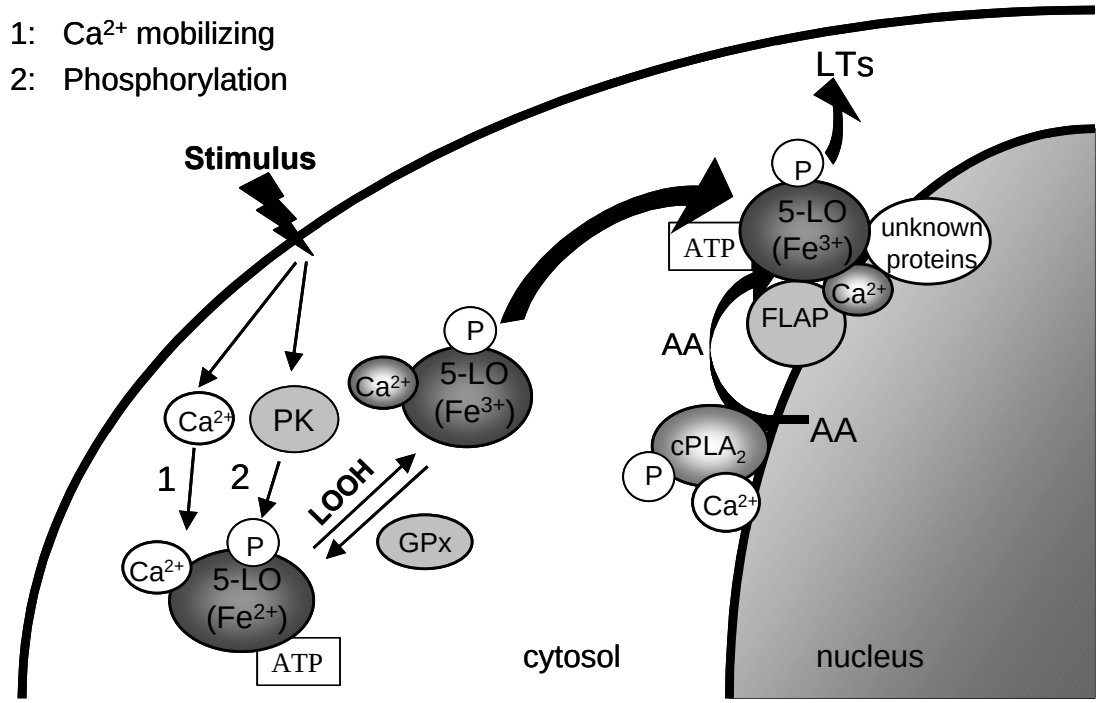


Fig. 1 . Activation of 5-LO and leukotriene synthesis in the cell

In resting cells 5-LO is localized in the cytosol or the soluble compartment of the nucleus depending on the cell type. Upon stimulation, the enzyme translocates to the

nuclear envelope, which seems to be prerequisite for LT synthesis ((Werz, 2002a) and references therein).

Protein phosphorylation is a signal transduction pathway, by which extracellular signals lead to the activation and redistribution of a variety of cellular enzymes and transcription factors. Several phosphorylation motifs have been identified in the primary sequence of human 5-LO for protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}-calmodulin-dependent kinase (CaMK II), mitogen-activated protein kinase-activated protein kinase (MAPKAPK, MK) -2 and -3, ribosomal S6 kinase, extracellular signal-regulated kinases1/2 (ERK1/2) and cyclin dependent kinase (Cdk1,Cdc2) (Lepley and Fitzpatrick, 1996). Several known stimuli of 5-LO product formation activate p38 mitogen-activated protein kinase (MAPK) (Werz, 2002b) and ERK1/2 (Werz et al., 2002). Phosphorylation of 5-LO and activation of LT formation by MK2 and ERK1/2 has been shown *in vitro* (Werz et al., 2002; Werz, 2002b).

A current model for 5-LO activation is shown in fig. 1.

1.4 Other proteins involved in leukotriene formation

1.4.1 5-Lipoxygenase-activating protein

5-Lipoxygenase-activating protein (FLAP) is an 18 kDa membrane-bound protein that is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) family (Miller et al., 1990). In intact cells, FLAP expression has been shown to be prerequisite for LT synthesis upon stimulation with ionophore A23187, wheras in cell homogenates FLAP is not required for 5-LO activity (Dixon et al., 1990). A model was proposed, suggesting a heteromerization of 5-LO with AA-binding FLAP and thereby facilitating the utilization of AA by 5-LO (Abramovitz et al., 1993; Ford-Hutchinson, 1994). The cellular distribution and regulation of 5-LO and FLAP appear to be similar (Silverman and Drazen, 1999).

Recently, a variant of the gene encoding FLAP has been associated to the pathogenesis of myocardial infarction and stroke by increasing LT production and inflammation in the arterial wall (Helgadottir et al., 2004). Thus, a role of the 5-lipoxygenase pathway in the development of cardiovascular diseases is indicated.

1.4.2 Cytosolic phospholipase A_2

A critical parameter for LT synthesis is the availability of the precursor, free AA. The key enzyme in the release of AA is the 85 kDa cytosolic Ca^{2+}-dependent phospholipase A_2 ($cPLA_2$). The phospholipase A_2 superfamily consists of a broad range of enzymes all specifically catalyzing the cleavage of the sn-2 ester bond of substrate phospholipids thus yielding free fatty acids and lysophospholipids (Qiu et al., 1998). The different PLA_2s are classified in three main types according to their molecular and biological properties: the secretory (s)PLA_2, the $cPLA_2$ and the intracellular Ca^{2+}-independent (i)PLA_2. $cPLA_2$ contains a C2 Ca^{2+}-binding domain and requires Ca^{2+} for translocation to the nuclear membrane. It can be phosphorylated at five different serine (Ser) residues (de Carvalho, 1996). Phosphorylation at Ser505 and Ser727 by ERK or p38 MAPK, depending on the cell type, is essential for full enzyme activation (Qiu et al., 1998). The activation of $cPLA_2$ bears a resemblance to the activation of 5-LO.

1.4.3 Leukotriene A_4 hydrolase and leukotriene C_4 synthase

Leukotriene A_4 hydrolase is a 69 kDa protein catalyzing the stereospecific hydrolysis of the instable epoxide LTA_4 to LTB_4 (Haeggström, 2002). LTA_4 hydrolase is expressed in almost all tissues and blood cells.

LTC_4 synthase is a glutathione-S-transferase catalyzing the conversion of LTA_4 to cysLTs. The 18 kDa membrane protein displays sequence homology to FLAP, both belonging to the MAPEG family (Jakobsson et al., 2000).

1.5 Proteins related to 5-LO

The 16 kDa coactosin-like protein (CLP) was shown to bind to F-actin filaments and to promote actin polymerization by counteracting capping of actin filaments, thus displaying profound similarity to coactosin (Rådmark, 2002). Binding of 5-LO to CLP has been shown in yeast two-hybrid-system and coimmunoprecipitation experiments. 5-LO and F-actin compete for CLP binding, and 5-LO interferes with actin polymerization. 5-LO activity is not affected by CLP binding, though (Provost et al., 2001).

The 96 kDa transforming growth factor β (TGFβ)-receptor-I-associated protein 1 (TRAP-1) is a cytosolic protein that has been shown to bind to TGFβ receptors (TβR).

Controversal studies have been published concerning the primary binding partner for TRAP-1. In one publication yeast two-hybrid assays were used to show binding of TRAP-1 to only activated TβRI (Charng et al., 1998). In another study immunoprecipitation experiments identified the inactivated TβRII as the primary binding partner for TRAP-1 (Wurthner et al., 2001). Also, TRAP-1 has been described as Sma and Mad related protein4 (Smad4) chaperone that facilitates interaction of Smad4 with Smad2/3 proteins by binding to Smad4 in the vicinity of the activated receptor and mediating its transfer to the phosphorylated Smad2/3. Beforehand, the activation of TβRII leads to dissociation of TRAP-1 from the receptor (Wurthner et al., 2001). Interaction of TRAP-1 with 5-LO has been shown in a yeast two-hybrid system (Provost et al., 1999). However, speculations, that TRAP-1 mediates the effects of TGFβ on 5-LO activity, have not been confirmed so far.

In the two-hybrid screening system with 5-LO as bait a novel human protein, containing ribonuclease III motifs, a double-stranded ribonucleic acid (RNA)-binding domain and helicase motifs, was identified. The C-terminus of this protein shows significant homology to a hypothetical helicase from *Caenorrhabditis elegans* (Provost et al., 1999). Highly homologous proteins of Drosophila (dicers) are involved in RNA interference. Thus, dicers cleave long double stranded RNA into 21-25-nucleotide sequences (small interfering (si) RNAs) which subsequently assemble with protein components into an RNA-induced silencing complex (RISC). RISC in turn binds to messenger RNA (mRNA) sequences complementary to the siRNAs and digests the bound mRNA. The consequent degradation by exonucleases results in gene silencing (Provost, 2002). The possible impact of 5-LO in RNA interference is of considerable interest, but the relevance of the 5-LO-dicer complex remains to be elucidated.

A C-terminal proline-rich region enables 5-LO to bind to the SH3 domain of Grb2, an "adaptor" protein for tyrosine kinase-mediated cell signalling. This interaction could be inhibited by a synthetic peptide containing the SH3-binding motif of 5-LO. The competitor also interfered with translocation of 5-LO from the cytosol to the nucleus in intact human neutrophils (Lepley and Fitzpatrick, 1994). Novel functional implications of 5-LO in tyrosine kinase signalling are indicated.

1.6 Biological functions of 5-LO products

Fig. 2 gives an overview of products derived from the LO pathway. LOs convert the substrate AA to hydroxyeicosatetraenoic acids (HETEs) and HpETEs. 12-HETE and 12-HpETE inhibit platelet aggregation, affect endocrine functions and may be second messengers in neurotransmission (Yamamoto et al., 1997). In addition 12-HETE has been shown to stimulate leukocyte chemotaxis (Turner, 1975) and to have modulatory effects on tumor metastasis (Tang, 1999). 15-HETE suppresses platelet 12-LO and neutrophil 5-LO (Vanderhoek, 1980). Specific receptors for HETEs remain to be identified. 5-HETE and 12-HETE have been shown to increase cell proliferation in various malignant cells (Avis et al., 2001; Ding et al., 1999). Cyclooxigenase-2 (COX2) and 5-LO are overexpressed in some cancer cells where 5-LO and FLAP inhibitors induce massive and rapid apoptosis (Ghosh, 1998; Gupta et al., 2001; Öhd, 2000; Yoshimura et al., 2004; Yoshimura et al., 2003). 5-HETE has been reported to induce anti-apoptotic signals in malignant mesothelial cells (Romano et al., 2001).

5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) stimulates chemotaxis, actin polymerization and Ca^{2+} mobilization in eosinophils and expression of CD11b/CD18 on polymorphonuclear leukocyte (PMNL) cell surface (Powell et al., 1999).

Lipoxins (lipoxygenase interacting products) (LX) are lipid mediators, synthesized from 12- and 15-HETE or LTA_4, serving as a substrate for 5-LO or 12- and 15-LOs, respectively. LXs exhibit anti-inflammatory effects and support repair and wound healing (Serhan et al., 1999).

5-LO further dehydrates its product 5-HpETE to the unstable epoxide LTA_4. In subsequent catalytic steps LTA_4 is converted to the biologically active LTs B_4, C_4, D_4 and E_4 (Samuelsson, 1983). LTs convey messages by interacting with specific membrane G-protein-coupled leukotriene receptors (Izumi et al., 2002) and can probably regulate transcription by binding to an intranuclear orphan receptor (Devchand et al., 1999). The proinflammatory LTB_4 stimulates chemotaxis and secretion of neutrophils and the adherence of leukocytes to the endothelium of venules (Haeggström, 2002). In addition, phagocyte aggregation, lysosomal enzyme release and superoxide formation can be stimulated (Samuelsson et al., 1987). In lymphocytes, LTB_4 stimulates the secretion of IgE, IgG and IgM (see (Werz, 2002a) and references therein). Thus, a major impact of LTB_4 in the pathogenesis of inflammatory diseases,

such as arthritis, psoriasis, inflammatory bowel disease and asthma, is indicated. LTA_4 synthase catalyzes the conjugation of LTA_4 with glutathione (GSH), forming the cysteinyl leukotriene (cysLT) LTC_4. Subsequent extracellular metabolism leads to LTD_4 and E_4 (Funk, 2001). CysLTs lead to smooth muscle contraction, thus promoting bronchoconstriction (Samuelsson, 1983). Furthermore, cysLTs increase mucus secretion and vascular permeability, causing swelling during acute inflammation. CysLTs have also been described to induce hypotension by leading to constriction of arterioles and subsequent decrease of coronary blood flow and myocardial contractility (Samuelsson et al., 1987). The synthesis and actions of LTs are summarized in figs. 2 and 3.

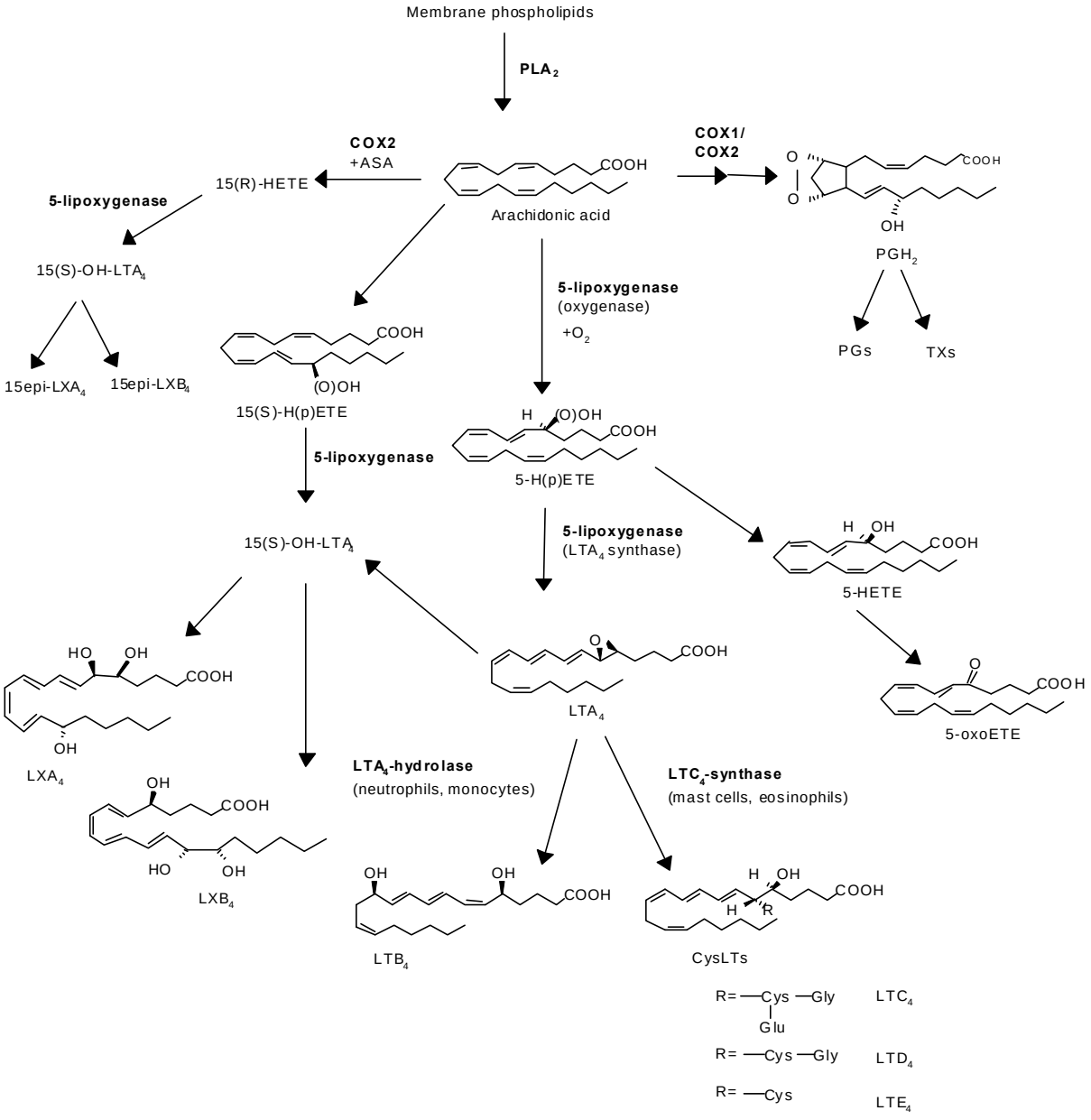


Fig. 2 . The arachidonic acid cascade with emphasis on 5-LO pathways.

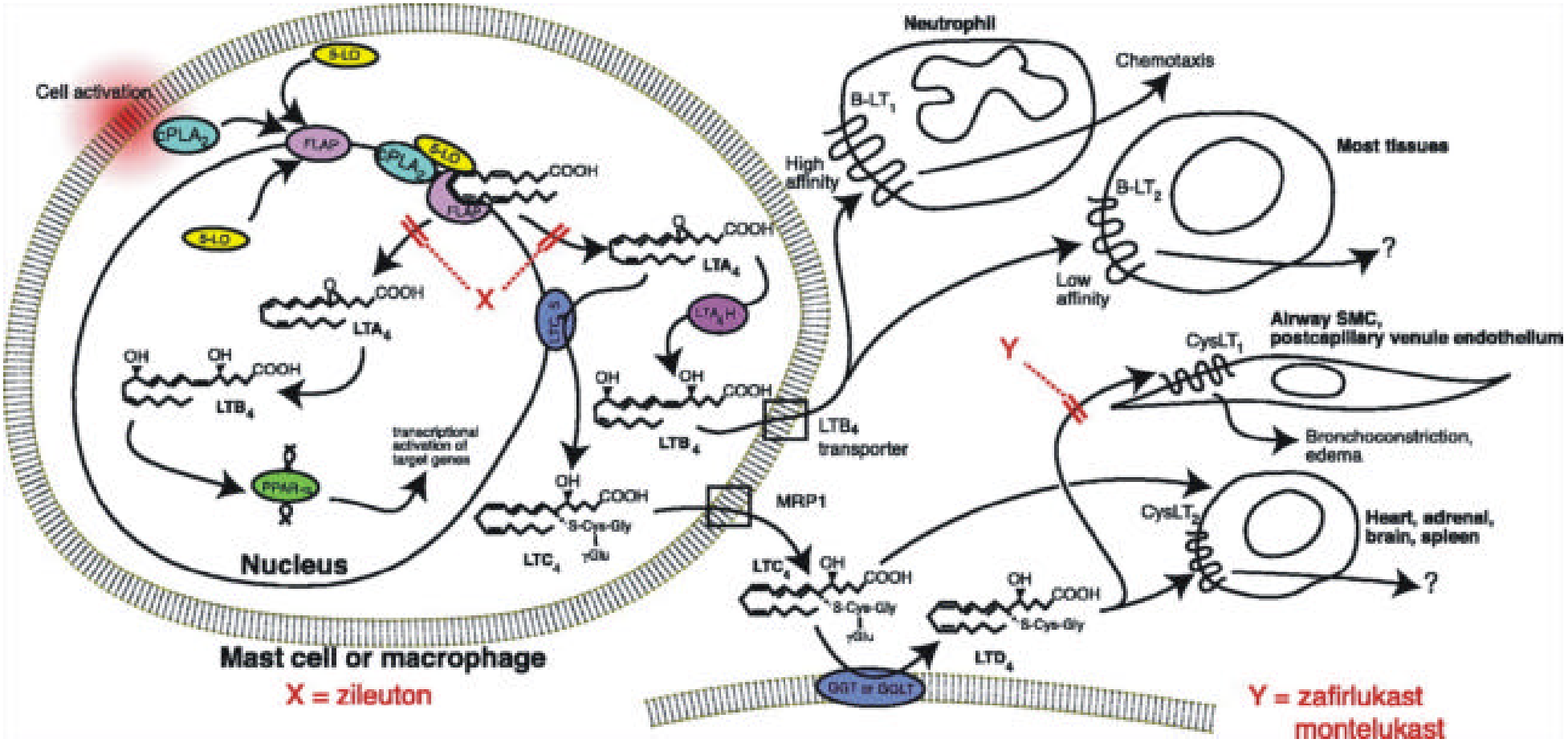


Fig. 3. Leukotriene synthesis and actions. *Upon cellular activation, 5-LO and $cPLA_2$ translocate to the nuclear envelope. FLAP facilitates presentation of AA to 5-LO for conversion to LTA_4. 5-LO activity is inhibited by zileuton. LTA_4 hydrolase forms LTB_4. Following transport out of the cell, LTB_4 can act on neutrophils through the B-LT_1 receptor or at the B-LT_2 receptor. LTB_4 may also act intracellularly on PPAR-α to induce target genes like those involved in β–oxidation. LTA_4 can also be converted to LTC_4 by LTC_4 synthase. The multidrug resistance-associated protein (MRP1) can facilitate transfer of LTC_4 out of the cell, where it is metabolized by γ-glutamyl transpeptidase (GGT) or γ-glutamyl leukotrienase (GGLT) to LTD_4. LTD_4 can act on airway smooth muscle cells (SMC) and postcapillary venule endothelial cells through $CysLT_1$ receptors, which is inhibited by zafirlukast and montelukast. LTC_4 or LTD_4 may also bind to $CysLT_2$ receptors that are found in a variety of tissues (Funk, 2001).*

Recent data suggest that 5-LO products play a role in the development of atherosclerosis (Mehrabian and Allayee, 2003) (for further information, see chapter 1.12).

In the arachidonic acid cascade 5-LO and COX1-3 are the key enzymes. The effects of the COX products prostaglandins, prostacyclins and thromboxanes comprise relaxation and contraction of smooth muscle, regulation of platelet activity, effects on the mucus layer in gastric mucosa, stimulation or inhibition of neurotransmitter release, temperature control, pain and inflammatory responses (Funk, 2001).

1.7 Endogenous inhibitors of 5-LO activity

GPxs are selenoproteins involved in the regulation of cellular peroxide levels. Fatty acid hydroperoxides, which are generated by the lipoxygenases, serve as redox partners in the oxidation of 5-LO active site iron from the ferrous to its active, ferric form (Ford-Hutchinson et al., 1994; Rouzer and Samuelsson, 1986). Glutathione-dependent peroxidase(s) suppress 5-LO activity by reducing lipid hydroperoxides (Straif et al., 2000). To date the four GPxs, classical cytosolic (c)GPx (GPx-1), gastro-intestinal (GI-) GPx (GPx-2), plasma (p)GPx (GPx-3), and phospholipid hydroperoxide (PH-) GPx (GPx-4) are known. Their substrates are soluble hydroperoxides like H_2O_2 or organic compounds like cumene or fatty acid hydroperoxides or even cholesterol hydroperoxides. Beside the predominantly utilized reducing agent GSH, other thiols like dithiothreitol (DTT), β-mercaptoethanol, thioredoxin, glutaredoxin or cysteine are used as cosubstrates. The four isoforms differ in their primary substrates and cosubstrates and their locations (for review on GPxs see (Arthur, 2000)).

Binding of adenosine to $A_{2\alpha}$ receptors leads to elevated intracellular cyclo AMP (cAMP) levels, which subsequently inhibit AA release. Thus, LTB_4 synthesis and 5-LO translocation are repressed (Surette et al., 1999). Also, several leukocyte functions like superoxide formation, phagocytosis and adherence are inhibited (Flamand et al., 2000; Krump et al., 1997).

Nitric oxide (NO) is a reducing agent that was shown to inactivate LOs by converting the active site iron to the ferrous form (Maccarrone et al., 1996). It is involved in the regulation of inflammatory processes (Coffey et al., 2000).

1.8 Pharmacological 5-LO inhibitors

The pathophysiological properties of LTs to cause allergic and inflammatory diseases gave reason to pharmacological intervention with the biosynthesis or the action of LTs. Although glucocorticoids have been shown to inhibit phospholipases and eicosanoid formation in many experimental systems, high dose glucocorticoid therapy did not significantly suppress generation of LTs (Claesson and Dahlen, 1999). LT receptor antagonists such as montelukast are established in asthma therapy (for review, see (Brooks and Summers, 1996)). Direct 5-LO inhibitors are divided into three groups: redox inhibitors, iron ligand inhibitors, and non-redox inhibitors. Redox inhibitors act by reducing the active site iron, thus keeping the enzyme in its inactive ferrous form. Compounds like nordihydroguaretic acid, caffeic acid, flavonoids, coumarins, AA-861, BW755C, or ZM-207968 efficiently inhibit 5-LO. However, unspecificity and severe side effects prevented bringing them onto the market (Ford-Hutchinson et al., 1994). Iron ligand inhibitors contain a hydroxamic acid or N-hydroxyurea group chelating the active-site iron. Zileuton is available in the USA for the treatment of asthma. ABT-761 and LDP-977 with improved potency and oral half-lives presently undergo clinical trials. Non-redox inhibitors with methoxyalkylthiazol or methoxytetrahydropyran structures compete with AA for binding at the active site of 5-LO. They seem to potently suppress various acute inflammatory responses but fail to inhibit chronic inflammation (Turner et al., 1996). A low cellular hydroperoxide level promotes non-competitive properties of these compounds, whereas high peroxide levels lead to a reduced efficacy (Werz et al., 1998).

3-Acetyl-11-keto boswellic acid (AKBA), the most potent 11-keto-BA was reported as the active principle of *Boswellia serrata* extracts, that have been traditionally used to cure inflammatory and arthritic diseases. BAs have been described as direct-type 5-LO inhibitors, without reducing or iron-chelating properties, that probably act by binding to a second regulatory binding site for AA which is distinct from the catalytic AA-binding site (Sailer et al., 1998). However, recently it has been found, that BAs in concentrations £ 10 µM potently enhanced the formation of reactive oxygen species (ROS), the release of AA and 5-LO product synthesis in PMNL (Altmann et al., 2004). Higher concentrations were required for the anti-inflammatory effects.

New compounds exhibiting dual acitivity towards COX and 5-LO like hyperforin (Albert, 2002) or ML3000 have been described. ML3000 entered clinical trials in osteoarthritis (Werz, 2002a).

Also, several FLAP inhibitors like MK886, Bay X1005, MK-0591 have been developed and have considerable potency in reducing LT synthesis in isolated neutrophils. However, in whole blood, their inhibitory potencies are low and *in vivo* they have no beneficial effect (Werz, 2002a).

1.9 The 5-LO gene organization

The human 5-LO gene was first characterized by Funk *et al.* (Funk, 1989) and Hoshiko *et al.* (Hoshiko, 1990) and has been cloned from bacteriophage and cosmid genomic libraries. It is located on chromosome 10q11.21 whereas all other LOs are clustered on chromosome 17p13 (Sun, 1998). The structure of the gene has been defined: it spans more than 84 kilobasepairs (kbp) and consists of a 6.1 kbp promoter and 14 exons divided by 13 introns (Funk, 1989). The sequences of the 5'-untranslated region (UTR), the promoter, the coding region and the 3'-UTR have been published some years ago (Dixon et al., 1988; Hoshiko, 1990; Matsumoto et al., 1988). Recently, with data from the human genome project (HUGO), even the intron sequences have become available. According to this data, the intron/exon structure published in (Funk, 1989) has been confirmed (fig. 4).

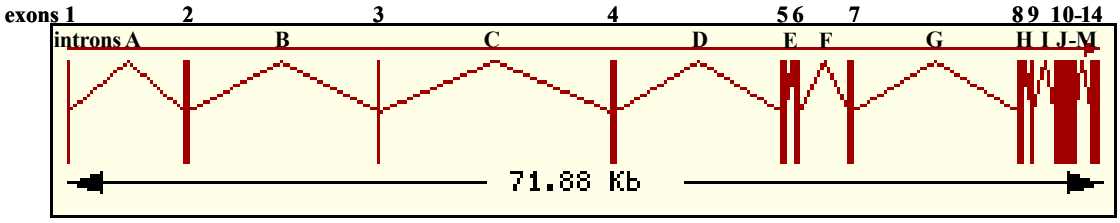


Fig. 4. Intron/exon structure of the 5-LO gene

For most introns the sizes reported by Funk are supported by the HUGO, only the size of intron C is significantly smaller (16.254 kbp instead of > 26 kbp) (tab. 1). Probably, sequencing is still not complete (18.02.04). Conspiciously, the first introns (A-D) are very large, whereas the last introns (H-M) are of minor length.

Exon/ Intron	bp (HUGO)	Exon/ Intron	bp (HUGO)	Exon/ Intron	bp (HUGO)
1	217	**6**	173	**11**	122
A	8000 (8053)	**F**	4000 (3485)	**K**	200 (190)
2	199	**7**	147	**12**	101
B	13000 (13173)	**G**	11000 (11665)	**L**	300 (287)
3	82	**8**	204	**13**	171
C	>26000 (16254)	**H**	1000 (710)	**M**	1300 (1221)
4	123	**9**	87	**14**	613
D	12000 (11727)	**I**	1700 (1607)		
5	107	**10**	179		
E	800 (812)	**J**	200 (198)		

Tab.1. Exon (numbers) and intron (capitals) sizes reported by (Funk, 1989) and from recent data of the human genome project (http://www.ncbi.nlm.nih.gov).

Transcription leads to a 2.7 kb mature mRNA. However, one group reported the expression of multiple 5-LO transcripts (2.7, 3.1, 4.8, 6.4, and 8.6 kb) in various brain tumors and in the differentiated promyelocytic leukemic HL-60 cells. The use of potential minor transcriptional initiation sites or aberrant splicing of the primary 5-LO transcript have been hypothesized to account for this observation (Boado et al., 1992).
Interestingly, in silico analysis revealed that some of the authentic 5’ splice sites differ from the consensus vertebrate 5’ splice site (tab. 2). Hence, these splice sites seem to be rather weak. Since weak splice sites might establish the possibility for alternative splicing reactions, the sequence was further analized for putative cryptic 5’ splice sites. As illustrated in figs. 5 and 6, several sequences with at least 85.7 % homology to the consensus vertebrate 5’ splice site can be identified on both strands. Most of these are found in intronic regions but some are also detected in exons 2, 8, and 10. It is not known, if these sites are of any relevance, though. Some of the accurate splice sites display a homology of less than 87.5 %, and thus have not been detected in this analysis. Moreover, a sequence element displaying high homology (85 %) to a

published purine-rich splicing enhancer (Yeakley et al., 1996) has been identified in exon 14.

Intron	5' splice site (exon intron)
A	AGC GUGGCG
B	CG GUGAGC
C	CG GUGAGU
D	AGC GUAAGU
E	TG GUGAGU
F	AG GUAGGG
G	AG GUAGGC
H	AG GUACAG
I	AG GUGGGU
J	AC GUGAGC
K	AG GUAGGG
L	AG GUAGGC
M	AG GUGAAG
consensus	AG GURAGU

Tab. 2. Functional 5' splice sites. *The functional 5-LO 5' splice sites in comparison with the consensus vertebrate 5' splice site (R = A/G).*

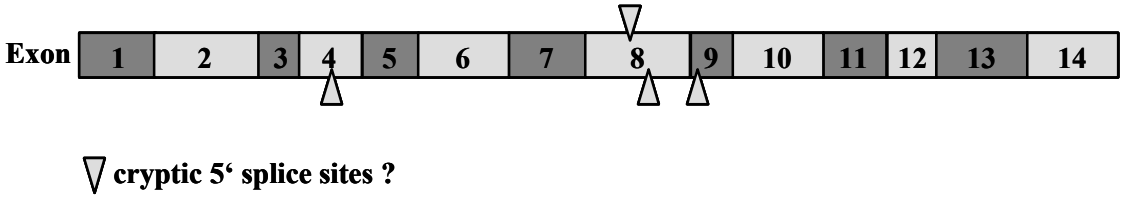


Fig. 5. Schematic representation of putative 5' splice sites in the 5-LO coding sequence. *Limit for detection was at least 85.7 % homology to the consensus vertebrate 5' splice site (G GURAGU; R = A/G).*

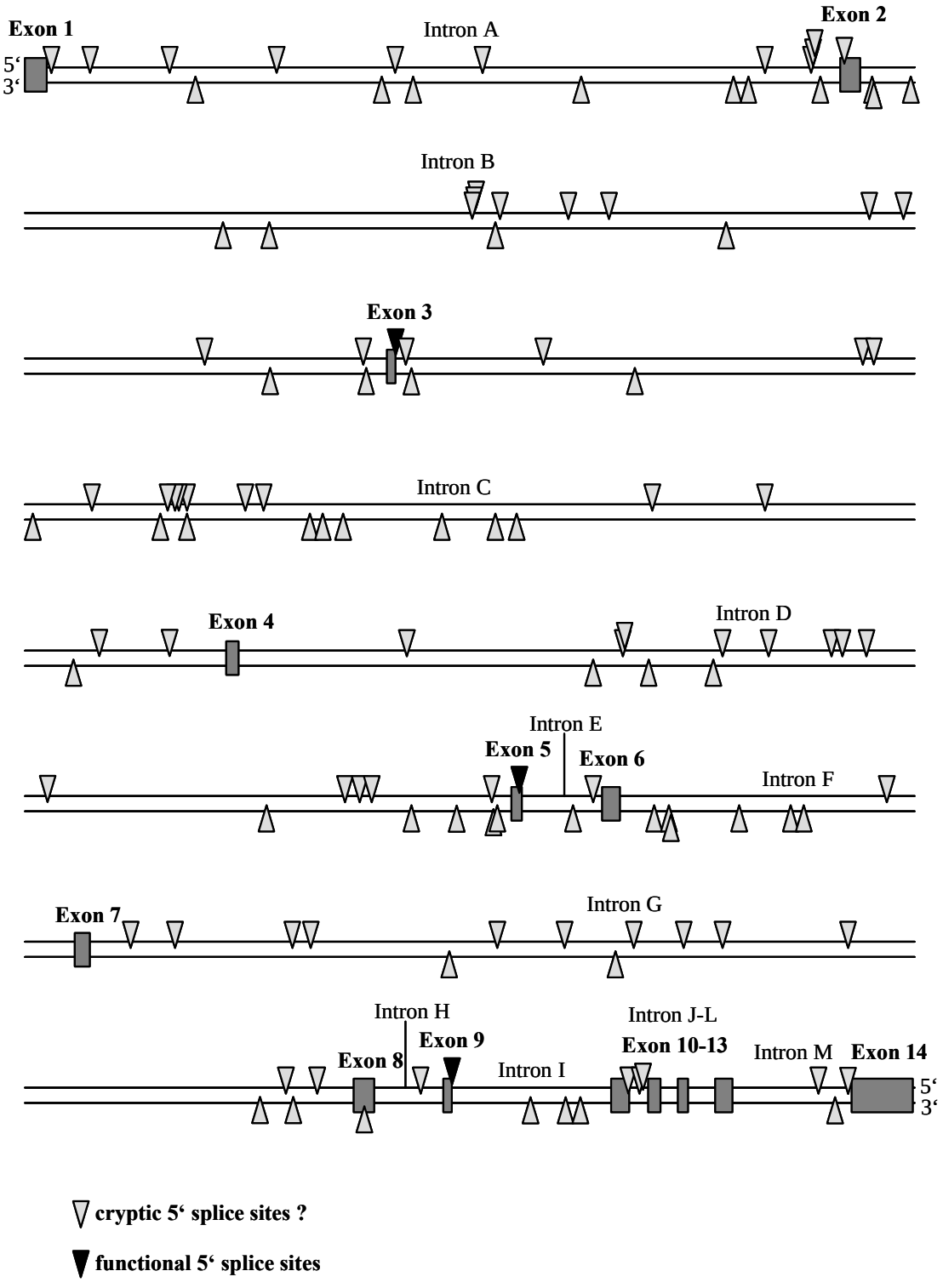


Fig. 6. Schematic representation of putative 5' splice sites in the 5-LO gene. *Limit for detection was at least 87.5 % homology to the consensus vertebrate 5' splice site (AG GURAGU; R = A/G).*

By sequence analysis multiple putative response elements (REs) can be identified on the direct as well as on the complementary strand. A remarkable cluster of consensus and nonconsenus serum protein1 (Sp1) binding sites is located in intron B. Also, various

putative Smad binding elements (SBEs) are detectable in some introns. In addition, several putative responsive elements for the vitamin D receptor (VDR), TGFβ, p53, Pu.1, early-growth response factor (Egr-1) and nuclear factor of κ-light polypeptide gene enhancer in B cells (NFκB) can be found throughout the whole coding and intron regions. Funk *et al.* (Funk, 1989) have proposed a putative mRNA processing signal (TGTGTTAT), located in the 3'-UTR. Interestingly, three additional identical sequences can be identified in intron C. The impact of some of these sequences has been investigated in this work.

Exon 11 represents a region of characteristically high sequence homology between all lipoxygenases. Thus, importance of the coded amino acids for protein functions such as subcellular localization, enzyme activity and substrate specificity is suggested.

1.10 The 5-LO promoter

The human 5-LO gene promoter was first characterized by Hoshiko *et al.* (Hoshiko, 1990). It is highly G+C rich and lacks TATAA or CCAAT boxes which is characteristic for house-keeping genes. Multiple transcriptional start sites have been reported and the major transcriptional start site was found to be located 65 bp upstream of the ATG. Deletion analysis revealed a core promoter region, comprising 292 bp upstream of the translational start site. By convention, all positions in the 5-LO gene are given in relation to the transcriptional start site. Two important cis-elements have been characterized in the promoter region: five overlapping tandem Sp1/Egr-1 consensus-binding sites (GGGCGG and GCG(T/G)GGGCG, respectively) between -210 and -244 bp and a c-Myb consensus site located between -1840 and -1852 bp (Hoshiko, 1990; Silverman et al., 1998). The transcription factor Sp1 is required for basal 5-LO transcription (Hoshiko, 1990), whereas the myb protein downregulates 5-LO gene expression and inhibits macrophage differentiation (Habenicht et al., 1989). In reporter gene experiments with HeLa and Drosophila SL2 cells it was found that overexpression of Sp1 and Egr-1 enhanced reporter gene activity. In SL2 cells, stimulation by Egr-1 seems to be mediated predominantly via the GC-box, whereas Sp1 transactivation mainly utilizes a solitary consensus binding site at -48 bp (Silverman et al., 1998). Egr family members are rapidly and transiently induced by a large number of growth

factors, cytokines, and injurious stimuli. Recently, Silverman and Drazen proposed a model, in which Egr-1, increased in inflammation, displaces Sp1 from the 5-LO promoter and further enhances transcription by recruiting the transcriptional coactivators CREB binding protein (CBP)/p300 (Silverman and Drazen, 2000). In addition, Hoshiko *et al.* have identified two positive regulatory regions between -3635 and –5835 bp and from -789 to -866 bp, and two negative regulatory regions from -1492 to -3335 bp and between -227 and -662 bp.

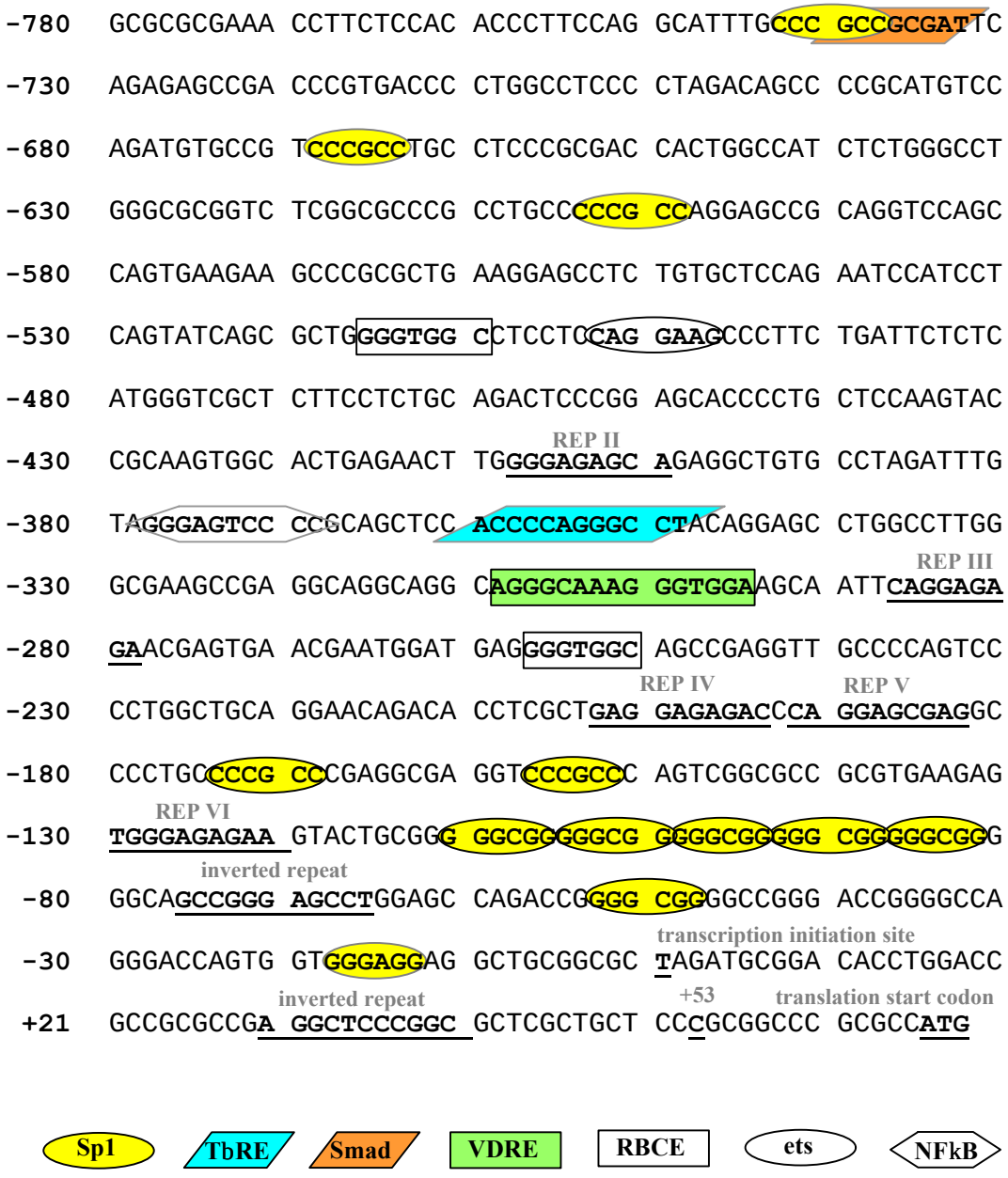


Fig. 7. The 5-LO promoter sequence from –780 to +65 bp with selected responsive elements (http://www.ncbi.nlm.nih.gov). *Transcription factor binding sites and response elements (putative REs with grey line) within the 5-LO promoter region. The plasmid pN10 comprises nucleotides –780 to +53 bp. All positions are defined in relation to the transcriptional start site.*

Moreover, the promoter contains numerous consensus-binding sites for transcription factors such as NFκB (involved in macrophage differentiation), glucocorticoid receptors (GRs), GATA, activating protein-2 (AP-2) and AP-1 family members. Six repeated sequences (REP I-VI) weakly homologous to the silencer regions I and II of the ε-globin gene and the chicken lysozyme gene have been found from –850 to –130 bp.

Important gene regulatory sequences are often conserved across species. Interestingly, 5-LO and the rabbit erythroid cell-specific 15-LO show an identical intron/exon organization and both gene promoters are G+C-rich and contain GC boxes (O'Prey et al., 1989).

Recently, the mouse 5-LO promoter has been cloned and analysed (Silverman et al., 2002). The human and mouse promoter sequences differ dramatically, although, there are some significant similarities. Both sequences lack TATAA motifs, transcription factor IIB (TFIIB) recognition elements, initiator sequences, and downstream core promoter elements. Both promoters have multiple transcriptional start sites and a major start site in similar regions. Also, the core promoter regions are of similar size (~300 bp). Finally, three highly homologous regions containing consensus-binding sites for transcription factors implicated in 5-LO regulation or leukocyte-specific gene expression (Sp1/Sp3, Ets/Pu.1/Spi-1, and GATA/GGAGA) have been detected. Binding to the indicated GC boxes of the human and mouse promoters and response to overexpression has been demonstrated for Sp1/Egr-1 and Sp1/Sp3, respectively (Hoshiko, 1990; Silverman et al., 2002). Sp1 is a transcriptional activator, whereas Sp3 is regarded as a suppressor of Sp1-mediated transcription (for review, see (Suske, 1999)). Binding of Pu.1 to the consensus-binding motif (GGAA/T) has been shown by electrophoretic mobility shift assays (EMSAs) (Silverman et al., 2002). The transcription factor Pu.1 is primarily expressed in hematopoietic cells and has been shown to regulate the expression of a variety of genes involved in inflammation, adaptive and innate immunity, and cell differentiation and proliferation. Thus, the conserved binding site may play a role in tissue-specific expression of 5-LO (Silverman et al., 2002). Although conserved in human and mouse, no binding of the transcription factors GATA/GGAGA to the respective consensus binding sites has been demonstrated so far (Silverman et al., 2002).

Furthermore, sequence analysis using consensus binding sites from databases and from the literature, reveals the existence of several putative REs for the nuclear receptors

(NRs) VDR, retinoid Z receptor α (RZRα) as well as the retinoic acid related orphan receptor α (RORα), TGFβ, Smads, AP-2, ATF-2, retinoblastoma control element (RBCE), hFast-1, Oct-1 and p53. In the 5-LO core promoter region, a binding site for VDR has been proposed previously (Carlberg, 1995). Binding of RORα1 and RZRα to a putative RZR RE in the 5-LO promoter (-1448 bp) has been demonstrated before, thus identifying human 5-LO as the first natural responding gene for RZRα. Also, 5-LO promoter activity has been shown to be repressed by the pineal gland hormone melatonin, the ligand for RZRα. The NRs RZRα and RORα are expressed in B lymphocytes, but not in granulocytes (Steinhilber et al., 1995).

1.11 5-LO promoter polymorphisms

A series of naturally occurring mutations in the 5-LO core promoter has been found, resulting in a variable number of two to eight Sp1/Sp3/Egr-1 consensus sites in the GC-box (Drazen and Silverman, 1999; Dwyer et al., 2004; In et al., 1997). Slight differences in Sp1/Sp3/Egr-1 binding affinity have been detected and promoter response of the mutant forms to transcription factor overexpression was significantly altered (In et al., 1997; Silverman et al., 1998). Interestingly, reporter gene assays in HeLa cells suggested, that every mutation decreases promoter activity, whereas experiments in Schneider Cells, which do not naturally express Sp1 or Egr-1, indicated, that promoter activity is proportional to the number of Sp1/Egr-1 binding sites (Silverman and Drazen, 2000). The polymorphisms are of particular interest, as asthma patients with sequence variants in the 5-LO gene promoter show a diminished response to treatment with 5-LO inhibitors (Drazen et al., 1999). Genotyping data and genetic association analyses performed for a Caucasian population indicated, that these polymorphisms do not constitute a genetic risk factor for the development of asthma, though (Sayers et al., 2003).

Very recently, the 5-LO promoter polymorphisms have been related to atherosclerosis susceptibility. In carriers of two variant 5-LO promoter alleles a profound increase in carotid intima-media thickness as compared to carriers of the wild type allele was detected. Moreover, the increased dietary intake of n-6 fatty acids enhanced the severity of atherosclerosis of carriers of two variant alleles, whereas the increased intake of

n-3 fatty acids blunted the atherogenic effect of the variant genotypes (Dwyer et al., 2004). These results are consistent with data obtained with 5-LO null mice, which were resistant to atherosclerosis (Mehrabian, 2002) and with the fact, that 5-LO is abundantly present in macrophages, foam cells, dendritic cells, and artery wall cells from atherosclerotic lesions (Spanbroek et al., 2003). Taken together, LT-mediated vascular inflammation in atherosclerosis is strongly suggested. However, the above mentioned reduced 5-LO gene expression of all naturally occuring mutants (Drazen and Silverman, 1999; Drazen et al., 1999; In et al., 1997; Silverman and Drazen, 2000) does not support this hypothesis, although nonhuman cells (Schneider) and tumor cells that do not express 5-LO (HeLa) might not be the right system for investigations on vascular inflammation.

Recently, it was hypothesized, that the decreased expression of 5-LO in the brain of carriers of variant promoter genotypes leads to reduced susceptibility and delayed onset of Alzheimer's disease (Manev, 2000). Aging has been reported to be associated with induced 5-LO gene expression in the brain and neuroprotection could be obtained by inhibition of the 5-LO pathway. Hence, a role of neuronal 5-LO in neurodegeneration is suggested and antiinflammatory treatment is likely to exert beneficial effects in aging-associated neurodegenerative diseases such as Alzheimer's (Manev et al., 2000).

1.12 5-LO gene expression

1.12.1 Cell type specificity

5-LO is mainly expressed in immune competent cells from myeloid origin including granulocytes, monocytes and macrophages, mast cells and B-lymphocytes (Steinhilber, 1999). Also, it is found in dendritic cells (Steinhilber, 1999) and epidermal Langerhans cells (Spanbroek et al., 1998). 5-LO expression often coincides with FLAP expression. Although T-cells express FLAP, they are negative for 5-LO. Interestingly, both, 5-LO and FLAP have been found in a number of non-hematopoetic cell types like differentiated human skin keratinocytes (Janssen-Timmen et al., 1995) and in neurons in various regions of the rat brain (Lammers et al., 1996).

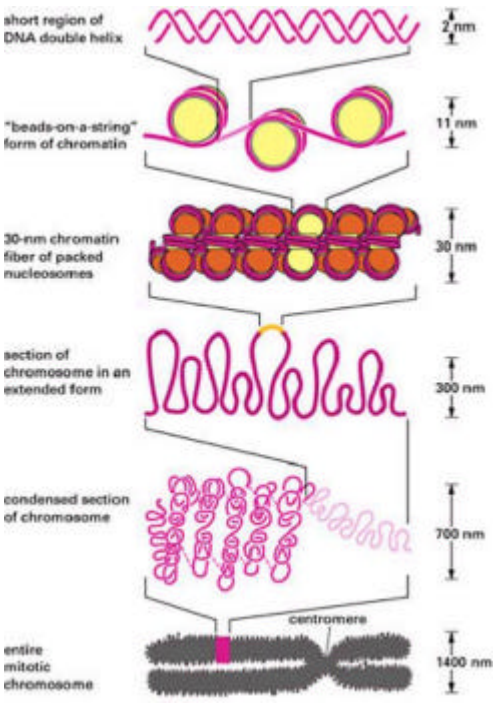
The mechanisms involved in the cell type specific 5-LO upregulation in response to differentiation signals and inflammatory stimuli have not been clarified yet. In the

human myeloid cell lines Mono Mac 6 (MM6) and HL-60, differentiation with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and TGFβ leads to a significant increase in 5-LO gene expression and activity (Brungs et al., 1994; Brungs et al., 1995; Janssen-Timmen et al., 1995). In contrast, U937 cells and HL-60TB cells express FLAP but not 5-LO (Claesson et al., 1993; Kargman et al., 1993). Silencing of tissue-specific genes is often due to strong methylation of their promoters and has been proved to be true for 5-LO (Chan et al., 2000; Uhl et al., 2002) (for more details, see chapter 1.12.2). Speculations about the impact of Pu.1 binding to its binding site in the 5-LO promoter in tissue-specific expression have already been mentioned in chapter 1.10 (Silverman et al., 2002).

1.12.2 Epigenetic mechanisms

Beside transcriptional and posttranscriptional modulation, epigenetic mechanisms are known to be involved in the regulation of gene expression. Epigenetic mechanisms are not related to genetic information encoded by the genetic code but refer to epigenetic, extrakaryotic information.

Fig. 8 . Chromatin organization.
(from http://www.accessexcellence.org).



In eukaryotes, DNA typically is arranged *in vivo* as a repeating array of nucleosomes, in which 146 bp of DNA are wound around a histone (H) octamer. The histone octamer consists of an $(H3, H4)_2$ tetramer and two dimers of (H2A, H2B). Extended chains of nucleosomes are compacted to form a chromatin fiber which in turn is folded into a chromosome (fig. 8). The nucleosomal organization of DNA seems to be prerequisite for functional synergism by enabling a coordinate binding of transactivating factors to otherwise distant sites (Beato et al., 1996). Chromatin can be divided into the decondensed, transcriptionally active euchromatin and the condensed, transcriptionally silent heterochromatin. Chromatin organization is

critical for many cellular processes such as transcription, replication, repair, recombination and chromosome segregation. The assembly of a gene into chromatin generally represses transcription. ATP-utilizing chromatin remodeling factors facilitate the binding and function of key components of the transcriptional apparatus by disruption and subsequent re-formation of histone-DNA contacts. Chromatin remodeling factors comprise ATPases from the SWI/SNF (mating type switch/sucrose non-fermenter), Mi-2/CHD and ISWI (imitation switch) family (Tyler and Kadonaga, 1999). The human homologs of components of the yeast SWI/SNF complex, human brahma (hbrm) and brahma related product 1 (BRG1), have been shown to interact with other factors of the transcriptional machinery including the GR and estrogen receptor (ER) in transient cotransfection assays. Posttranslational, covalent modifications of the histone tails, the so-called histone code, lead to dynamic changes in the chromatin structure due to altered interactions with DNA or chromatin-associated proteins. Histone modifications include acetylation of lysines, phosphorylation of serines and threonines, poly(ADP-ribosylation) of glutamic acids, ubiquitination of lysines, sumolation of lysines and methylation of lysines and arginines (Khorasanizadeh, 2004; Rice and Allis, 2001; Wolffe and Guschin, 2000).

Histone acetylation is a very dynamic, reversible process, that is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Although at low level rather global, acetylation of the core histones occurs locally on target promoters at specific lysines in particular histones. Acetylated N-terminal histone tails bind DNA and other histones with reduced affinity, thus leading to less tightly wrapped nucleosomes. This effect might be enhanced by other chromatin remodelling factors or components of the transcriptional machinery. Hence, access and binding of transcription factors to their recognition sites is facilitated and transcription is activated (Wolffe and Guschin, 2000). Moreover, histone acetylation specifically can regulate DNA replication, histone deposition, and DNA repair by recruiting regulatory proteins that contain an acetyllysine binding bromodomain (Khorasanizadeh, 2004).
HATs are widely spread and can be found among proteins with other known functions in trancription, such as transcription initiation factors (e.g. TFIIIC), transcript elongation factors (e.g. Elp3), general transcription factors (e.g. TATA-binding protein (TBP)-associated factor ($TAF_{II}250$)), coactivators (e.g. p300/CBP, p300/CBP-associated factor (PCAF)) and NR coactivators (e.g. steroid receptor coactivator-1

(SRC-1)/NCoA1, ACTR). Multiple interactions between HATs and regulatory proteins, the transcription apparatus and/or other HATs modulate their influence on transcription. HATs transfer an acetyl group from acetyl-coenzyme A to the ε-amino group of the lysine (Sterner and Berger, 2000). Several nuclear HATs contain a bromodomain, thus they bind to already acetylated lysines.

HDACs as well as HATs occur in multi-protein complexes, and among these proteins are those that regulate substrate specificity and others functioning in recruitment, corepression or chromatin remodeling. To date, 13 classical HDAC are known and can be divided into the phylogenetic classes I and II. HDAC function seems to be regulated by its intrinsic features, abundance, cellular compartmentalization and association with cofactors. Beside an essential Zn^{2+} ion, other cofactors are required for HDAC activity, including histone binding proteins, recruiters (e.g. Sp1/3, NFκB, methyl-CpG-binding protein (MeCP2), GATA-2, other HDACs), nuclear hormone receptor binding proteins (e.g. NCoR, silencing mediator for retinoic acid and thyroid hormone receptors (SMRT)), chromatin remodeling factors (e.g. Mi-2) and others (Ballestar and Wolffe, 2001; de Ruijter et al., 2003).

Inhibition of HDACs by compounds such as trichostatin A (TsA), trapoxin, valproate or butyrate by displacing the Zn^{2+} ion and/or blocking access to the active site leads to a general hyperacetylation of histones, which is followed by transcriptional activation. Since histone hypoacetylation has been linked to the pathogenesis of certain types of cancer, HDAC inhibitors (HDACis) have great potential for the treatment of malignant disease. Some HDACis entered clinical trials. Recently, HDACis have been suggested to prevent oxidative neuronal death and may therefore be useful in the therapy of neurological diseases like Parkinson's disease and stroke (Ryu et al., 2003). Increased histone acetylation is suggested to lead to re-expression of silenced genes and/or the silencing of downstream genes due to the regained acess of their promoters to other regulatory factors. General effects of HDAC inhibition are apoptosis, necrosis, differentiation, inhibition of proliferation and cytostasis. Interestingly, Sp1 sites in the promoter seem to be essential for full efficiency of HDACis. Possible explanations are the ability of Sp1 to bind HDAC1/2 and to protect DNA against methylation (Choi et al., 2002; de Ruijter et al., 2003; Doetzlhofer et al., 1999; Jung, 2001; Nakano et al., 1997).

Hormone receptors recognize their REs in chromatin, while the access of many other transcription factors to promoters is hindered by its nucleosomal organization.

Unliganded nuclear hormone receptors such as the thyroid hormone and retinoic acid receptor (RAR) have been shown to bind the nuclear corepressor (NCoR) which interacts with the transcriptionally repressive Sin3 (SWI independent) and recruits HDAC. Binding of the ligand to the chromatin-bound receptor leads to recruiting of coactivators and displacement or rearrangement of the nucleosome and enables free access of transcription factors to their binding sites (Beato et al., 1996; Wolffe and Guschin, 2000). In consensus with these reports, hormone treatment led to increased histone acetylation and selective changes in the chromatin structure in the promoters of ER, GR, RAR and VDR target genes (Chen et al., 1999; Shen et al., 2002). Further more, HDACis have been shown to synergistically enhance cell differentiation by $1,25(OH)_2D_3$ (Gaschott et al., 2001; Kosugi et al., 1999) and the VDR has been identified as mediator of butyrate-induced differentiation (Gaschott et al., 2001).
An involvement of chromatin structure and histone acetylation in the regulation of 5-LO expression has been discussed for some time. In HL-60 cells, differentiation with dimethyl sulfoxide (DMSO) led to changes of the repressive chromatin conformation alongside with transcriptional activation of the 5-LO gene (Ponton et al., 1996). Also, 15-LO-1 mRNA expression and 5-LO mRNA and protein expression are induced in Caco-2 cells following butyrate-induced cell differentiation (Kamitani et al., 2000; Wächtershäuser et al., 2000). The underlying mechanism, possibly being histone acetylation, has not been investigated in these studies, though. Recently, valproate has been reported to significantly increase histone acetylation and 5-LO protein in the mouse hippocampus, *in vivo* (Yildirim et al., 2003). For further information, see paper IV/chapter 4.5.

Methylation of cytosines within the CpG dinucleotide is the most prevalent epigenetic modification of DNA in mammalian genomes. In vertebrates, the genomes of somatic cells are globally methylated. However, discrete regions, including most repetitive and parasitic DNA, are hypermethylated, whereas the GC-rich CpG islands are hypomethylated. CpG islands contain promoters and binding sites for proteins involved in transcription and are typically found in house-keeping genes and widely expressed genes. Both methylated and unmethylated DNA is assembled into nucleosomes. Methylated DNA is generally associated with transcriptionally silent heterochromatin. DNA methylation is involved in many processes such as transcription, chromatin structure modulation, genomic stability, differentiation, genomic imprinting,

X chromosome inactivation, ageing and the silencing of parasitic DNA elements. DNA methylation outside of promoter regions probably contributes to the silencing of cryptic transcriptional start sites. Aberrant DNA methylation patterns play a role in the pathogenesis of diseases like cancer, ICF (immunodeficiency, centromere instability, facial anomalies), Rett, ATRX (X-linked, α-thalassemia mental retardation) and Fragile X syndromes (Leonhardt and Cardoso, 2000; Robertson, 2002).

(Cytosine-5) DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the 5' position of cytosine. Currently, the five mammalian DNMTs DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L are known but only DNMT1, DNMT3A and DNMT3B have been shown to be catalytically active. Due to its preference for hemimethylated double-strand DNA, DNMT1 is commonly termed maintenance methyltransferase. It is suggested, that DNMT1 is required to maintain methylation patterns following replication and for genomic imprinting and X chromosome inactivation. Targeted disruption of the DNMT1 gene indicated the contribution of other methyltransferases to the homeostasis of DNA methylation patterns, though. Alternative splice variants of DNMT1 have been reported that might exert tissue-specific roles. DNMT3A and DNMT3B are capable of methylating unmethylated and hemimethylated DNA *in vitro*. Since they are essential for *de novo* methylation *in vivo*, they are usually referred to as *de novo* methyltransferases. They presumably have independent, but overlapping functions in the establishment of cellular DNA methylation patterns during embryonic development. Several DNMT inhibitors are known, including 5-azacytidine (AC), 5-aza-2'-deoxycytidine (AdC), S-adenosylhomocysteine (SAH), dibutyryl-cyclo-AMP (dbcAMP) and sodiumarsenite (Attwood et al., 2002; Robertson, 2002).

Since DNMTs do not seem to have any sequence specificity, other mechanisms must exist to target DNA methylation to certain DNA sequences. Several different proteins have been reported to interact with the catalytically active DNMTs. Among these proteins are chromatin remodeling enzymes (SNF2-like proteins), histone deacetylases (HDAC1/2), the presumably catalytically inactive DNMT3L, methyl-CpG binding proteins (MBD2/3), proliferating cell nuclear antigen (PCNA), a transcription elongation factor (E2F1), a trancriptional repressor (RP58), corepressors (DNMT1-associated protein (DMAPI), TSG101), an oncogenic transcription factor (PML-RAR fusion protein) and a tumor suppressor (retinoblastoma protein (pRb)). Methylcytosine binding proteins are components of macromolecular repressor complexes like MeCP1

and Mi-2/nucleosome remodeling histone deacetylase complex (NuRD), which can preferentially bind, remodel, and deacetylate methylated DNA-containing nucleosomes *in vitro*. Thus, chromatin remodeling, histone acetylation (and possibly other histone modifications), DNA methylation and transcription are tightly connected. In fig. 9 a proposed model depicts the reliance of these activities on each other.

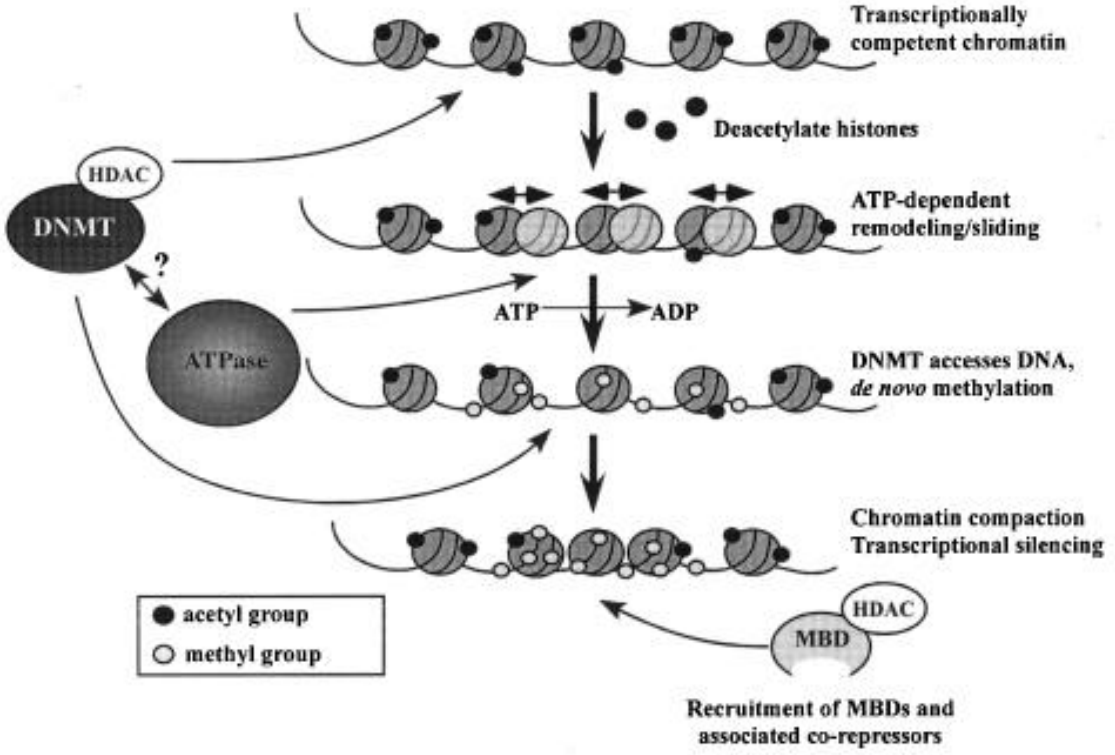


Fig. 9. Model for how HDACs, ATP-dependent chromatin remodeling enzymes, and DNA methyltransferases may cooperate to set up region-specific DNA methylation patterns. *The histones within a transcribed or transcriptionally competent region destined for silencing (top) may first be deacetylated (and potentially methylated) and this likely initiates transcriptional silencing. The chromatin remodeler can now recognize the chromatin and mobilize nucleosomes in an ATP-dependent manner to allow the DNMT access to its target DNA sites or create a particular chromatin signature or 'epitope' that is recognized by the DNMT or DNMT-containing complex. Once the region is methylated, the methylated cytosines will recruit methyl-CpG binding proteins (MBD) and their associated co-repressors to further reinforce transcriptional silencing and chromatin compaction (Robertson, 2002).*

Histone deacetylation of a transcriptionally active region may lead to transcriptional shutdown and enables chromatin remodeling enzymes to bind and modulate chromatin structure, which in turn facilitates either the access of DNMTs to the nuclesosomal DNA or the recognition of the region by DNMTs. Methylated CG dinucleotides may directly interfere with the binding of transcription factors to their binding sites. The gene silencing exhibited by DNA methylation is further reinforced by the recruitment of

competing methylcytosine binding proteins and their associated repressive activities. This model is supported by the fact that the HDACi TsA leads to cytosine hypomethylation at specific sequences. CpG islands are thought to be protected from methylation through binding of Sp1 to sites in or near these regions of DNA, and also through the action of an embryo-specific factor (Attwood et al., 2002; Ballestar and Wolffe, 2001; Newell-Price et al., 2000; Richards and Elgin, 2002; Robertson, 2002).

5-LO gene expression has been shown to be regulated by DNA methylation. The 5-LO promoter was heavily methylated in the 5-LO-negative myeloid cell lines U937 and HL-60TB, whereas it was completely unmethylated in the 5-LO-positive cell line HL-60. Furthermore, reporter gene assays with a 5-LO promoter plasmid revealed an almost complete loss of transcriptional activity by methylation of the GC-rich core region by *SssI* methylase. Treatment of U937 and HL-60TB cells with the DNMT inhibitor AdC led to a prominent promoter hypomethylation and a correlating induction of 5-LO pre-mRNA and mature mRNA (Uhl et al., 2002). Further, it was found that incubation of rat cerebellar neurons with AdC or valproate induced 5-LO mRNA. Both agents have also been reported to induce 5-LO mRNA and to decrease DNA methylation in human NT2 and NT2-N cells. It can be speculated, that aging-altered DNA-methylation may cause aging-associated 5-LO upregulation in the brain (Manev and Uz, 2002; Zhang et al., 2004).

1.12.3 Gene regulation by $1,25(OH)_2D_3$

The nuclear hormone vitamin D is a major component in the maintenance of calcium and phosphate homeostasis, bone formation, small lipophilic compound metabolism, and cellular growth. Further, the hormone can induce growth inhibition, differentiation and apoptosis of a number of cell types and also has immune-suppressive effects. Vitamin D_3 (cholecalciferol) can either be derived from dietary sources or synthesized in the skin from 7-dehydrocholesterol by exposure to ultraviolet light. The functionally active compound, the seco-steroid $1,25(OH)_2D_3$ (calcitriol), is generated by 25-hydroxylation in the liver and subsequent 1α-hydroxylation in the kidney. Like other nuclear hormones, $1,25(OH)_2D_3$ is a lipophilic compound that easily passes biological membranes and enters the nucleus. Most of the known biological effects of $1,25(OH)_2D_3$ are “genomic” effects that are mediated through specific and high affinity binding to its NR VDR. Also, rapid, “non-genomic” actions of $1,25(OH)_2D_3$ are known

that have been suggested to be mediated via putative membrane receptors. They have been reported to involve activation of Ca^{2+} channels, G-protein coupled receptors, and downstream PKC and MAPK pathways (Carlberg and Polly, 1998; Issa et al., 1998).

Most primary $1,25(OH)_2D_3$ target genes are involved in calcium endocrinology or bone formation such as osteocalcin, osteopontin, human parathyroid hormone (PTH), CYP24 (cytochrome P450, subfamily XXIV, vitamin D 24-hydroxylase), CYP1α, and the calbindin genes. However, VDREs have also been identified in other genes like 5-LO, β_3 integrin, fibronectin, atrial natriuretic factor (ANF), tumor necrosis factor α (TNFα), c-fos or $p21^{WAF1/CIP1}$.

The 52-60 kD protein VDR is a member of the NR superfamily of transcription factors. According to amino acid sequence homology and RE preference, the VDR can be classified together with the T_3R and RAR into the second subclass of this family. The first subclass contains the steroid hormone receptors ER, progesterone receptor (PR), GR, mineralocorticoid receptor (MR), and androgen receptor (AR). Orphan receptors, for which no ligand was known at the time of their discovery, form the third subclass of the NR superfamily. According to their function, VDR, RAR, T_3R, ER, PR, AR, GR, and MR form the classical endocrine receptor subgroup of the NR superfamily. The ligand-bound VDR predominantly resides within the nucleus, but substantial amounts of unoccupied receptor have been detected in the cytoplasm (Carlberg and Polly, 1998; Issa et al., 1998).

Recently, the three-dimensional structure of the VDR has been solved (Rochel et al., 2000). For a schematic representation, see fig. 10. Like other NRs it is characterized by an N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). The LBD contributes to ligand binding, dimerization, nuclear import signalling, interaction with the transcriptional machinery, and transcriptional activation/inhibition. It contains 12 α-helices and two β-sheets and at least three different dimerization interfaces. Ligand binding modifies the orientation of helix 12 containing the activation function-2 (AF-2) domain which results in the closing of the ligand binding pocket (“mouse trap model”) and the formation of a new binding interface by helices 3, 5, and 12 that allows interaction with transcriptional cofactor proteins. The AF-2 domain is essential for effective receptor-ligand interaction. Corepressors have been suggested to interact with regions within helices 1, 10, and 11. The DBD is characterized by two zinc fingers, formed by two zinc atoms that are coordinated by eight conserved cysteine

residues. Further, it contains several positively charged amino acids, favouring electrostatic interactions with the DNA backbone and two nuclear import regions. The hinge region D between DBD and LBD confers flexibility to the protein. Unlike other NRs, the VDR lacks a ligand-independent AF-1 motif in its aminoterminal A/B domain. Hormone dependent phosphorylation at distinct serine residues has been reported for VDR and might be involved in the regulation of DNA binding, ligand binding, nuclear localization and gene transactivation (Issa et al., 1998; Polly et al., 2000; Quack and Carlberg, 2000; Rachez and Freedman, 2000).

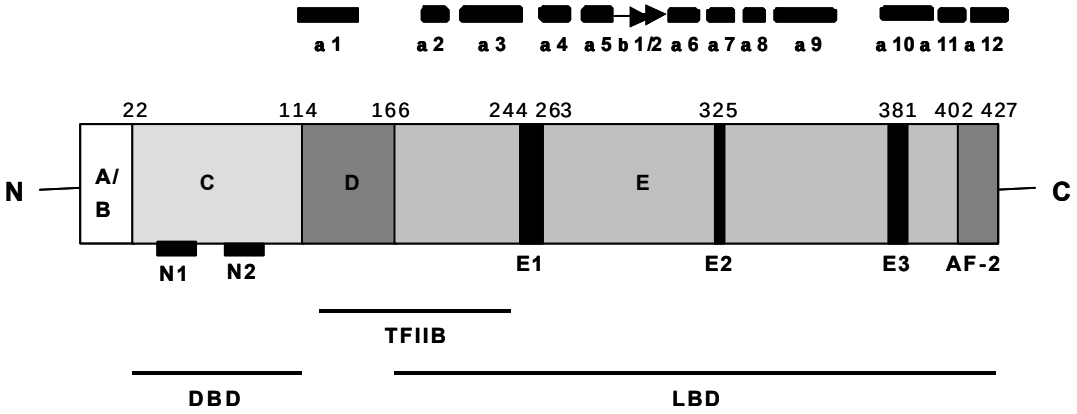


Fig. 10. Modular structure of the VDR. *The DNA binding domain (DBD, C) contains two potential nuclear transfer signals (N1 and N2). The ligand binding domain (LBD, E) contains three putative heterodimerization interfaces (E1, E2, and E3). A TFIIB interface is also indicated. The 12* a*-helices and 2* b*-sheets of the LBD are shown above as tubes and arrows, respectively (adapted from (Issa et al., 1998)).*

Transcriptional transactivation by a ligand-activated nuclear hormone receptor requires the close proximity to the basal transcription machinery, which is achieved through specific binding to REs. Thus, primary $1,25(OH)_2D_3$ responding genes contain within their promoter region a VDRE. Since VDR monomer affinity to VDREs is rather weak, efficient DNA binding requires homo- and/or heterodimerization with an accessory partner receptor, being predominantly RXR. In fact, many NRs heterodimerize with RXR. In these various heterodimeric complexes, RXR seems to assume different conformations and displays an altered sensitivity to its ligand 9-cis retinoic acid (RA). VDR-RXR, similarly to T_3R-RXR and RAR-RXR, cannot be activated by 9-cis RA to a large extent, unless it is already stimulated by $1,25(OH)_2D_3$. The synergistic action between VDR and RXR appears to depend on sufficient amounts of RXR, since otherwise other competing, RXR interacting receptors can lead to transrepressive effects. Moreover, at limiting concentrations of RXR, 9-cis RA mediates the

dissociation of heterodimer complexes and induces homodimerization, thus leading to a squelching effect. The VDR bound to a specific VDRE exibits distinct preferences for particular RXR isoforms, which influences the positive response to $1,25(OH)_2D_3$ (Carlberg and Polly, 1998; Carlberg and Saurat, 1996; Thompson et al., 1999).

Simple REs for a dimerizing NR, like the VDR, are formed by two hexameric core binding motifs that are arranged as a direct repeat (DR), a palindrome or an inverted palindrome (IP). The VDR consensus core binding motif has the sequence RGKTCA (R = A/G, K = G/T). Nevertheless, several natural VDREs carry a rather degenerated motif, such as the VDRE identified in the human 5-LO promoter (AGGGCA AAG GGTGGA). These class II elements are generally weaker than the perfect or reasonable class I VDREs when isolated from their natural promoter context. According to the "3-4-5 rule" proposed by Umesono *et al*, optimal VDREs are direct repeats spaced by three nucleotides (DR3) (Umesono et al., 1991) and the majority of the naturally occurring VDREs that have been identified so far, are DR3-type elements. VDR heterodimers have been shown to adopt a predefined polarity on VDREs. Since the receptor, which is in the 3' position is more easily activated (at lower ligand concentrations), the heterodimerization confers a polarity-directed ligand sensitivity. The polarity of binding and the optimal distance of core binding motifs are based on the spacing requirements of the DBDs and the location of their dimerization interfaces. Binding of the first ligand presumably leads to conformational changes in both heterodimeric partners and thereby increases the sensitivity to the second ligand, which is termed allosteric synergistic ligand interaction. Beside the simple VDREs, several natural VDREs are complex VDREs that are composed of more than two core binding motifs and/or overlap with binding sites for other transcription factors like AP-1, TBP, cAMP-regulated enhancer-binding protein (CREB) or CTF/NF-1 (Carlberg, 1996; Carlberg, 2003).

The VDR has been shown to interact with various compounds of the transcriptional machinery. Many coactivators contain three copies of a homologous LXXLL motif through which they bind to the AF-2 domains and a lysine residue in helix 3 of the LBDs of DNA-bound NRs. They enhance ligand-dependent transcriptional activation (fig. 11). The main members of the NR coactivator gene family SRC/p160 SRC-1/NCoA-1, transcriptional intermediary factor (TIF2)/ GRIP1/ SRC-2/ NCoA-2, and receptor associated coactivator 3 (RAC3)/ ACTR/ p300/CBP integrator protein (PCIP)/ SRC-3 appear to be interacting with many NRs and binding to the VDR has

already been experimentally proven. In glutathione S-transferase (GST) pull-down assays VDR did not display any coactivator selectivity (Carlberg, 2003; Carlberg and Polly, 1998; Freedman, 1999; Issa et al., 1998; Jones et al., 1998; Rachez and Freedman, 2000).

Further, the corepressor proteins NCoR, RIP13Δ1, SMRT and Alien can interact with VDR and mediate ligand-independent transrepression (fig. 11). NCoR has been shown to bind to the hinge region of RAR and T_3R. In COS-1 and yeast cells binding of unliganded VDR-RXR heterodimers to rat CYP24 VDREs exerts transcriptional repression via interaction with the corepressor RIP13Δ1, whereas ligand activation leads to dissociation of the corepressor and binding of a coactivator. VDR-RXR-Alien complexes mediate repression only through DR3-type VDREs and not through IP9-type VDREs (Carlberg, 2003; Carlberg and Polly, 1998; Jones et al., 1998; Polly et al., 2000; Rachez and Freedman, 2000).

The third category within the cofactor group are the cointegrator proteins such as CBP/p300. They can cooperate with NRs, coactivators, corepressors, and the preinitiation complex, thus forming larger coactivator complexes (fig. 11). As already mentioned in chapter 1.12.2., coactivators such as SRC-1 and RAC3 and cointegrators like CBP/p300 contain intrinsic HAT activities, whereas transcriptional repressive complexes include for instance NCoR, Sin3 and HDAC. Thus, a link between NR signalling and chromatin organization is provided. Presumably, coactivator function is primarily the recruitment of CBP/p300, that appears to be the primary source of HAT activity (Carlberg and Polly, 1998; Freedman, 1999; Issa et al., 1998; Jones et al., 1998; Rachez and Freedman, 2000).

Further NR coactivator complexes without HAT activity are VDR interacting proteins (DRIP) and the analogous TR associated proteins (TRAP)/SMCC, activator-recruited co-factor (ARC), negative regulator of activated transcription (NAT), CRSP and mammalian Mediator. They are the target not only of NRs but also of many other transcription factors such as Sp1 and form a bridge from the LBD to the basal transcription machinery through a single subunit (DRIP205/TRAP220) (fig. 11). Since Mediator binds to the carboxy-terminal repeat domain (CTD) of RNA polymerase II, it has been assumed, that this complex targets RNA polymerase II holoenzyme to promoters. Since the DRIP-mediated potentiation of ligand-dependent VDR-RXR transcription activity depends on the presence of chromatin, it can be assumed, that the DRIP complex contains or recruits chromatin-remodelling activities other than HATs.

Ligand-activated VDR-RXR heterodimers seem to change rapidly between coactivators of the p160-family and those of the DRIP/TRAP family (Carlberg, 2003; Freedman, 1999; Issa et al., 1998; Rachez and Freedman, 2000).

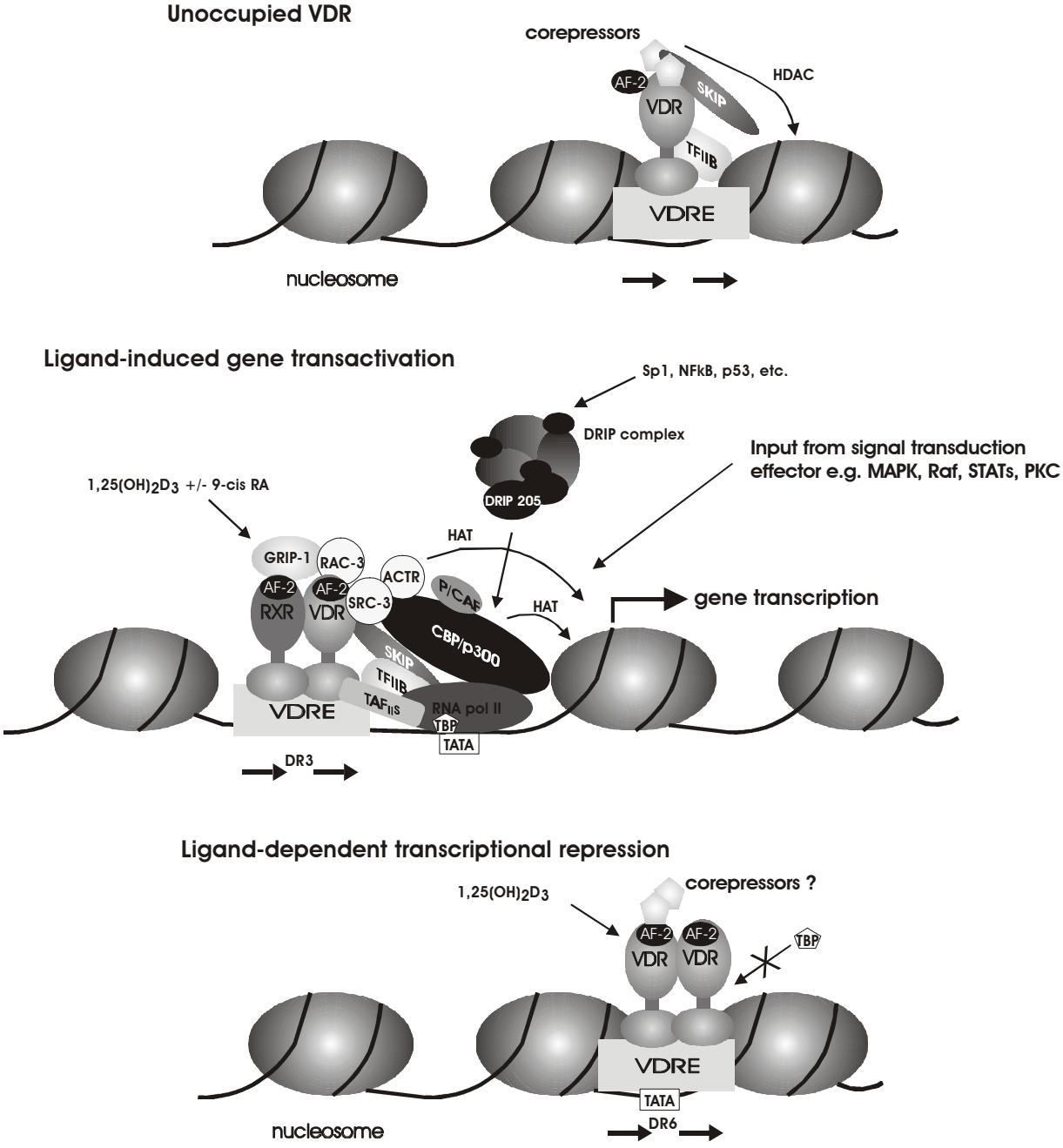


Fig. 11. Model for VDR regulation of gene transcription. *The various coregulators (middle panel) do not necessarily assemble at the same time; in fact, a cyclic association and dissociation has been reported for some cofactors (adapted from (Issa et al., 1998))*

In addition, direct protein-protein interactions of the VDR with components of the basal transcriptional machinery, like TFIIA and TFIIB and the TBP-associated factors

$TAF_{II}135$, $TAF_{II}55$, and $TAF_{II}28$ have been reported. Ligand binding causes dissociation of corepressors and TFIIB, thereby relieving repression (fig. 11) (Carlberg and Polly, 1998; Issa et al., 1998; Jones et al., 1998; Masuyama et al., 1997; Rachez and Freedman, 2000).

Recently, the coregulator Ski-interacting protein/Nuclear coactivator-62 kDa (SKIP/NCoA62) whose primary sequence is unrelated to other NR coactivators has been described to interact with the VDR. Like DRIP/TRAP it appears to lack chromatin modifying activity. SKIP/NCoA62 binds the VDR ligand-independently via a helix 10 interface that is functionally and physically distinct from the region bound by the AF-2-dependent p160 coactivators. The interaction is required for full VDR transactivation activity and the interface contains residues that are also involved in TFIIB and RXR binding. It was suggested, that SKIP/NCoA62 may regulate the exchange between TFIIB and RXR at the VDR helix 10 interface. SKIP/NCoA62 preferentially interacts with the VDR-RXR heterodimer as compared with the VDR monomer or homodimer and addition of ligand leads to a profound stabilization of the complex. GRIP1 and SKIP/NCoA62 form a ternary complex with VDR *in vitro* and *in vivo* (chromatin immunoprecipitation assays), SKIP/NCoA62 entering last, and synergistically enhance ligand-dependent VDR transcriptional activity in transient reporter gene assays. SKIP/NcoA62 also interacts with the Ski (Sloan-Kettering-Institute) oncoprotein and with other nuclear proteins such as the NRs RAR, ER, and GR, the repressor CBF-1, and the coregulators SMRT, Sin3A, HDAC2, and SRC-1. Further, it enhances TGFβ-dependent transactivation by interacting with Smad proteins. Thus, via specific interactions with corepressors or coactivators in a ligand-dependent mode, SKIP/NCoA62 might mediate a switch from transcriptional repression to activation. In addition, SKIP/NCoA62 has been shown to interact with components of the splicing machinery like pre-mRNA processing factor 8 (Prp8), U5 snRNP 200-kDa helicase, and Prp28, and with putative components of the nuclear matrix like matrin 3 and tubulin β 5. Expression of a dominant negative SKIP/NCoA62 led to a $1,25(OH)_2D_3$-dependent transient accumulation of unspliced growth hormone mRNAs transcribed from a VDRE-driven reporter gene cassette containing five exons and introns, each. Thus, SKIP/NCoA62 seems to be required for correct mRNA processing and might provide a functional link between VDR-mediated transcription and RNA splicing (Barry et al., 2003; Leong et al., 2001; Makarov et al., 2002; Zhang et al., 2001; Zhang et al., 2003).

To further complicate the complex network of regulatory mechanisms, the transcriptional regulator Yin-Yan-1 (YY1), that is located in the nuclear matrix, has been described to competitively block the binding of VDR-RXR heterodimers to the VDRE from the rat osteocalcin gene. Dependent on the cellular protein context, YY1 can activate or repress transcription via interactions with compounds of the basal transcription machinery, such as TFIIB, transcription factors like Sp1, CREB, and CBP/p300, and HDACs (Carlberg and Polly, 1998; Doetzlhofer et al., 1999; Issa et al., 1998; Yang et al., 1997).

Beside VDREs conferring transcriptional activation upon ligand stimulation, also repressor VDREs have been described for instance for the IL-2 and PTH and PTH-related peptide (PTHrP) gene promoters. In the case of the repression of IL-2 gene expression the VDR is suggested to act as a DNA-independent modulator of nuclear signalling pathways (Issa et al., 1998; Jones et al., 1998; Quack and Carlberg, 2000).

$1,25(OH)_2D_3$ has not only been described to augment transcription of target genes but also to enhance mRNA stability as reported for the epidermal growth factor receptor (EGFR) (González et al., 2002).

Because of its above described effects, $1,25(OH)_2D_3$ has been identified as a potential therapeutic in diseases like osteoporosis, psoriasis, multiple sclerosis, cancer, rheumatoid arthritis, diabetes, hyperparathyroidism, and in transplantation. However, severe side effects led to the development of several analogs aiming to dissociate antiproliferative and calcemic activities (fig. 12). The first generation of compounds contains the prodrugs 1-OH-D_3 and 1-OH-D_2 that are currently used to treat osteoporosis. The main target for structure modification was the C17 side chain, attempting to increase the metabolic clearance rate by decreasing interactions with the serum protein vitamin D-binding protein (DBP). Only those analogs are VDR agonists that cause both an efficient dissociation of corepressors from the receptor as well as a specific binding of coactivators. All agonists show an identical mode of action (EC_{50} values of about 0.1 nM), but interestingly some superagonists display a RE-type selectivity. Among the most potent agonists are the antipsoriatic drugs calcipotriol (MC903) and 22-oxacalcitriol or the highly antiproliferative seocalcitol (EB1089). Limited protease digestion assays with various $1,25(OH)_2D_3$ analogs led to the differentiation of an agonistic, an antagonistic and a non-agonistic conformation of the VDR. They differ in the orientation of helix 12. Antagonists bind to the LBD of VDR with sufficient affinity and stabilize the VDR in the antagonistic conformation, in which

the association of coactivators is blocked. VDR antagonists (i.e. ZK159222) have relatively bulky ring structures in their side chains. However, corepressor dissociation seems not to be prevented. The differentiation of the analogs into agonists and antagonists is somewhat difficult, though, as many of them are partial agonists and/or function as agonists in certain tissues and antagonists in others. This is possibly due to different expression levels of coactivator and corepressor proteins. Therefore, the term selective VDR modulator has been proposed. Analogs with two side chains at C20 such as Gemini stabilize the VDR in a conformation, in which corepressors do not dissociate from the receptor, thus blocking interaction with coactivators. These compounds are referred to as non-agonists. In the case of exposure of DNA-bound VDR-RXR heterodimers to low corepressor levels versus an excess of coactivator proteins, non-agonists turn into agonists or even superagonists (Berg and Haug, 1999; Carlberg, 2003; Issa et al., 1998; Jones et al., 1998).

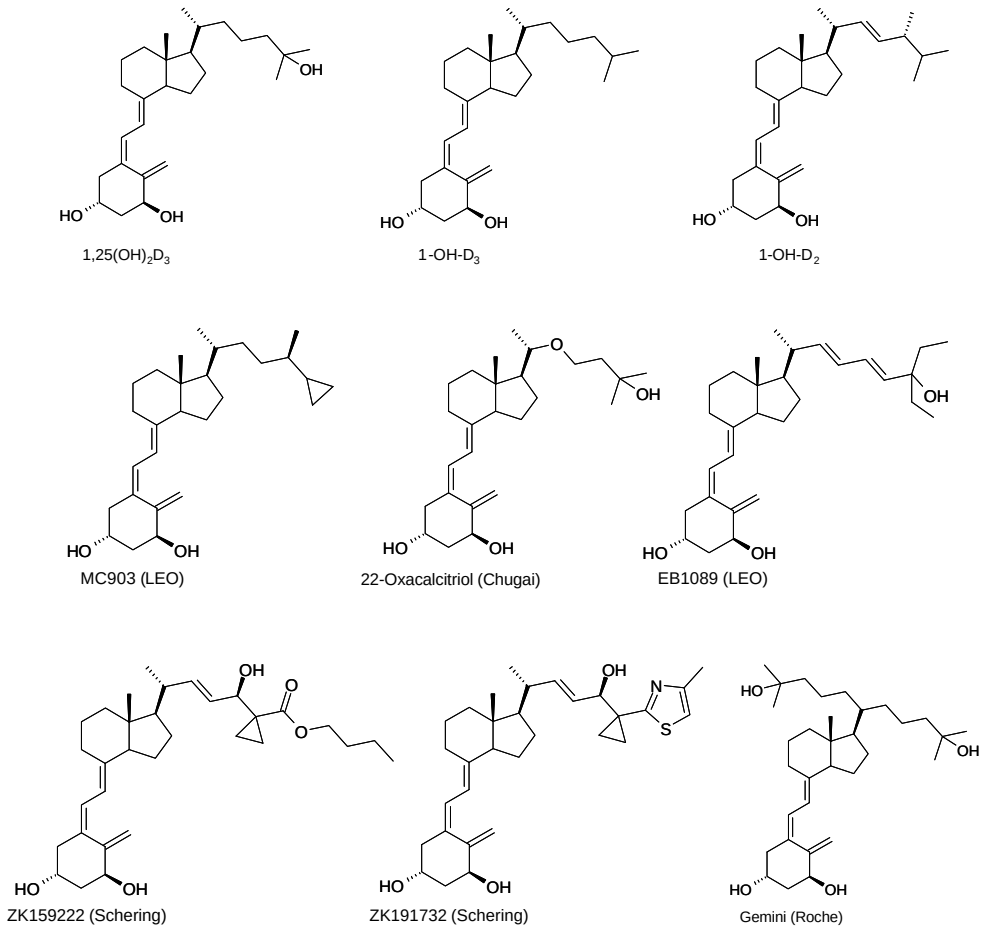


Fig. 12. Structure of 1,25(OH)$_2$D$_3$ and selected analogs.

5-LO activity, protein expression and mRNA strongly increase upon differentiation of myeloid cell lines such as MM6 and HL-60 with $1,25(OH)_2D_3$/TGFβ (Brungs et al., 1994; Brungs et al., 1995). In MM6 cells, treatment with $1,25(OH)_2D_3$ and TGFβ led to an up to 5-fold increase in primary transcripts, a 64-fold increase in mature 5-LO mRNA, a 128-fold increase in protein expression, and a more than 500-fold increase in 5-LO activity. No significant induction of 5-LO transcription was found in nuclear run-off assays, though (Härle et al., 1999). Both $1,25(OH)_2D_3$ and TGFβ were required for these profound responses. Either agent alone induced small amounts of 5-LO protein and mRNA but only very low 5-LO activities (Härle et al., 1998). As will be further discussed in chapter 1.12.4, the regulation of 5-LO gene expression by $1,25(OH)_2D_3$/TGFβ is but one example for several lines of evidence for an interplay between the $1,25(OH)_2D_3$ and TGFβ signalling pathways.

The induction of 5-LO activity in MM6 cells by $1,25(OH)_2D_3$/TGFβ could be suppressed by 75 to 85 % when the $1,25(OH)_2D_3$ antagonist ZK191732 was added (Gaschott et al., 2001). In contrast, three new potent VDR agonists have been identified due to their induction of 5-LO activity in MM6 cells (Werz et al., 2000).

1.12.4 The TGFb/Smad signalling system

As mentioned above (chapter 1.12.3), 5-LO activity, protein expression and mRNA strongly increase upon incubation of myeloid cell lines with TGFβ/$1,25(OH)_2D_3$ (Brungs et al., 1994; Brungs et al., 1995). Hence, transcriptional control by the TGFβ/Smad signalling system is of particular interest.

The TGFβ superfamily of secretory peptides is composed of many multifunctional cytokines including various forms of TGFβ, the bone morphogenetic proteins (BMPs), the nodals, the activins, the inhibins, the anti-Müllerian hormone (AMH), myostatin and many others. These factors regulate cell migration, adhesion, multiplication, differentiation and death and their effects differ depending on the type and state of the cell. The highly similar isoforms TGFβ1, TGFβ2, and TGFβ3 potently inhibit cellular proliferation of many cell types, whereas they stimulate growth of most mesenchymal cells. In addition, TGFβs strongly induce extracellular matrix synthesis and integrin expression, inhibit hematopoietic functions and modulate immune responses (Massagué and Wotton, 2000; Piek et al., 1999). Thus, disruptions of TGFβ signalling pathways

underlie many human disorders such as cancer, fibrotic or inflammatory disorders (Massagué, 1998).

TGFβ is synthesized as a prohormone. After cleavage, the mature hormone remains bound to the propeptide latency-associated protein (LAP). Recognition by the signalling receptors requires a multistep activation process that is currently poorly understood. The bioactive forms of TGFβ and related factors are dimers.

Smad proteins are the downstream effectors of TGFβ signalling and can be divided into three groups. The receptor-regulated Smads (R-Smads) comprise the human members Smad1, 2, 3, 5 and 8 and their cooperating Smad (or common-partner/collaborating/co-mediator Smad, Co-Smad) is Smad4. The human inhibitory Smads (I-Smad/Anti-Smads) are Smad6 and 7. Smads1, 5 and 8 transmit signalling by BMP receptors, while Smads2 and 3 mediate signalling by TGFβ and activin receptors (Massagué and Wotton, 2000).

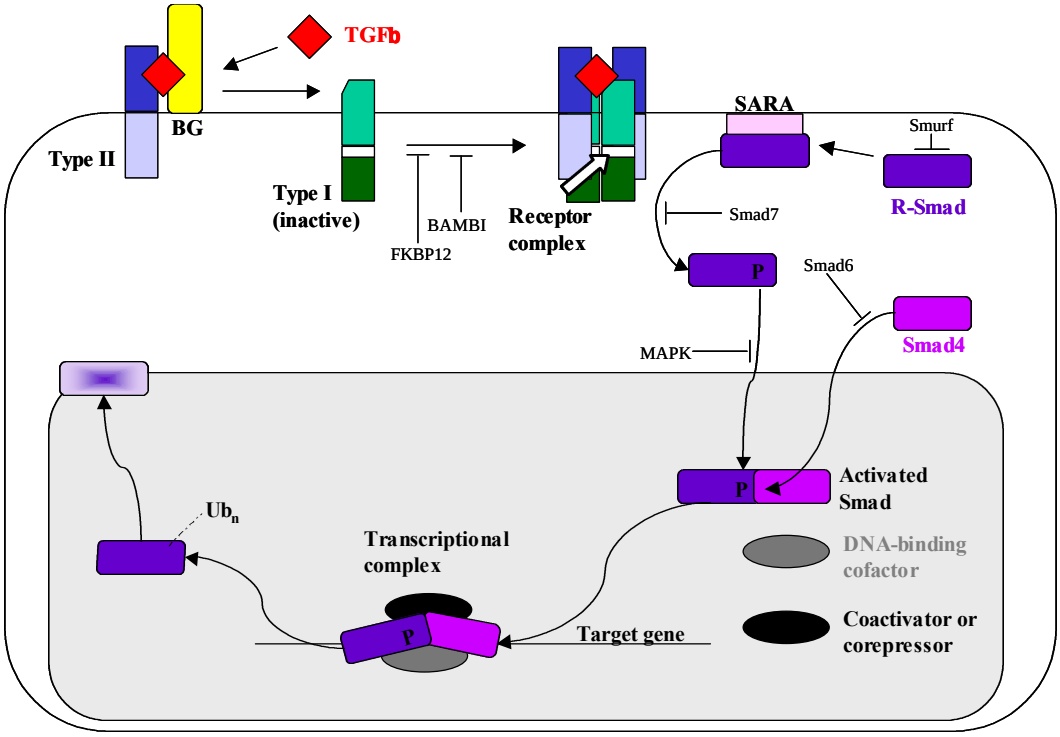


Fig. 13. The TGFb/Smad signalling pathway. *The two-step transcriptional activation process involves two families of membrane receptor protein kinases and their substrates, the Smad proteins. The Smad complex acts as transcription factor. (Adapted from (Massagué and Wotton, 2000)).*

Binding of the ligand TGFβ to its type II receptor (facilitated by betaglycan (BG)/TGFβ type III receptor) leads to assembly with the type I receptor, both of which are transmembrane protein serine/threonine kinases. In this ligand-induced complex, the constitutively active TGFβ receptor II (TβRII) activates the TβRI by phosphorylation of its GS region (the complex containing two TβRIIs and TβRIs, each). The immunophilin FKBP12 suppresses receptor phosphorylation by binding to the unphosphorylated GS regions of TβRIs. BMP exerts negative-feedback control through the protein BMP and activin membrane-bound inhibitor (BAMBI) that forms heterodimers with type I receptors and interferes with their activation. TβRIs specifically phosphorylate R-Smads in their SSxS (x = M/V) motif, leading to activation, complex formation with Co-Smads and nuclear accumulation. The membrane associated protein Smad anchor for receptor activation (SARA) promotes the interaction of Co-Smads with the activated TβRs, whereas the inhibitory Smads Smad6 and Smad7 block activated Smad1 and the activated receptors, respectively. Basal levels of Smad1 and Smad5 are regulated by the E3 ubiquitin ligase Smad ubiquitination regulatory factor-1 (Smurf1), administering them to ubiquitination and degradation. Following translocation to the nucleus, the heteromeric Smad complex binds to DNA via SBEs (CAGAC or even AGAC) and certain G/C-rich sequences, recruits transcriptional coactivators (CBP/p300, PCAF) and activates transcription (Itoh et al., 2000). Phosphorylation of R-Smads by Ras-activated ERKs attenuates nuclear accumulation (Fig. 13, (Massagué and Chen, 2000; Massagué and Wotton, 2000)).

Because of the weak affinity of Smad MH1 domains to SBEs, probably additional DNA contacts via transcriptional partners are required. These cofactors bind to their corresponding cognate sequences located at specific distances from the SBEs and to the Smad MH2 transactivation domains. They determine the choice of target genes and comprise DNA-binding adaptors (e.g. forkhead activin transducers (FAST-1/-2), olf-associated zink finger (OAZ)), constitutive (e.g. transcription factor binding to immunoglobulin heavy constant μ enhancer 3 (TFE3), core-binding factor A (CBFA)) and signal-regulated (e.g. AP-1) transcription factors (Massagué and Wotton, 2000). Among the transcription factors reported to interact with Smad proteins are some of which binding sites have been identified in the 5-LO promoter, such as Sp1 (Feng et al., 2000; Moustakas and Kardassis, 1998; Pardali et al., 2000), Sp3 (Lai et al., 2000), AP-1 (Brodin et al., 2000; Takeshita et al., 1998; Wong et al., 1999), AP-2, NFκB (López-

Rovira et al., 2000) and the VDR. The VDR has been shown to physically and functionally interact with Smad3 and Smad7 *in vitro* and *in vivo*, thus crosslinking TGFβ and $1,25(OH)_2D_3$ signalling (Aschenbrenner et al., 2001; Yanagi et al., 1999; Yang et al., 2001b). At least one member of the SRC-1/TIF2 protein family has been shown to be required for the interaction (Yanagi et al., 1999; Yanagisawa et al., 1999). The spacing between VDRE and SBE significantly affects the synergistic transcriptional activation by TGFβ and $1,25(OH)_2D_3$ (Subramaniam et al., 2001). In one study, multiple mechanisms responsible for TGFβ/$1,25(OH)_2D_3$ crosstalk have been reported. Thus, ligand-binding to the VDR induces TGFβ mRNA expression, the VDR enhances TβRII expression in certain cell types, Smad3 activates the VDR and the crosstalk is dependent on the PI 3-kinase pathway (Yang et al., 2001b).

Also, negative regulation of TGFβ signalling by interactions with transcription factors such as the GR (Song et al., 1999), Smad interacting protein 1 (SIP1) or Evi-1 has been reported (Watanabe and Whitman, 1999).

In addition, Smad proteins are suggested to activate transcription by relieving the action of transcriptional repressors. Furthermore, suppression of transcription by the recruitment of corepressors (e.g. TG3-interacting factor (TGIF), the proto-oncogene cSKI, Ski-related novel gene N (SnoN)), has also been described (Massagué and Wotton, 2000). The nuclear hormone receptor-interacting cofactor SKIP has been described to associate with Ski and Sno and to act as coactivator of TGFβ-dependent transcription. Thus, SKIP appears to modulate both TGFβ and NR signalling pathways (Leong et al., 2001) (also see chapter 1.12.3).

Recently, two proteins that are involved in RNA metabolism have been identified as Smad binding proteins by yeast two-hybrid and GST pull-down assays. Splicing factor 3b subunit 2 (SF3b2) is involved in the excision of introns from pre-mRNA. The role of eukaryotic translation initiation factor 4E nuclear import factor 1 (4-ET) is the nucleocytoplasmic shuttling of eukaryotic translation initiation factor 4E (eIF4E). These interactions may provide a functional link between TGFβ signalling and RNA processing (Warner et al., 2003).

Cell type specificity of Smad-mediated gene responses are probably due to different expression patterns of Smad-interacting proteins.

For the relevance of TRAP1 in TGFβ signalling and as a protein binding to 5-LO, see chapter 1.5.

Finally, a crosstalk between the TGFβ and the kinase pathways is suggested. Antagonistic as well as cooperative interactions between TGFβ and Ras signalling have been described (Massagué and Chen, 2000). In addition, TGFβ and BMP can probably activate various MAPK signalling pathways. TGFβ can enhance the activity of AP-1 (a dimer of c-Jun and c-Fos) complexes through phosphorylation of c-Jun by c-jun NH_2-terminal kinase (JNK), and as well the activity of CREB complexes through phosphorylation of activating-transcription factor (ATF2) by p38. Activated Smads can associate with Jun or ATF2, *in vitro*. The mitogen-activated protein kinase kinase kinase (MAPKKK) family member TGFβ-activated kinase1 (TAK1) likely links the receptors with these pathways by direct interaction with MAPK kinases (MKKs) that subsequently activate JNK or p38 (Massagué, 2000; Massagué et al., 2000; Massagué and Chen, 2000). In addition, without Smad proteins being involved, TGFβ can directly activate MAPKs, PI 3-K, and other mediators (Engel et al., 1999; Kracklauer et al., 2003; Yang et al., 2003).

Recently, based on genome-level analysis, a hierarchical model of gene regulation by TGFβ has been proposed (fig. 14) (Yang et al., 2003). In this model, Yang and co-authors suggest, that Smad3 directly activates immediate-early target genes of TGFβ, encoding regulator proteins including signal transducers and transcriptional regulators. As the characteristic Smad3/Smad4 DNA-binding motif repeats are not present in secondary gene targets, these are probably regulated through lower affinity SBEs or by immediate-early target gene-encoded transcriptional regulators. Smad2 and ERKs might predominantly transmodulate immediate-early gene and intermediate gene regulation by TGFβ/Smad3.

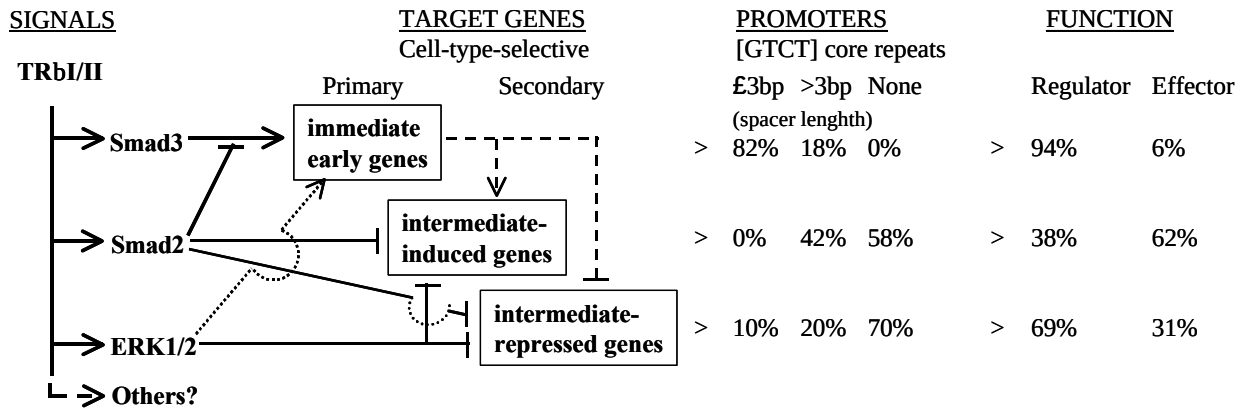


Fig. 14. Hierarchical model of gene regulation after ligand-induced activation of the TGFb receptor complex (TRbI/II)*. (Adapted from (Yang et al., 2003))*

1.12.5 Other inducers of 5-LO gene transcription

Depending on the cell type, several cytokines have been shown to be inducers of the 5-LO pathway. In granulocytes, 5-LO expression and AA liberation are stimulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) in response to platelet-activating factor (PAF) (McColl et al., 1991). IL-3 increases the expression of 5-LO, FLAP, $cPLA_2$ and LTC_4 synthase in mouse mast cells (Murakami et al., 1995).

Furthermore, enhanced steady state levels of 5-LO mRNA have been detected in response to differentiation and activation by RA, glucocorticoids, human chorionic gonadotropin, dbcAMP, DMSO, phorbol-12-myristate-13-acetate (PMA), okadaic acid, oxidized low density lipoproteins or Ca^{2+}-ionophore in various leukocyte cell lines (Bigby, 1999; Silverman and Drazen, 1999; Zhang et al., 1999). In HL-60 cells the upregulation is, at least in part, caused by TGFβ in serum (Steinhilber et al., 1993).

2 Aims of the present investigation

Due to the pharmacological relevance of 5-LO products the regulation of 5-LO gene expression is important for the pathogenesis of inflammatory diseases (Steinhilber, 1999; Werz, 2002a). Thus, regulatory mechanisms that influence 5-LO expression on transcriptional or posttranscriptional levels are of interest (Drazen and Silverman, 1999; Drazen et al., 1999; Silverman and Drazen, 2000).

In this study the contribution of epigenetic mechanisms (Uhl et al., 2002) as well as of the signalling pathways of the potent 5-LO stimuli TGFβ and $1,25(OH)_2D_3$ (Brungs et al., 1994; Brungs et al., 1995) to the induction of 5-LO gene expression should be characterised. A previously performed functional analysis of the 5-LO promoter (paper I; dissertation N. Klan) failed to identify sequence elements in the promoter that were required for the profound induction of 5-LO mRNA, protein, and activity during the differentiation of myeloid cell lines induced by TGFβ and $1,25(OH)_2D_3$ (Härle et al., 1999). Hence, the main aim of this thesis was to identify sequences outside of the promoter that might confer the activation of 5-LO gene expression by TGFβ and $1,25(OH)_2D_3$. For this purpose, several reporter gene plasmids containing different parts of the 5-LO gene were cloned and analysed in reporter gene assays.

Furthermore, possible crosstalks between the signalling pathways of 5-LO relevant transcription factors, epigenetic mechanisms and posttranscriptional events were subjects of the present investigation.

3 Methods

The following table lists the methods used in this thesis and indicates in which paper the respective method is described.

Method	**Paper**
Cloning of reporter gene plasmids (using PCR-methods and restriction enzymes)	II, III
Transient transfections (standard calcium phosphate method)	II, III
Reporter gene assays (luciferase and SEAP assays)	(I), II, III
RT-PCR	I, II
SDS-PAGE and Western blot	(I)

The following human cell lines have been used:

Cell line	**Paper/ chapter**
Mono Mac 6 cells (monocytes/macrophages)	II, 4.2.7, 4.5.2
U937 cells (lymphoma cells)	4.5.1
HeLa cells (cervix carcinoma cells)	II, III
Caco-2 cells (colon carcinoma cells)	4.2.2, 4.2.3, 4.4
HL-60 cells (promyelocytic leukemia cells)	I

4 Results

4.1 Vitamin D-responsive elements within the human 5-LO gene promoter bind VDR, but do not confer vitamin D-dependent transcriptional activation

Among the putative VDREs within the 5-LO promoter, the sequence located at positions -291 bp to -276 bp (AATTCA GGAG AGAACG) shows some homology to the DR4-type mouse phosphate intestinal transporter (mPit-1) VDRE (AGTTCA TGAG AGTTCA) and the sequence -309 bp to -294 bp (AGGGCA AAG GGTGGA) was previously proposed for possible VDR binding (Carlberg, 1995). DNase I footprinting assays and EMSAs that were performed by coauthors, revealed that the purified VDR binds to putative VDREs within the 5-LO promoter as a heterodimer together with RXR. EMSAs with nuclear extracts from TGFβ/1,25$(OH)_2D_3$-differentiated MM6 cells also indicated binding of additional protein(s) induced by TGFβ/1,25$(OH)_2D_3$ to the VDR/RXR/5LO-56 complex. Interestingly, TGFβ and 1,25$(OH)_2D_3$ had no effect on 5-LO promoter activity in transient reporter gene assays in different cell types (also performed by coauthors), which is consistent with results obtained by nuclear run-off assays using nuclear extracts from MM6 cells (Härle et al., 1999). Transient transfections and subsequent luciferase assays with systematically truncated 5-LO promoter reporter constructs plus expression vectors for VDR and RXR indicated the presence of two negative (-5814 bp to -5395 bp and -913 bp to -778 bp) and two positive regulatory regions (-778 bp to -229 bp and -141 bp to -78 bp) in MM6 cells. In HeLa cells, the inhibitory region from -913 bp to -778 bp and the positive regulatory region from -778 bp to -229 bp were not detected. In both cell lines the pN10 construct (-778 bp to +53 bp) caused the most profound induction of transcriptional activity. Coexpression of the receptors VDR and RXR did not significantly affect 5-LO promoter activity when the DNA sequence from -778 bp to -229 bp, which contains the putative VDREs, was present, but reduced 5-LO promoter activity in reporter gene constructs that lack the VDREs.

4.1.1 Transcription of the stably transfected 5-LO promoter CAT reporter gene in HL-60 cells is independent of TGFb/1,25(OH)$_2$D$_3$

In order to investigate whether integration of the 5-LO promoter into the genome is required for the upregulatory effects of TGFβ and 1,25(OH)$_2$D$_3$, HL-60 cells were stably transfected with 5-LO promoter-chloramphenicol acetyltransferase (CAT) constructs leading to the cell lines HL60-6079-5LO, which contains a stably integrated CAT gene under the control of the 5-LO promoter (-6079 to +81 bp), HL60-SV40 (where the CAT reporter gene is under the control of an SV40 promoter), and HL60-Basic (no promoter). Cells were cultured with or without 1 ng/ml TGFβ in combination with 50 nM 1,25(OH)$_2$D$_3$ and after 24 h, CAT and endogenous 5-LO mRNA expression were determined by RT-PCR. TGFβ and 1,25(OH)$_2$D$_3$ did not significantly affect CAT expression in HL60-6079-5LO cells and only slightly stimulated CAT mRNA expression in HL60-SV40 and in HL60-Basic cells (1.9- and 1.7-fold, respectively) (paper I: fig. 8A). In contrast, TGFβ and 1,25(OH)$_2$D$_3$ caused a 13.8-fold, 11.1-fold and 7.1-fold increase of endogenous 5-LO mRNA in HL60-SV40, HL60-Basic, and HL60-6079-5LO, respectively (paper I: fig. 8B).

4.2 The coding sequence mediates induction of 5-LO expression by 1,25(OH)$_2$D$_3$ and TGFb

The results reported in paper I (chapter 4.1) indicated, that the strong increase in 5-LO mRNA expression induced by TGFβ and 1,25(OH)$_2$D$_3$ are not mediated by the promoter but possibly by REs that are located outside of the 5-LO promoter region. Hence, the contribution of the coding region, the 3'-UTR and introns J, K, L and M to the induction of 5-LO gene expression was investigated.

4.2.1 1,25(OH)$_2$D$_3$ and TGFb induce accumulation of mature 5-LO mRNA

The 5-LO positive human myeloid cell line MM6 was incubated with 1 ng/ml TGFβ in combination with 50 nM 1,25(OH)$_2$D$_3$ for 24 hours. Subsequently, nuclear and cytosolic RNA was isolated and subjected to RT-PCR analysis, using either oligo(dT)$_{12-18}$ or random hexamer primers for cDNA synthesis for the determination of mature and pre-mRNA, respectively. The time course experiment revealed a prominent

increase of mature 5-LO mRNA in the nucleus as well as in the cytosol following incubation with $1,25(OH)_2D_3$ and TGFβ, whereas pre-mRNA levels were not significantly elevated (paper II: fig. 1).

4.2.2 Cotransfection of VDR and RXR induces reporter gene activity when the 5-LO coding sequence is present

Reporter gene assays with plasmid constructs containing the 3'-UTR, the coding sequence (cds) and the last four introns (introns J, K, L and M) were performed in the presence and absence of expression vectors for VDR and RXR. For that purpose, the 3'-UTR, the cds and the last four introns were cloned into the 5-LO promoter reporter gene construct pN10 and in the pGL3 vectors (Promega) in such a way that the 5-LO cds was in frame to the luciferase sequence. Thus, in the cell, fusion proteins were generated. Two sets of luciferase reporter gene plasmids were constructed, the first containing the 5-LO core promoter (-778 to +53 bp) in the plasmid pGL3-Basic plus the different parts of the 5-LO gene, and the second containing the 5-LO cds plus the last four introns in front of the luciferase gene using the pGL3-Basic, pGL3-Promoter and pGL3-Control vector, respectively (paper II: fig. 2). The core promoter-containing pN10 construct was chosen for cloning, as it has previously been shown to cause the most profound reporter gene activity (chapter 4.1/paper I).

HeLa cells that do not express endogenous 5-LO were transiently transfected with the aforementioned reporter gene plasmids in the presence or absence of VDR and RXR expression constructs. Reporter gene activity of constructs containing the 5-LO promoter alone or in combination with the 3'-UTR was not upregulated by cotransfection of RXR/VDR (paper II: fig. 3). However, inclusion of the 5-LO cds led to an approximately 3-fold induction of luciferase activity by NR overexpression and addition of introns J-M even led to an about 6-fold induction. This upregulation seemed to be independent from the 5-LO promoter since it was also observed with plasmids containing no promoter, an SV40-promoter or an SV40-promoter plus -enhancer (pGL3-ba-cdsInJM, pGL3-prom-cdsInJM and pGL3-ctrl-cdsInJM, respectively).

All experiments have been performed using 800 ng reporter gene plasmid and 100 ng expression vector for the NRs, each (8/1). Various amounts of expression plasmids have been investigated revealing a dose-dependent upregulation (fig. 15). Although 200 ng expression vector (4/1) led to an even more profound induction of reporter gene

activity, 100 ng were considered to ensure the availability of sufficient amounts of VDR and RXR and were used for all subsequent experiments.

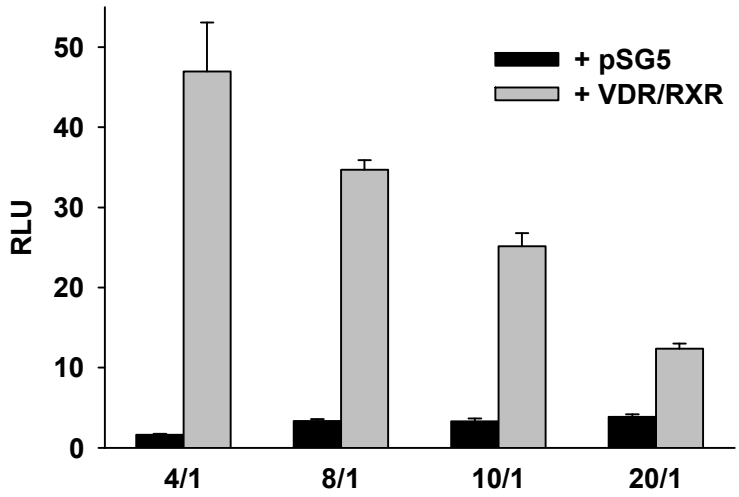


Fig. 15. VDR/RXR dose-dependently enhance luciferase activity of a coding sequence-containing reporter gene construct in transiently transfected HeLa cells. *The plasmid pN10-cds3UTR was transiently transfected into HeLa cells with or without increasing amounts of expression vectors for VDR and RXR. Luciferase activity was measured 24 h after transfection. Depicted are the relative light units (RLU) of the respective ratios of reporter to NR expression vectors. Each experiment was performed in triplicates and normalization for transfection efficiency was performed by cotransfection of pCMV-SEAP. Displayed is one representative out of at least three independent experiments (mean ± SE).*

Consistent with the data from reporter gene assays, RT-PCR with HeLa cells that were transiently transfected with pN10-cdsInJM revealed a 2.2- (±0.4) fold VDR/RXR-mediated induction of 5-LO pre-mRNA (random hexamer priming) and 4.6- (±1.8) fold induction of mature mRNA (oligo(dT)$_{18\text{-}20}$ priming).

In the 5-LO positive Caco-2 cells the plasmids pN10, pN10-cdsInJM and p(DR3)4tkluc led to 2.5-, 7.7- and 1.0-fold inductions by cotransfection of RXR/VDR, respectively (fig. 16).

In summary, the data show that the 5-LO cds mediates induction by VDR/RXR. Interestingly, in both cell lines no effect of VDR/RXR overexpression was observed with the p(DR3)4tkluc plasmid that contains a 4-times concatemerized rat atrial natriuretic factor (rANF) VDRE.

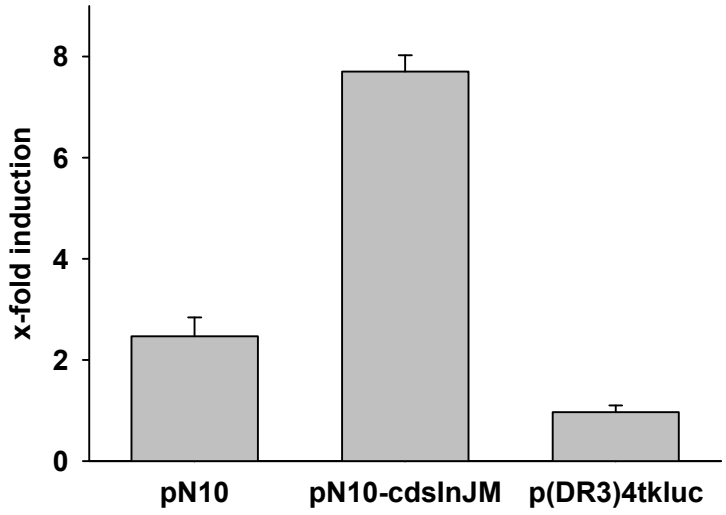


Fig. 16. Effects of VDR/RXR on the transcriptional activity of luciferase reporter gene constructs in transiently transfected Caco-2 cells. *The indicated plasmids were transiently transfected into Caco-2 cells with or without expression vectors for VDR and RXR. p(DR3)4tkluc, containing a four times concatemerized DR3-type VDRE in front of the thymidine kinase promoter, was used as positive control. Luciferase activity was measured 24 h after transfection. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to pSG5-cotransfected cells.*

4.2.3 Induction of reporter gene activity by 1,25(OH)$_2$D$_3$ and TGFb is also mediated by the 5-LO coding sequence

In order to investigate whether the effect of 1,25(OH)$_2$D$_3$ and TGFβ also depends on sequences located outside of the promoter like the coding region and 3'-UTR, reporter gene assays with the corresponding plasmid constructs (paper II: fig. 2) were performed. HeLa and Caco-2 cells were incubated with 1 ng/ml TGFβ for 24 hours before transfection. After exchange of medium, cells were incubated with 1 ng/ml TGFβ and/or 50 nM/1 µM of 1,25(OH)$_2$D$_3$ for another 8/22 hours (HeLa and Caco-2 cells, respectively) before luciferase activity was determined. In both cell lines TGFβ alone only slightly increased reporter gene activity. However, in HeLa cells the combination of both agents revealed a significant synergistic effect with reporter gene constructs containing the coding sequence (paper II: fig. 4). The upregulation by 1,25(OH)$_2$D$_3$ and TGFβ varied for the used constructs. With pN10 and pN10-3UTR, induction by TGFβ and 1,25(OH)$_2$D$_3$ did not differ from the negative control

pGL3-Basic, whereas the presence of the 5-LO cds (pN10-cds) led to a 5.1-fold induction and inclusion of the cds plus introns J-M (pN10-cdsInJM) to a 12.4-fold upregulation. The reporter gene activity of the promoterless plasmid pGL3-ba-cdsInJM was also induced (about 16-fold) and the plasmid p(DR3)4tkluc (containing a 4-times concatemerized VDRE) employed as positive control was induced by 9-fold. Interestingly, introduction of the cds plus the introns J-M downstream of the translational stop codon of the luciferase gene (pN10-cdsInJM-inv) led to a significant reduction of inducibility (3.6-fold). Preincubation with TGFβ was required to obtain the prominent synergistic effects, indicating that the effect represents a secondary response (data not shown). Incubation of the transiently transfected cells with increasing amounts of $1,25(OH)_2D_3$ revealed an explicit dose-dependency (fig. 17). Since the concentration of 50 nM is the standard concentration used in previous studies, all subsequent experiments have been performed with 50 nM $1,25(OH)_2D_3$.

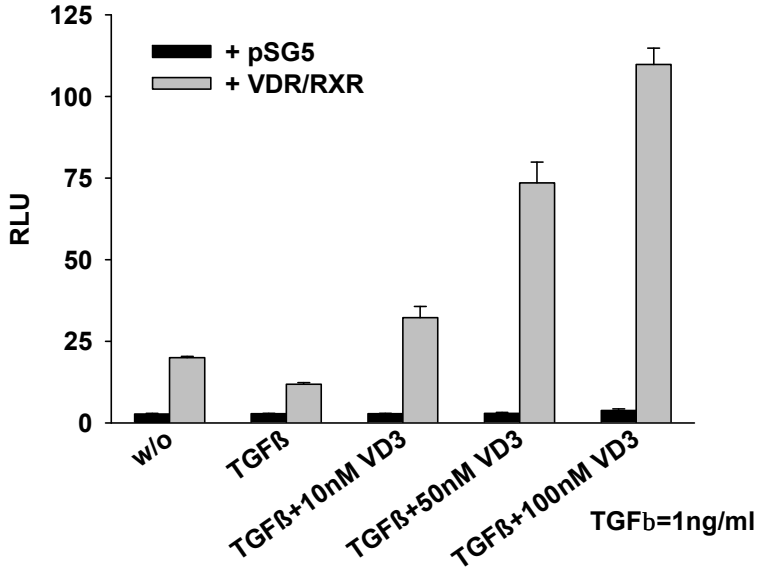


Fig. 17. $1,25(OH)_2D_3$ dose-dependently enhances luciferase activity of a coding sequence-containing reporter gene construct in transiently transfected HeLa cells. *The plasmid pN10-cdsInJM was transiently transfected into HeLa cells with or without expression vectors for VDR and RXR. 16 h after transfection 1 ng/ml TGFb and increasing amounts of $1,25(OH)_2D_3$ (VD3) were added for another 8 h before luciferase activity was determined. Each experiment was performed in triplicates and normalization for transfection efficiency was performed by cotransfection of pCMV-SEAP. Displayed is one representative out of at least three independent experiments ± SE (Cells were not preincubated with TGFb!) (RLU: relative light units).*

In Caco-2 cells (fig. 18) considerably higher amounts of $1,25(OH)_2D_3$ (1 µM) were required to obtain significant inducing effects (2.9-fold for pN10-cdsInJM). No

synergistic action of TGFβ and $1,25(OH)_2D_3$ was observed. Interestingly, $1,25(OH)_2D_3$-mediated induction of the control plasmid p(DR3)4tkluc was considerably higher than in HeLa cells (16.5-fold).

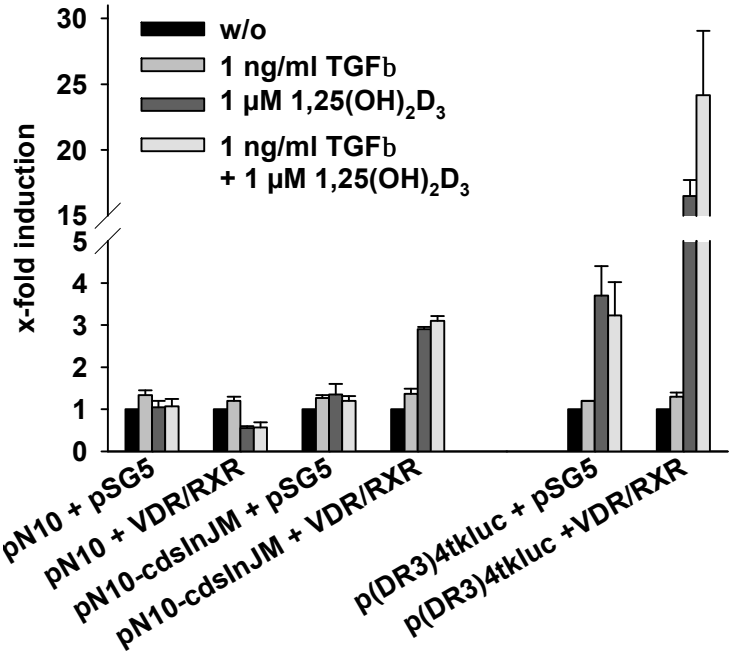


Fig. 18. In Caco-2 cells $1,25(OH)_2D_3$ mediates upregulation of luciferase activity, but no synergistic action of $1,25(OH)_2D_3$ and TGFb can be observed. *The indicated plasmids were transiently transfected into Caco-2 cells with or without expression vectors for VDR and RXR. Before transfection, cells were preincubated with TGFb (1 ng/ml) for 24 h and 16 h after transfection $1,25(OH)_2D_3$ (1 µM) and/or TGFb (1 ng/ml) were added for another 22 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP.*

In agreement with the well-known fact that VDR binds to DNA predominantly as heterodimer with RXR, overexpression of both NRs was required for upregulation of 5-LO expression by TGFβ and $1,25(OH)_2D_3$ (fig. 19).

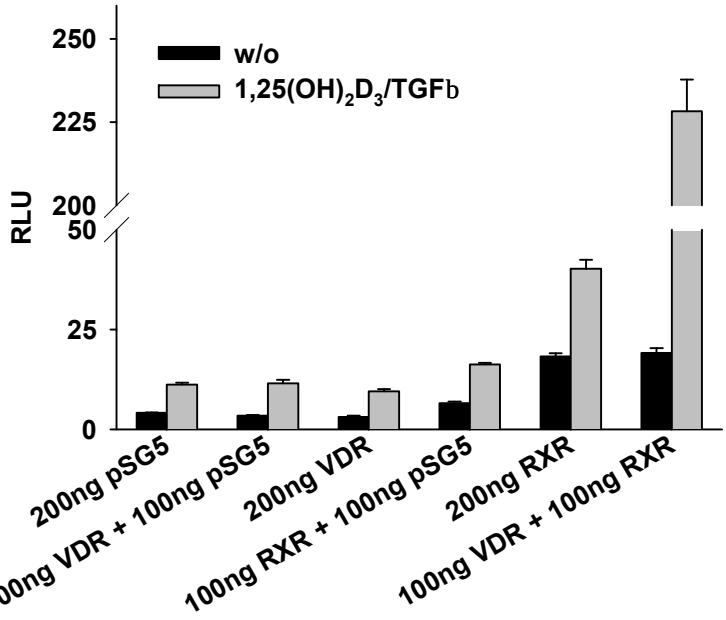


Fig. 19. Cotransfection of VDR and RXR is required for full upregulation of pN10-cdsInJM by 1,25(OH)$_2$D$_3$ and TGFb in transiently transfected HeLa cells. *pN10-cdsInJM was transiently transfected into HeLa cells with or without expression vectors for VDR and/or RXR. Before transfection, cells were preincubated with TGFb (1 ng/ml) for 24 h and 16 h after transfection 1,25(OH)$_2$D$_3$ (50 nM) and TGFb (1 ng/ml) were added for another 8 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP (RLU: relative light units).*

Taken together, the data show that the 5-LO cds mediates induction of reporter gene activity by 1,25(OH)$_2$D$_3$ and TGFβ.

4.2.4 1,25(OH)$_2$D$_3$ and TGFb regulate 5-LO mRNA processing

In order to determine whether the effects of 1,25(OH)$_2$D$_3$/TGFβ occur on the mRNA level, HeLa cells were transiently transfected with pN10-cdsInJM, pSG5-VDR and pSG5-RXR. The formed mRNA transcripts were subsequently analysed by RT-PCR. Cells were preincubated with TGFβ (1 ng/ml) for 24 h and after transfection 1,25(OH)$_2$D$_3$ (50 nM)/TGFβ (1 ng/ml) were added together with or without cycloheximide (CHX) (10 µM) for 8 h. After extraction of total RNA, RT-PCR analysis was performed using either oligo(dT)$_{18\text{-}20}$ or random hexamer priming for cDNA synthesis and different sets of primers for PCR reactions (paper II: tab. 1, fig. 5). With primer pairs that are located in the beginning or the end of the coding sequence, 28 PCR cycles were sufficient to obtain defined PCR products of correct size. In contrast, 35

cycles were required when the respective primer pairs were located between exons 8 and 13 indicating that central parts of the cds are removed during mRNA processing. Interestingly, when a primer pair spaning the complete coding region was used (cds_start/Asurev), multiple PCR products were obtained, indicating that mRNA processing seems to occur at several sites. The $1,25(OH)_2D_3$/TGFβ-mediated induction of transcripts varied significantly (given as fold induction, paper II: fig. 5) depending on the RNA position that was analysed by PCR. The most prominent increase in 5-LO mRNA was observed when primers that span intron M like 5-LO1/Asurev (14.6-fold) and InJM_5/Asurev (9.8-fold) or primers that are located within intron M (7.8-fold) were used. This increase roughly corresponds to the measured enhancement of luciferase activity (12.4-fold). Interestingly, the induction obtained with these primer pairs was significantly inhibited when CHX was added together with $1,25(OH)_2D_3$ and TGFβ, indicating that protein synthesis was required (paper II: figs. 6B and D). Strikingly, when using primers cds_start/1883_rev or Ncofor1/Asurev that amplify exons 1 to 8 or parts of exon 14 and the luciferase gene, respectively, (paper II: fig. 5) only a weak increase in the respective PCR signals (6.0-fold and 2.5-fold, respectively) was obtained that was not inhibited by CHX (paper II: figs. 6A and C). These parts of the mRNA were prominently expressed since 28 cycles were sufficient to obtain clear PCR signals of correct size.

In summary, the characterization of the transcribed RNA species by RT-PCR leads to the suggestion that central parts of the 5-LO cds are removed during mRNA processing and that $1,25(OH)_2D_3$ and TGFβ induce the inclusion of exon 13 into the mature mRNA.

4.2.5 Upregulation of 5-LO mRNA processing by $1,25(OH)_2D_3$/TGFb does not depend on a distinct part of the coding sequence

In order to identify sequences in the coding region that participate in the $1,25(OH)_2D_3$/TGFβ-mediated regulation of 5-LO expression, deletion constructs of the vector pN10-cds were cloned and analyzed in reporter gene assays. The coding region was divided into four parts which were subsequently deleted (paper II: fig. 7). Domain A ranges from exon 1 to 4, domain B from exon 4 to 10, domain C from exon 10 to 14 and domain D represents the last part of exon 14 (paper II: fig. 5). The respective deletion of parts A to D reduced induction of reporter gene activity by

1,25(OH)$_2$D$_3$/TGFβ (paper II: figs. 8A and B). A significant decrease in 5-LO upregulation by 1,25(OH)$_2$D$_3$/TGFβ could be seen when parts B+C, A+B+C or B+C+D were deleted (from 5.1-fold to 2.6-, 2.0- and 2.2-fold, respectively). In constructs in which large parts of the cds are deleted (pdcdsABC and pdcdsBCD), the 1,25(OH)$_2$D$_3$-mediated induction of luciferase activity was completely lost and only the minor TGFβ-mediated enhancement could be measured.

Sequence analysis revealed the existance of some putative VDREs in the 5-LO coding sequence and even in the introns. The two most promising putative VDREs that are located in exon 2 and intron J were deleted and the resulting constructs were subjected to transient transfection studies as described above. As can be seen in figs. 20 A+B, deletion of the putative VDREs does neither significantly affect the VDR/RXR- nor the TGFβ/1,25(OH)$_2$D$_3$-mediated upregulation of 5-LO expression.

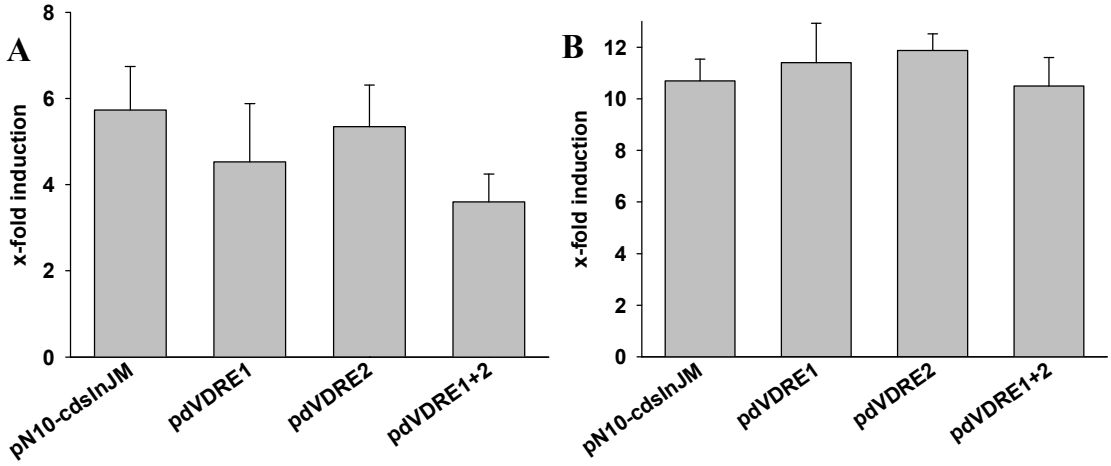


Fig. 20. Deletion of putative VDREs does neither affect the VDR/RXR- (A) nor the TGFb/1,25(OH)$_2$D$_3$- (B) mediated upregulation of 5-LO expression. *The indicated plasmids were transiently transfected into HeLa cells with or without expression vectors for VDR and RXR. Before transfection, cells were preincubated with TGFb (1 ng/ml) for 24 h and 16 h after transfection 1,25(OH)$_2$D$_3$ (50 nM) and TGFb (1 ng/ml) were added for another 8 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to pSG5-cotransfected (A) or to untreated cells (B).*

In summary, the deletion analysis demonstrates that for an almost complete loss of the response to 1,25(OH)$_2$D$_3$/TGFβ, removal of large parts of the cds is required. This suggests that the regulation of the 5-LO mRNA processing by TGFβ and 1,25(OH)$_2$D$_3$ involves multiple parts of the cds rather than being mediated by distinct REs.

4.2.6 Inclusion of the 5-LO coding sequence plus introns J-M changes the kinetics of 1,25(OH)$_2$D$_3$/TGFb-mediated upregulation of reporter gene activity

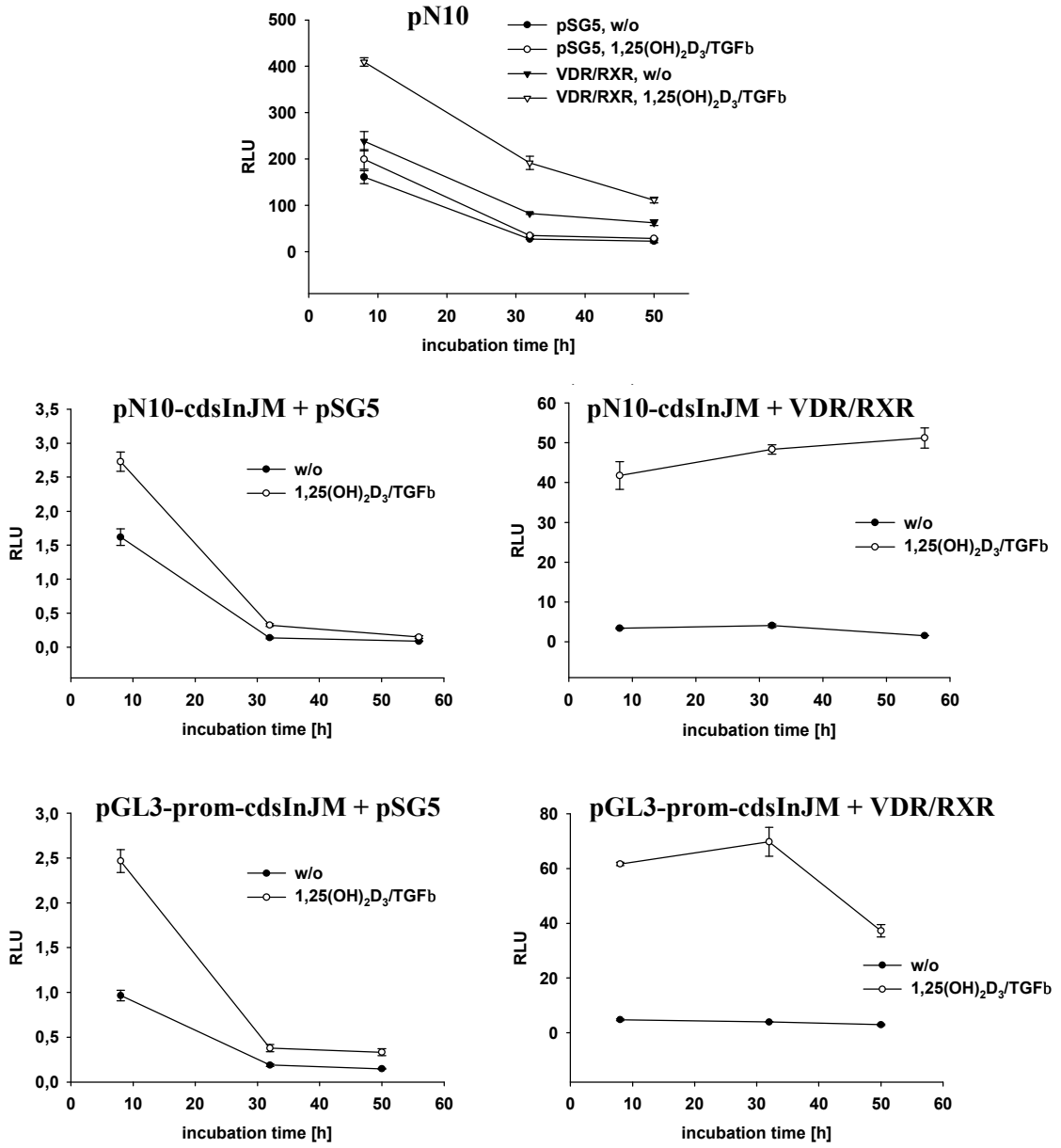


Fig. 21. Long-term kinetics of TGFb/1,25(OH)$_2$D$_3$-mediated 5-LO upregulation. *The indicated plasmids were transiently transfected into HeLa cells with or without expression vectors for VDR and RXR. Before transfection, cells were preincubated with TGFb (1 ng/ml) for 24 h and 16 h after transfection 1,25(OH)$_2$D$_3$ (50 nM) and TGFb (1 ng/ml) were added for the indicated times before luciferase activity was determined. Each experiment was performed in triplicates. Results are normalized for transfection efficiency by cotransfection of pCMV-SEAP (RLU: relative light units).*

To further determine the 5-LO upregulating effect long-term kinetics were measured. HeLa cells were preincubated with TGFβ (1 ng/ml) for 24 hours and were transfected

with the plasmids pN10, pN10-cdsInJM or pGL3-prom-cdsInJM. After change of medium, the cells were incubated with TGFβ (1 ng/ml) and 1,25$(OH)_2D_3$ (50 nM) for up to 56 hours. Remarkably, luciferase activity of cells transfected with pN10 decreased in a time-dependent manner, independently from cotransfection with VDR/RXR and incubation with 1,25$(OH)_2D_3$/TGFβ (fig. 21 A). In contrast, luciferase activity of cells transfected with pN10-cdsInJM (figs. 21 B+C) or pGL3-prom-cdsInJM (figs. 21 D+E) scarcely decreased, when the NRs were cotransfected and cells were incubated with 1,25$(OH)_2D_3$/TGFβ. Thus, interaction of VDR/RXR heterodimers with the 5-LO cds and/or introns J-M seem to prolong the response to 1,25$(OH)_2D_3$ and TGFβ.

4.2.7 Depending on cell type and assay system, the 1,25$(OH)_2D_3$ analogon ZK191732 displays agonistic or antagonistic action on 5-LO expression

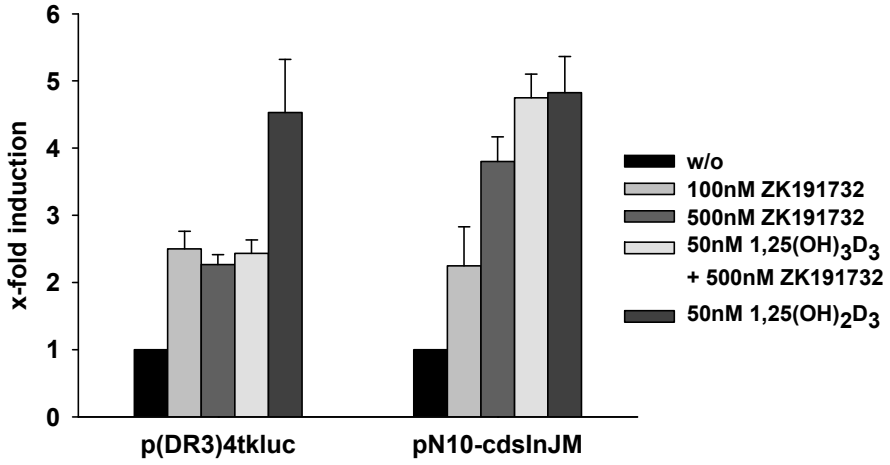


Fig. 22. Agonistic effect of the 1,25$(OH)_2D_3$ analogon ZK191732. *The indicated plasmids were transiently transfected into HeLa cells with expression vectors for VDR and RXR. 16 h after transfection the indicated amount of ZK191732 and/or 1,25$(OH)_2D_3$ (50 nM) were added for 8 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to untreated cells.*

To antagonize the 1,25$(OH)_2D_3$-mediated regulation, HeLa cells were transiently transfected with the reporter gene construct containing both 5-LO promoter and cds plus introns J-M or the positive control p(DR3)4tkluc and the expression plasmids for VDR and RXR and were incubated with the 1,25$(OH)_2D_3$-analogon ZK191732 (100/500 nM) for 8 hours (the structure is depicted in fig. 12, chapter 1.12.3). Interestingly, the

compound that has previously been determined to act antagonistically on 5-LO activity in MM6 cells (Gaschott et al., 2001), showed an explicit dose-dependent agonistic activity in this assay (fig. 22). Strikingly, with p(DR3)4tkluc the effect was rather low and no dose-dependency could be observed. Consistent with the above described results these data suggest that the pathway of $1,25(OH)_2D_3$-mediated regulation of 5-LO gene expression is distinct from the classical $1,25(OH)_2D_3$ signalling pathway through VDREs.

In the previous experiments mentioned above, the antagonistic activity of ZK191732 in MM6 cells has been characterized by 5-LO activity assays. To address, whether the $1,25(OH)_2D_3$ analogon diminishes 5-LO activity by inhibiting the protein expression 5-LO protein levels have been determined by means of Western blot. MM6 cells were grown with or without $1,25(OH)_2D_3$ (50 nM)/TGFβ (1 ng/ml) and/or ZK191732 (500 nM or 1 µM) for 4 days until cells were harvested and subjected to SDS-PAGE and Western blot. Fig. 23 illustrates that 1 µM ZK191732 induces 5-LO protein expression (lane 5). However, 500 nM ZK191732 are not sufficient to significantly upregulate 5-LO protein level (lane 3). Interestingly, in combination with $1,25(OH)_2D_3$/TGFβ the analogon dose-dependently reduces the prominent induction of 5-LO protein mediated by $1,25(OH)_2D_3$/TGFβ (lanes 2, 6, and 7). Thus, in MM6 cells the $1,25(OH)_2D_3$ analogon ZK191732 displays agonistic as well as antagonistic activities.

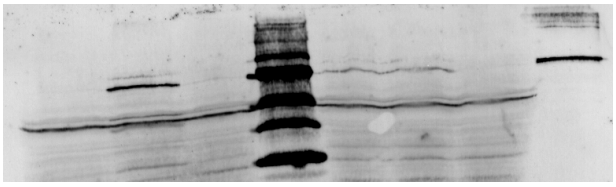


Fig. 23. Agonistic and antagonistic effects of the $1,25(OH)_2D_3$ analogon ZK191732. *Mono Mac 6 cells were grown in the absence of additives (lane 1) or in the presence of $1,25(OH)_2D_3$ (50 nM)/TGFb (1 ng/ml), ZK191732 (500 nM/1 µM), or their combinations for 4 days. The samples (3 x 10^5 cells, solubilized in SDS-PAGE loading buffer) were analysed for 5-LO protein by Western blot. Lane 4: protein size marker.*

4.3 The coding sequence mediates induction of 5-LO expression by Smads3/4

Since Smad proteins have been described as downstream effectors of TGFβ, the role of TGFβ/Smad signalling in 5-LO gene regulation was investigated.

4.3.1 Effects of TGFb on 5-LO reporter gene activity

HeLa cells were transiently transfected as described above with the above mentioned luciferase reporter gene constructs (for overview see paper III: fig. 1) in the absence of VDR/RXR and Smad3/Smad4 expression constructs. TGFβ (1 ng/ml) led to an about 1.5-fold induction of reporter gene activity with the control pGL3-Basic (paper III: fig. 2). A similar induction was observed with pN10, pN10-3UTR and pN10-cdsINJM-inv. Interestingly, slightly increased effects (about 2-fold induction) were observed with plasmids that contain the 5-LO coding sequence (pN10cds, pN10-cdsInJM, pGL3-ba-cdsInJM and pGL3-prom-cdsInJM). With all plasmids tested, $1,25(OH)_2D_3$ (50 nM) alone did not significantly enhance transcriptional activity under these experimental conditions, i.e. in the absence of cotransfected VDR/RXR. A slight induction of transcriptional activity was obtained with the p(DR3)4tkluc plasmid when $1,25(OH)_2D_3$ and TGFβ were combined.

4.3.2 Smad3/Smad4 strongly induce reporter gene activity when the 5-LO coding sequence is present

HeLa cells were transiently transfected with luciferase reporter gene constructs (paper III: fig. 1) and cotransfected with either pCGN-Smad3 and pCGN-Smad4 or an empty cytomegalovirus (CMV) promoter construct as control. In order to determine the optimal amount of Smad expression vectors, different amounts were tested. As shown in fig. 24, 100 ng of each expression vector per 800 ng reporter gene plasmid (8/1) was sufficient for maximal reporter gene activation. Thus, all subsequent experiments were performed with these amounts.

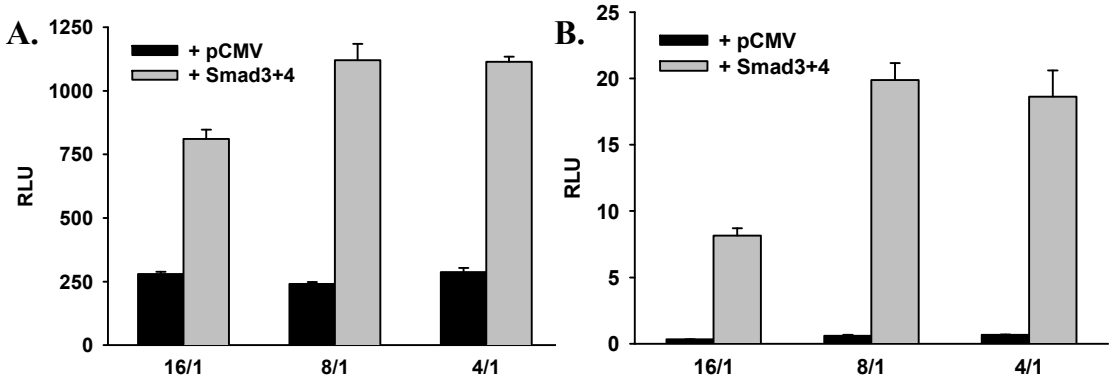


Fig. 24. Determination of optimal amounts of Smad3/4 expression vectors for cotransfection in HeLa cells. *The plasmids pN10 (A) and pN10-cdsInJM (B) were transiently transfected into HeLa cells with or without increasing amounts of expression vectors for Smads3 and 4. Luciferase activity was measured 24 h after transfection. Each experiment was performed in triplicates and normalization for transfection efficiency was performed by cotransfection of pCMV-SEAP. Depicted are the relative light units (RLU) of the respective ratios of reporter to Smad expression vectors (mean ± SE). Displayed is one representative out of at least three independent experiments.*

Coexpression of Smads increased reporter gene activity obtained with pN10 (containing the 5-LO core promoter) by 3.7-fold. Interestingly, the TGFβ-responsive plasmid p3TP-Lux, carrying three TGFβ tetradecanoyl phorbol acetate REs from the plasminogen activator inhibitor 1 (PAI-1) gene, was only induced by 1.6-fold which was comparable to the negative control pGL3-Basic (2.2-fold). The effects of the Smad proteins on 5-LO gene expression was strongly enhanced by the presence of the cds (paper III: fig. 3). Induction of pN10-cds and of pN10-cdsInJM (containing also introns J-M) was 17.5-fold and 24.0-fold, respectively. Interestingly, the upregulation of the 3'-UTR-containing plasmid pN10-3UTR was significantly higher than with pN10 (8.8-fold versus 3.7-fold) but inclusion of the 3'-UTR did not further enhance the induction of the constructs containing the coding sequence (pN10-cds3UTR and pN10-cdsInJM3UTR). When the 5-LO coding and intronic sequences were inserted behind the luciferase stop codon (pN10-cdsInJM-inv) there was a strong reduction of the upregulation by cotransfection of Smad3/Smad4 to pN10 levels (3.1-fold). Induction of reporter gene activity by Smad3/Smad4 also occurred with pGL3-ba-cdsInJM and pGL3-prom-InJM (without promoter and with SV 40 promoter, respectively), which suggests that the Smad3/Smad4 effects do not depend on the 5-LO promoter.

4.3.3 TGFb effects are only marginally enhanced by Smad3/Smad4

In the absence of coexpressed Smads, TGFβ enhanced reporter gene activity by 1.5- to 2.7-fold for 5-LO promoter constructs and up to 4.1-fold for the positive control p3TP-Lux (paper III: fig. 4). Interestingly, in cells that were cotransfected with Smad3 and Smad4 there was no significantly altered response to TGFβ indicating that the TGFβ effects are independent of Smad overexpression.

4.3.4 Synergism between RXR/VDR and Smad3/Smad4

Cotransfection of expression plasmids for VDR/RXR and/or Smad proteins 3/4 profoundly enhanced absolute reporter gene activity (paper III: fig. 5). In cells cotransfected with VDR/RXR, addition of TGFβ (1 ng/ml) or 1,25$(OH)_2D_3$ (50 nM) upregulated reporter gene activity and both ligands displayed a synergistic effect. In cells cotransfected with Smads3/4 only, TGFβ enhanced reporter gene activity whereas 1,25$(OH)_2D_3$ was without effect. The combined cotransfection of Smads3/4 and VDR/RXR led to additive effects on absolute reporter gene activity. As expected, addition of either 1,25$(OH)_2D_3$ or TGFβ increased reporter gene activity and combination of both agents had a synergistic effect (paper III: fig. 5). Interestingly, coexpression of Smads3/4 and VDR/RXR increased reporter gene activity by 12.6-fold, whereas the ligand-mediated inductions by 1,25$(OH)_2D_3$ or TGFβ and the combination of both agents were 2.4-, 2.6- and 5.9-fold, respectively, suggesting a remarkable constitutive activity of Smads and VDR/RXR, respectively.

4.3.5 Identification of sequence elements with significance for Smad signalling in 5-LO gene regulation

In order to identify sequences in the coding region and introns that participate in Smad3/4 and TGFβ-mediated enhancement of 5-LO expression, the above described deletion constructs from the vector pN10-cds were analyzed in reporter gene assays. As shown in fig. 6A (paper III) deletion of parts A, B or D reduced Smad3/4-mediated induction of 5-LO expression from 14.7-fold (pN10-cds) to 8.8-, 8.6- and 9.0-fold, respectively. A prominent decrease to 3.9-fold was observed, when part C was deleted. Comparably, a 3.7-fold upregulation of 5-LO gene expression by Smad3/4 coexpression was determined for the 5-LO promoter plasmid pN10. Deletion of two or three parts of the cds rather decreased the inhibitory effect (paper III: fig. 6B). In contrast to the

Smad3/4 mediated effects, transcriptional activation by TGFβ was not affected by the deletion of parts of the coding region (paper III: figs. 7A+B).

Sequence analysis revealed the existence of several putative SBEs in the 5-LO coding sequence and introns. Deletion of a 286 bp sequence in intron M from the vector pN10-cdsInJM comprising 4 putative SBEs (paper III: fig. 8A) led to a construct, which is less responsive to coexpression of Smad proteins (pdSBE2 10.4-fold, pN10-cdsInJM 18.4-fold) (paper III: fig. 8B), suggesting that these SBEs might be involved in the regulation of 5-LO mRNA expression by Smads. The minor TGFβ-mediated upregulation of 5-LO expression was not altered by the deletion (paper III: fig. 8C).

Furthermore, the two most promising putative TGFβ responsive elements (TREs), located in exon 10 and intron J, were deleted from plasmid pN10-cdsInJM (paper III: fig. 8A). Deletion of TRE 1 significantly reduced the Smad-mediated enhancement of 5-LO expression from 16.2- to 6.7-fold (paper III: fig. 8B), whereas deletion of TRE 2 or of both led to no marked changes. The TGFβ-mediated transcriptional activation of 5-LO was not affected by either deletion (paper III: fig. 8C).

4.4 Cotransfection of Sp1 or Ap2a does not significantly affect reporter gene activity

As already mentioned in chapter 1.10, numerous putative and some functional REs for various transcription factors have been detected throughout the whole sequence of the 5-LO gene. One of these is Sp1, for which the characteristic binding sites (GC boxes) are known in the promoter and which is required for basal 5-LO expression (Hoshiko, 1990). Also, putative binding sites for Ap2α can be found in the 5-LO promoter. To address, whether both transcriptional regulators influence 5-LO promoter activity and/or like VDR and Smads mediate any effect through the cds, cotransfection studies have been performed.

HeLa and Caco-2 cells were transiently transfected with luciferase plasmids as described above and cotransfected with an expression vector for either Sp1 or Ap2α. In HeLa cells the reporter gene activity of the 5-LO promoter driven construct pN10 was not affected by overexpression of either Sp1 or Ap2α. Insertion of the 5-LO cds plus introns J-M slightly enhanced the Ap2α-mediated effect from 1.1-fold to 2.1-fold,

whereas Sp1 inducibility was not significantly changed (1.4-fold and 1.8-fold, respectively) (fig. 25 A). Similarly, but to an even lower extent, in Caco-2 cells the Ap2α-mediated induction is slightly higher for pN10-cdsInJM compared to pN10 (1.6-fold versus 1.1-fold) (fig. 25 B).

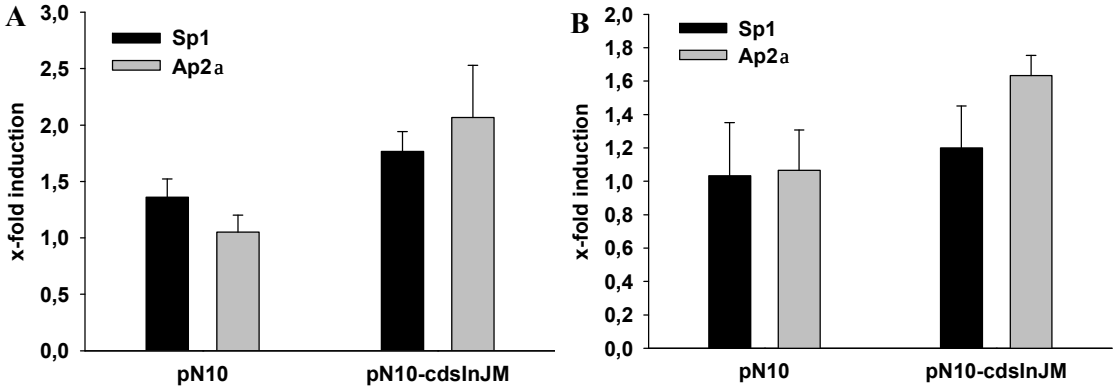


Fig. 25. Cotransfection of Sp1 or Ap2a. *The indicated plasmids were transiently transfected into HeLa (A) or Caco-2 (B) cells with or without expression vectors for Sp1 or Ap2a. 24 h after transfection luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to cells cotransfected with empty vector.*

4.5 TsA and structurally related HDACis induce 5-LO (and 15-LO) promoter activity

As reported in chapter 1.12.2 the 5-LO promoter is regulated by DNA methylation. Since DNA methylation and histone acetylation are tightly connected processes and recent reports indicate an involvement of chromatin remodeling and histone acetylation in the regulation of 5-LO and 15-LO gene expression (for further information, see chapter 1.12.2), the impact of histone acetylation on 5-LO and 15-LO promoter activity has been investigated.

4.5.1 TsA induces 15-LO mRNA expression in U937 cells

Since upregulation of 15-LO expression by the HDACi sodium butyrate has already been shown in Caco-2 cells (Kamitani et al., 2000), the effects of the more potent HDACi TsA on 15-LO mRNA expression in the myeloid cell line U937 has been

investigated. U937 cells were cultured with TGFβ (1 ng/ml) and 1,25$(OH)_2D_3$ (50 nM) for 24 h, with or without TsA (330 nM) for the last 8 h. Following cell harvest, total RNA was extracted and reverse transcribed into cDNA. 15-LO and β-actin expression were analyzed by PCR employing 35 and 24 cycles, respectively. TsA alone induced 15-LO mRNA expression by 5.8-fold, whereas 1,25$(OH)_2D_3$/TGFβ rather decreased 15-LO mRNA generation. Combination of TsA with 1,25$(OH)_2D_3$/TGFβ lessened the TsA-mediated upregulation (3.2-fold) (fig. 26). Thus, 15-LO mRNA expression is not increased by 1,25$(OH)_2D_3$/TGFβ in U937 cells and in combination with TsA, 1,25$(OH)_2D_3$/TGFβ seem to suppress TsA-mediated 15-LO mRNA induction.

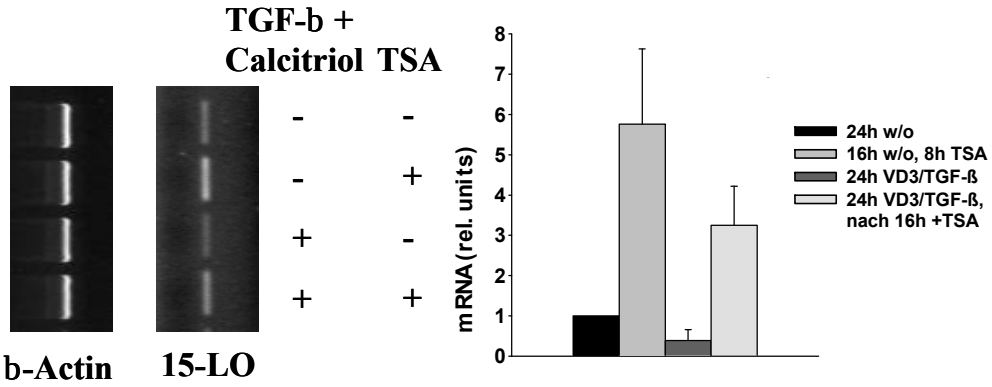


Fig. 26. Induction of 15-LO mRNA expression in U937 cells. *Cells were cultured for 24 h in the presence or absence of TGFb (10 ng/ml) and 1,25$(OH)_2D_3$ (VD3) (50 nM). TsA (330 nM) was added after 16 h for another 8 h, before cells were harvested and analyzed by RT-PCR. 35 and 24 cycles were used for 15-LO and b-actin, respectively (mean ± SE).*

4.5.2 TsA induces 5-LO mRNA expression in MM6 cells

In order to study the effects of TsA on 5-LO mRNA expression, MM6 cells were cultured with TsA (330 nM) and/or TGFβ (1 ng/ml) and 1,25$(OH)_2D_3$ (50 nM) for 24 h. Following cell harvest, total RNA was extracted and reverse transcribed into cDNA. 5-LO and β-actin expression were analyzed by PCR employing 28 and 24 cycles, respectively. TsA alone led to an about 11-fold increase in 5-LO mRNA expression, whereas induction by 1,25$(OH)_2D_3$/TGFβ was 43-fold (paper IV: fig. 1). Interestingly, combination of TsA with 1,25$(OH)_2D_3$/TGFβ did not further increase the effect of 1,25$(OH)_2D_3$/TGFβ but rather inhibited (62 %) the induction mediated by both agents and led to an only 16-fold upregulation of 5-LO mRNA compared to the control. Hence,

TsA alone strongly increases 5-LO mRNA generation in MM6 cells but diminishes the 1,25$(OH)_2D_3$/TGFβ-induced 5-LO mRNA upregulation.

Experiments performed by coauthors revealed that TsA strongly induces 5-LO promoter activity in transiently transfected MM6 and HeLa cells (paper IV). Further, as already mentioned above, 5-LO promoter activity was independent from 1,25$(OH)_2D_3$/TGFβ in reporter gene assays. Combination with TsA and/or overexpression of VDR/RXR did not alter the response to 1,25$(OH)_2D_3$/TGFβ.

Since the involvement of multiple Sp1 binding sites in the transcriptional activation of genes such as $G\alpha_{i2}$ and WAF/Cip1 by HDACis like sodium butyrate, TsA or HC (*Helminthsporium carbonium*) toxin has been suggested recently (Nakano et al., 1997; Yang et al., 2001b), the GC boxes have been deleted from the 5-LO promoter reporter gene construct pN10. Reporter gene assays with the resulting plasmids indicated that the TsA-mediated induction of 5-LO promoter activity does not depend upon the five tandemized Sp1 sites.

Furthermore, transient transfection experiments with *in vitro* methylated plasmid DNA demonstrated that DNA methylation dominates over histone acetylation in the regulation of 5-LO promoter activity. TsA only partially reversed methylation-dependent inhibition of 5-LO promoter-driven luciferase activity.

Finally, a series of recently synthesized HDACis that are structurally related to trapoxin B and TsA have been studied in the transient transfection system. Since the EC_{50} values obtained in this model are consistent with reported potencies in other *in vitro* systems, this assay seems to be a useful tool for the screening for and evaluation of HDACis.

4.6 Interplay of 1,25$(OH)_2D_3$/TGFb signalling and histone acetylation in the regulation of 5-LO gene regulation

5-LO has been shown to be induced by HDAis (chapter 4.5/paper IV), HDACis have been reported to synergistically enhance cell differentiation by 1,25$(OH)_2D_3$ (Gaschott et al., 2001), and the VDR interacts with cointegrators containing intrinsic HAT activities (chapter 1.12.3). Thus, the possible crosslinking of the 1,25$(OH)_2D_3$/TGFβ

signalling pathway and histone acetylation has been further investigated. It was of particular interest, if histone acetylation also affects the cds and/or introns J-M.

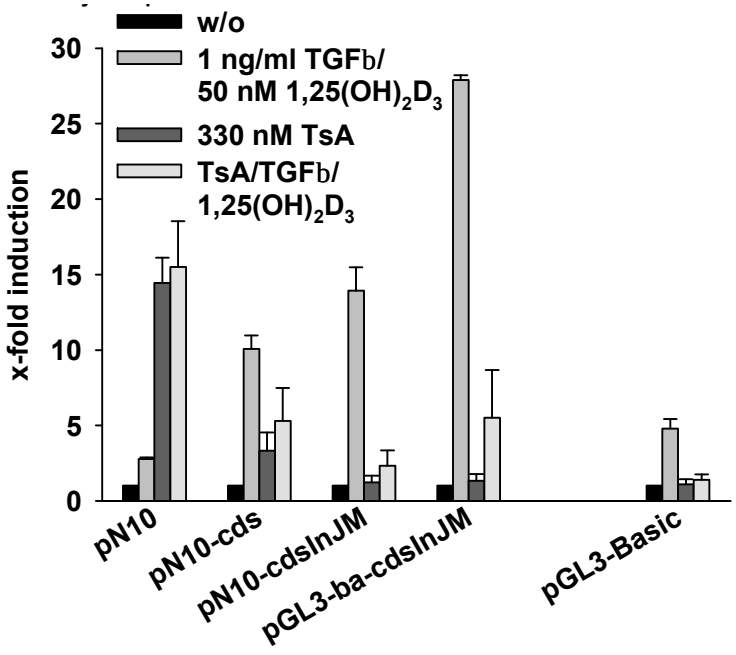


Fig. 27. Interaction of 1,25(OH)$_2$D$_3$/TGFb signalling and histone acetylation. *The indicated plasmids were transiently transfected into HeLa cells, untreated or preincubated for 24 h with TGFb (1 ng/ml), and cotransfected with expression vectors for VDR and RXR. 16 h after transfection 1,25(OH)$_2$D$_3$ (50 nM)/TGFb (1 ng/ml) and/or TsA (330 nM) were added for 24 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to untreated cells.*

Some of the above described (5-LO promoter) reporter gene plasmids were again analyzed in the transient transfection system. HeLa cells, untreated or preincubated with TGFβ (1 ng/ml), were transiently transfected with luciferase reporter vectors and cotransfected with expression plasmids for VDR and RXR. 16 h after transfection, medium was changed and 1,25(OH)$_2$D$_3$ (50 nM)/TGFβ (1 ng/ml) and/or TsA (330 nM) were added for 24 h, until cells were harvested and luciferase activity was measured. As displayed in fig. 27, 1,25(OH)$_2$D$_3$/TGFβ alone only slightly induced reporter gene activity of the 5-LO promoter containing plasmid pN10 or the negative control pGL3-Basic containing no promoter (2.8- and 4.8-fold, respectively). In contrast, as already reported above, the cds (and introns) containing plasmids pN10-cds, pN10-cdsInJM and pGL3-ba-cdsInJM (for overview, see paper II: fig. 2) are highly inducible by both agents (10.1-, 13.9-, and 27.9-fold, respectively). Consistent with the results reported in chapter 4.5/paper IV, the 5-LO core promoter (pN10) is activated by TsA (14.4-fold).

Inclusion of the cds and introns J-M suppresses the TsA-mediated upregulation to 3.3-, 1.2-, and 1.3-fold for the plasmids pN10-cds, pN10-cdsInJM and pGL3-ba-cdsInJM, respectively. The combined incubation with $1,25(OH)_2D_3$/TGFβ/TsA does not affect the TsA-mediated induction of pN10. However, the response of the cds-containing plasmids to $1,25(OH)_2D_3$/TGFβ/TsA is drastically reduced compared to $1,25(OH)_2D_3$/TGFβ alone (pN10-cds 5.3-fold, pN10-cdsInJM 2.3-fold, and pGL3-ba-cdsInJM 5.5-fold). Thus, TsA seems to mediate an inhibitory effect on 5-LO upregulation by $1,25(OH)_2D_3$/TGFβ through the cds.

4.7 Sp1 and Sp4, but not Sp3 synergistically with TsA enhance coding sequence-mediated upregulation of 5-LO gene expression

Recent reports indicate a crosslink between chromatin organization and the action of transcription factors of the Sp family. For instance, binding of Sp1 and Sp3 to HDAC1/2 has been described to be required for HDAC activity and Sp1 sites in promoters seem to be essential for full efficiency of HDACis (de Ruijter et al., 2003; Nakano et al., 1997; Yang et al., 2001a). The Sp family of transcription factors consists of the closely related proteins Sp1, Sp2, Sp3 and Sp4. Sp1, Sp3 and Sp4 can bind to and act through GC-boxes (for review, see (Suske, 1999)). Sp1 is an ubiquitous factor, known to be a transcriptional activator of a large number of genes. It belongs to the limited list of transcription factors that have been shown to interact with nucleosomal DNA ((Li et al., 1994) and references therein) and is required to prevent methylation of CpG islands. Its function is severely affected by nucleosome structure (Hodny et al., 2000). Sp1 interacts with its binding site in the form of multiple stacked tetramers, which contain several interacting surfaces for associating proteins (Davie, 2003). Sp2 function has not been elucidated, yet. Sp3, like Sp1, is ubiquitiously expressed. Its action is not fully understood, either, but it seems that dependent on the structure and arrangement of the binding sites it can strongly activate transcription or repress Sp1-mediated activation by competing with Sp1 for their common binding sites. Finally, Sp4 is a tissue-specifically expressed transcriptional activator. Sp knockout experiments indicate that Sp family members have individual as well as overlapping functions.

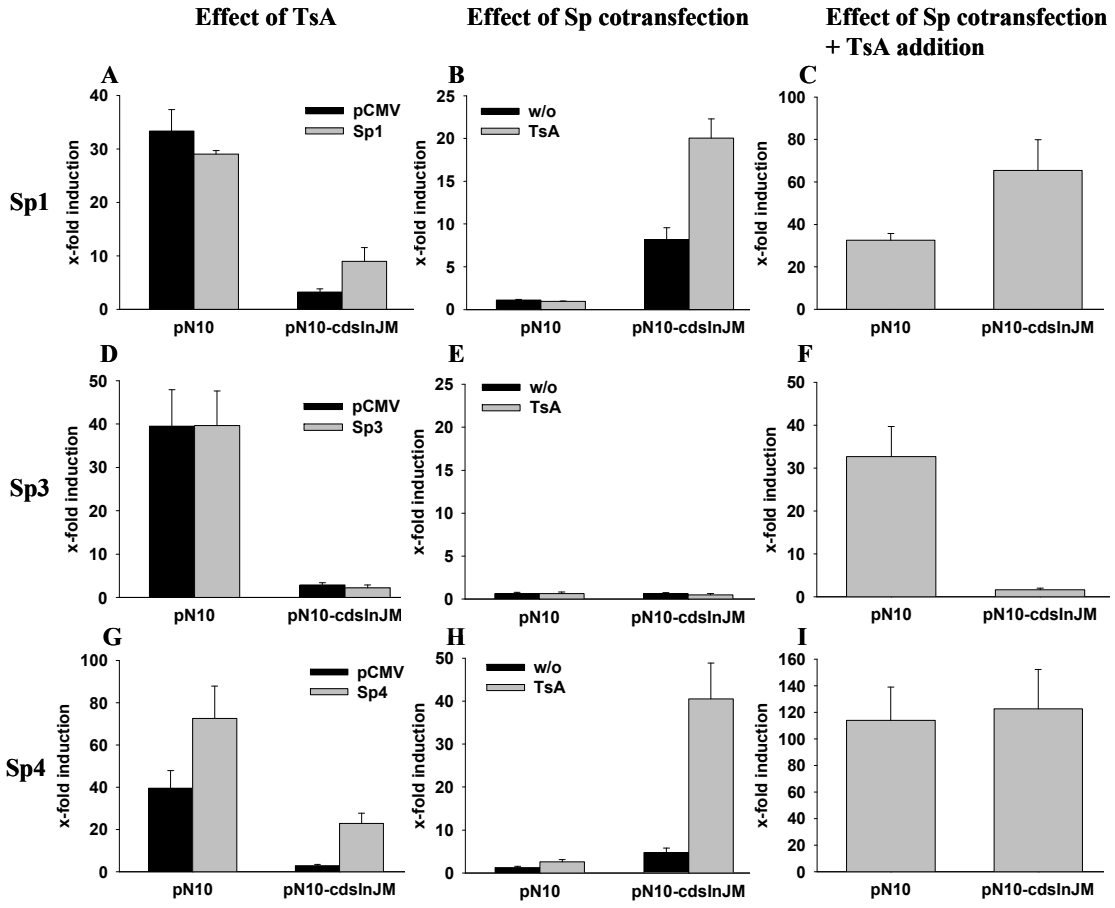


Fig. 28. Crosstalk of Sp1/Sp3/Sp4 signalling and histone acetylation. *The plasmids pN10 or pN10-cdsInJM were transiently transfected into HeLa cells with or without expression vectors for Sp1 (A, B, C), Sp3 (D,E,F) or Sp4 (G, H, I). 16 h after transfection TsA (330 nM) was added for 24 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions mediated by Sp proteins (A, D, G), TsA (B, E, H) or both (C, F, I) are expressed with respect to untreated cells, cells cotransfected with empty vector and both, respectively.*

For information on the relevance of Sp1 for basal 5-LO promoter activity, see chapters 1.10 and 1.11. Sp1 cotransfection studies have already been described in chapter 4.4. To characterize the crosstalk of Sp family members and histone acetylation in the regulation of 5-LO gene expression, Sp1/Sp3/Sp4 cotransfections have been combined with TsA treatment in transient transfection experiments. To further address, whether sequences in the cds and introns are involved in this regulation, not only the 5-LO promoter reporter gene plasmid pN10 but also the cds and introns J-M-containing construct pN10-cdsInJM was used for transfections. HeLa cells were transiently transfected with the reporter gene plasmids and cotransfected with expression vectors for the transcription factors Sp1, Sp3 or Sp4. 16 h after transfection, medium was

changed and cells were incubated with TsA (330 nM) for 24 h. Subsequently, the cells were harvested and the luciferase assay was performed. Fig. 28 shows fold inductions calculated for TsA-treatment, Sp cotransfection or the combination of both. As already mentioned above, the 5-LO promoter is highly responsive to TsA. Independently from cotransfection with Sp1 or Sp3, pN10 luciferase activity was induced by about 30-fold (figs. 28 A+D). However, overexpression of Sp4 enhanced the TsA-mediated activation of pN10 from 39.5- to 72.6-fold (fig. 28 G). Consistent with fig. 27, inclusion of cds and introns J-M strongly reduced the TsA-mediated induction. Interestingly, cotransfection of Sp1 or Sp4 also increased the TsA-mediated upregulation of pN10-cdsInJM from 3.2- to 8.9-fold and from 2.9- to 22.8-fold, respectively (figs. 27 A+G) (note: Sp1-mediated induction is higher than depicted in fig. 27 due to the prolonged incubation time). Consistent with previous results, Sp1 overexpression did not induce 5-LO promoter activity (fig. 28 B), although the transcription factor is required for basal promoter activity. Likewise, Sp3 and Sp4 did not increase pN10 luciferase activity (figs. 28 E+H). Interestingly, insertion of the coding sequence and introns J-M increased the induction by Sp1 (8.1-fold compared to 1.1-fold for pN10) and Sp4 (4.8-fold compared to 1.3-fold for pN10), which was not observed for Sp3 (figs. 28 B+H+E). Similar to the above described enhancement of TsA-mediated activation by Sp1 and Sp4, Sp1- and Sp4-mediated upregulation of pN10-cdsInJM was prominently increased by TsA treatment (figs. 28 B+H: from 8.1- to 20.1-fold and from 4.8- to 40.5-fold, respectively). Thus, Sp1/Sp4 and histone hypoacetylation seem to have additive effects on the coding sequence-mediated induction, whereas Sp3, independently from histone acetylation does not affect luciferase activity. Figs. 28 C+F+I show the total inductions mediated by the transcription factor Sp plus TsA. Interestingly, inclusion of the cds and introns J-M enhanced the induction mediated by Sp1+TsA from 32.6- to 65.4-fold. Since Sp3 did not induce luciferase activity of either plasmid, the total effects corresponded to the TsA effects (pN10: 37.7-fold, pN10-csdInJM: 1.7-fold). The very high Sp4+TsA-mediated induction was not different for both plasmids (pN10: 113.9-fold, pN10-csdInJM: 122.6-fold), indicating that the different intensities of the single effects compensated each other.

5 Discussion

5.1 Functional interaction of the VDR with the 5-LO promoter does not mediate induction of 5-LO gene expression by 1,25(OH)$_2$D$_3$

Since the 5-LO promoter contains several putative VDREs (within -313 bp to -258 bp), it was reasonable to assume that the prominent upregulatory effects of 1,25(OH)$_2$D$_3$ on 5-LO mRNA expression in myeloid cell lines during maturation are due to ligand-dependent transactivation of the VDRE by VDR. In fact, the binding of VDR together with RXR to 5-LO promoter fragments spanning the putative VDREs *in vitro* with similar magnitudes compared to the well-documented VDRE of the mouse osteopontin promoter could be shown. Also, differentiation of MM6 cells by 1,25(OH)$_2$D$_3$ and TGFβ caused markedly enhanced protein binding to VDREs from nuclear extracts compared to untreated cells. However, luciferase reporter gene assays failed to demonstrate significant transcriptional activation of the 5-LO promoter upon stimulation with 1,25(OH)$_2$D$_3$ (plus TGFβ) in various myeloid cell lines. Expression of a reporter construct containing a 4-times concatemerized rANF VDRE in front of the thymidine kinase promoter (p(DR3)4tkluc) was strongly increased after treatment of these cell lines with 1,25(OH)$_2$D$_3$ and TGFβ, though. Moreover, in stably transfected HL-60 cells endogenous 5-LO mRNA was strongly upregulated by 1,25(OH)$_2$D$_3$ and TGFβ, whereas the CAT reporter gene driven by the entire 5-LO promoter fragment (-6079 to +81 bp), was not responsive. Hence, the simple lack of bridging factors or coactivators in transient transfections could be excluded. Taken together, it can be concluded that the strong induction of 5-LO mRNA expression by 1,25(OH)$_2$D$_3$ and TGFβ in myeloid cell lines seems to be mediated by regulatory elements located outside of the 5-LO promoter and apparently does not involve the VDR/RXR interaction with the VDREs located within the 5-LO promoter.

By means of reporter gene assays using HeLa and MM6 cells, previous findings that the promoter region containing the five tandemized Sp1-sites is essential for basal promoter activity (Silverman et al., 1998) were confirmed. For both cell lines positive and negative regulatory regions were identified. In agreement to the presented results, Hoshiko *et al.* identified a positive regulatory region (-6079 to -3635 bp), which comprises the sequence between -5814 and -5395 bp that has been reported in paper I.

In HeLa cells the presence of a negative regulatory region at -662 to -227 bp that was reported before (Hoshiko, 1990) could not be confirmed. The reason for this discrepancy is unknown but could be due to different transfection protocols, different deletion constructs or experimental conditions. In addition, a new inhibitory region (from -5395 to -4894 bp), which contains two putative binding sites for the transcription factor p53 that match the consensus sequence (GAACATGTCC) to more than 80 % was identified. Using MM6 cells, a positive regulatory region (-778 to -229 bp) that is not functional in the 5-LO negative HeLa cells was found. Interestingly, putative VDREs are located in that region, but as stated above, in reporter gene experiments TGFβ/1,25$(OH)_2D_3$ did not alter 5-LO promoter activity in any cell type investigated. These findings are in agreement with results from nuclear run-off assays (Härle et al., 1998; Härle et al., 1999) and previous transfections (Uhl et al., 2002) where increased transcription of the 5-LO gene could not be demonstrated.

Of interest, the coexpression of VDR and RXR was found to be essential for high level induction of reporter gene activity of the plasmid p(DR3)4tkluc that was used as positive control. Low expression of VDR and RXR (no expression vectors added), caused only marginal effects of TGFβ/1,25$(OH)_2D_3$, except for RBL-1 cells, which could be due to a high expression level of VDR and/or VDR related coactivators.

5-LO promoter deletion studies revealed that coexpression of the receptors VDR and RXR does not significantly affect 5-LO promoter activity when the VDREs are present. In contrast, it reduces 5-LO promoter activity in reporter gene constructs that lack the DNA sequence from -778 to -229 bp, which contains the putative VDREs. One possible explanation could be that VDR/RXR recruit coactivators that are required for high 5-LO promoter activity. However, the VDR does not bind to 5-LO promoter constructs lacking the VDREs, and instead sequesters coactivators from the 5-LO promoter. This conclusion is supported by DNA footprinting and EMSA analyses, where a strong interaction of VDR/RXR with the VDREs at -309 to -294 bp and at -290 to -275 bp with homology to the VDRE of the mPit-1 gene, could be demonstrated.

In summary, it was strongly suggested that the effects of TGFβ/1,25$(OH)_2D_3$ on 5-LO gene expression are mediated by further regulatory elements outside of the 5-LO promoter, which were absent in transient and stable reporter gene transfections. Considered were exons, introns or the 3'UTR which might contain sequences like mRNA processing signals, sequences that affect mRNA stability (Dixon et al., 2000;

Lemm and Ross, 2002; Tierney and Medcalf, 2001), or transcription factor binding sites.

5.2 The upregulation of 5-LO gene expression by 1,25(OH)$_2$D$_3$/TGFb depends upon the coding sequence

In order to investigate, whether sequences in the exons, introns or 3'UTR of the 5-LO gene confer the prominent response to 1,25(OH)$_2$D$_3$/TGFβ, the above described reporter gene constructs were cloned. On the one hand reporter gene assays demonstrated that induction of luciferase activity by 1,25(OH)$_2$D$_3$ and TGFβ depends on the 5-LO cds. This upregulation was independent of the promoter (5-LO, CMV, no promoter) indicating that at least under the *in vitro* conditions of reporter gene assays, the 5-LO promoter is not required for the 1,25(OH)$_2$D$_3$ and TGFβ effect. On the other hand, the induction of reporter gene activity mediated by the cds depended on the coexpression of VDR/RXR which indicates that the VDR is involved in this signalling. Coexpression of VDR/RXR was also required for 1,25(OH)$_2$D$_3$ signalling in the control experiments, i.e. the stimulation of reporter gene activity of the p(DR3)4tkluc plasmid that contains a four times concatemerized DR3 VDRE in front of the thymidine kinase promoter. However, there was a significant difference in the reporter gene assays between classical vitamin D signalling and the induction of reporter gene activity mediated by the cds. Whereas coexpression of VDR/RXR did not affect reporter gene activity of the p(DR3)4tkluc plasmid in the absence of 1,25(OH)$_2$D$_3$ and TGFβ, it led to a 3-fold stimulation of luciferase activity of vector constructs containing the 5-LO cds in HeLa cells (paper II: fig. 3). This suggests that there is a considerable ligand-independent activity of the VDR regarding the induction of reporter gene activity mediated by the 5-LO cds. Inclusion of the last four introns (J, K, L and M) further enhanced the inducibility of the reporter gene constructs.

In agreement with the fact that 1,25(OH)$_2$D$_3$ predominantly acts by binding to VDR/RXR heterodimers, overexpression of both NRs is required for full induction of luciferase activity by 1,25(OH)$_2$D$_3$/TGFβ (fig. 19). Hence, although 1,25(OH)$_2$D$_3$ action through the cds displays evident differences from classical 1,25(OH)$_2$D$_3$ signalling, VDR/RXR heterodimers are clearly involved.

Compared to the 5-LO negative cell line HeLa, in the 5-LO permissive Caco-2 cells the $1,25(OH)_2D_3$/TGFβ mediated activation of 5-LO expression is rather low and non-synergistic (fig. 18), which might be due to different tissue-specific expression levels of $1,25(OH)_2D_3$/TGFβ and/or required cofactors.

As it was previously found in MM6 cells, $1,25(OH)_2D_3$ and TGFβ augment 5-LO mRNA expression mainly by posttranscriptional mechanisms (Härle et al., 1998). RT-PCR data obtained with MM6 cells (paper II: fig. 1) revealed a prominent induction of mature 5-LO mRNA but not pre-mRNA by $1,25(OH)_2D_3$/TGFβ, which suggests enhanced mRNA processing as mechanism of 5-LO upregulation. This conclusion is also supported by the increased inducibility of intron-containing plasmids by $1,25(OH)_2D_3$/TGFβ in reporter gene assays, suggesting that splicing processes are involved. Generally, the presence of introns in the transcription unit was shown to activate gene expression in yeast and mice (Ares et al., 1999; Fong and Zhou, 2001). Another possible mechanism could be transcriptional arrest mediated by the cds that is overcome by $1,25(OH)_2D_3$ and TGFβ. However, such a mechanism is rather unlikely because RT-PCR data show that there is only low expression of the middle part of the 5-LO cds but not of the 3'-end. Furthermore, inducibility by $1,25(OH)_2D_3$/TGFβ was rather low with the pN10-cdsInJM-inv plasmid, which contains the 5-LO cds behind the luciferase reporter gene. However, a transcriptional stop site should also be operative under these conditions.

It can only be speculated, whether the first (very long) introns of the 5-LO gene also participate in its regulation. It was not possible to include these introns in the reporter gene vectors because of their huge size. Thus, it is very well possible that these introns also participate in the regulation of the 5-LO gene expression. In consequence, the observed $1,25(OH)_2D_3$/TGFβ effects on 5-LO expression in MM6 cells and in the reporter gene assays performed in this investigation might be due to slightly different mechanisms.

The partial inhibition of the $1,25(OH)_2D_3$/TGFβ effects by CHX suggests that the response to both agents requires at least in part protein biosynthesis. Thus, it is possible that $1,25(OH)_2D_3$/TGFβ induce the biosynthesis of proteins involved in 5-LO mRNA splicing and/or other processing reactions. This circumstance would explain the observed induction of luciferase activity without concomitant increases in the mRNA level in the reporter gene assays. In the constructs, the 5-LO and luciferase coding

sequences are merged in frame so that translation of the transcribed mRNA yields a 5-LO-luciferase fusion protein. Thus, aberrant RNA processing that leads to a frame shift in the 5-LO coding region prevents correct translation of the luciferase. Consequently, induction of correct 5-LO mRNA processing by $1,25(OH)_2D_3$/TGFβ generates correctly translated luciferase protein and subsequent activity.

Taking the existance of some rather weak 5' splice sites in the 5-LO gene (chapter 1.9, tab. 2) into consideration, it appears likely that auxiliary sequences help to specify the appropriate splice sites. Usually, the pairing of the correct splice sites is thought to be achieved by either exon or intron definition. Dependent on the sizes of exons and introns, bridging factors between the spliceosome components U1 snRNP (bound to the 5' splice site) and U2 auxiliary factor (U2AF, bound to the polypyrimidine tract) span either the exon or the intron (Berget, 1995). SR proteins belonging to the superfamily of arginine and serine rich splicing factors have been suggested to act as exon-bridging proteins in constitutive splicing. Their ability to bind exon sequences via their RNA binding domains and to interact with U2AF and U1 snRNP through their RS domains supports this hypothesis. Interactions between SR proteins bound to neighboring exons are also likely to be involved in juxtaposing exons (Berget, 1995; Fu, 1995). The definition of corresponding splice sites across the exon or intron cannot occur on terminal exons, but instead is suggested to involve interactions with the 5' cap structure and poly-A site, respectively (Black, 1995). Sequences have been identified that can activate the use of weak splice sites independently from exon-bridging interaction. Most of these are purine-rich splicing enhancers located in the exon downstream of the stimulated intron that are involved in the recruitment of U2AF. Complexes that assemble on an exonic splicing enhancer contain U1 snRNP and SR proteins. SR proteins play a role in the recognition of splicing enhancers, in stabilizing U1/5' splice site interactions and in defining a functional 5' splice site. Furthermore, they stimulate and/or stabilize U2-U6 interaction at the 3' splice site and also at later stages of spliceosome assembly. Splicing enhancers have been described to be involved in constitutive as well as in alternative splicing. In addition, positive-regulatory sequences that are not purine-rich exist in introns (Black, 1995; Fu, 1995; Graveley, 2000; Mount, 2000; Schaal and Maniatis, 1999; Yeakley et al., 1996). Heterogeneous nuclear RNA particle (hnRNP) proteins have been proposed to bind to intronic consensus sequences (silencers), to affect 5' splice site recognition antagonistically to SR protein function, and to be involved in differential splicing (Berget, 1995; Fu, 1995; Yang et al., 1994).

As already mentioned in chapter 1.9, a sequence highly homologous to an exonic splicing enhancer composed of repetitive GAA elements has been detected in exon 14 of the 5-LO gene. This splicing enhancer has been reported to be recognized by a complex of nuclear proteins including a specific SR protein and a GAA-binding protein and to recruit additional SR proteins (Yeakley et al., 1996). It is tempting to speculate about the relevance of such a sequence in the 5-LO cds.

Interestingly, bifunctional proteins displaying features of classical transcription factors and coactivators as well as of SR protein splicing factors (e.g. PGC-1, a coactivator of many NRs) or of interaction with RNA or RNA-binding proteins (e.g. Pu.1) have been discovered (Bentley, 2002; Monsalve et al., 2000). Strikingly, Pu.1-binding to the 5-LO promoter has been reported (Silverman et al., 2002) and putative Pu.1 binding sites are also located in the 5-LO coding and intron sequences (chapter1.9).

As already mentioned in chapter 1.12.3, NRs such as VDR have recently been shown to be involved in coupling of transcription to splicing (Zhang et al., 2003). mRNA processing events that have been observed to be affected by steroid hormones include intron retention, 5' splice site selection, exon skipping and inclusion, terminal exon selection, and polyadenylation. Ligand-activated steroid hormone receptors have been proposed to bind to target DNA REs and recruit cell-specific coregulators. Some of these like the hnRNP-like protein coactivator activator (CoAA) are involved both in transcriptional and splicing regulation (Auboeuf et al., 2002). CoAA also enhances the activity of various transcription factors and in turn CoAA activity may be influenced by other promoter-recruited coregulators (Auboeuf et al., 2004). Interestingly, CoAA action has been reported to be not promoter-specific (Iwasaki et al., 2001), which would be consistent with the results obtained by reporter gene assays in this study. In chapter 1.12.3 the NR coactivator SKIP/NCoA62 has already been described as another candidate for interconnecting VDR-mediated transcription and mRNA splicing. The binding interface located in VDR helix 10 is thus distinct from the interface in helix 12 bound by p160 coactivators. Consistent with VDR-SKIP/NCoA62 interactions having been described to occur ligand-independently (Barry et al., 2003) a $1,25(OH)_2D_3$/TGFβ-independent effect has also been measured by VDR/RXR cotransfection in reporter gene assays with plasmids containing the 5-LO cds (paper II: fig. 2; fig. 15). Alternatively, endogenous $1,25(OH)_2D_3$ and/or low corepressor levels might be responsible for this induction. Inhibition of VDR-TFIIB interactions (thereby releasing TFIIB into the transcription preinitiation complex) and facilitation of VDR-RXR

interactions by the ligand and the concurrent binding of the coactivator GRIP-1 to the AF-2-dependent hydrophobic cleft (Barry et al., 2003) may account for the 1,25$(OH)_2D_3$/TGFβ-mediated effect in our transient transfection experiments (paper II: fig. 4; fig.18). Zhang *et al* reported a 1,25$(OH)_2D_3$-induced increase in the interaction of endogenous VDR, SRC-1, and SKIP/NCoA62 with the VDREs in the native rat 24-hydroxylase promoter as determined by ChIP analysis (Zhang et al., 2003). Hence, the regulation of 5-LO gene expression seems to differ from classical signalling through VDREs at least with respect to the observed promoter-independency. Nevertheless, the assembly of VDR and several coregulators in multi-protein complexes presumably contributes to 1,25$(OH)_2D_3$/TGFβ-action in 5-LO regulation. Interactions of SKIP/NCoA62 with key components of the spliceosome that are critical for proper excision of introns from pre-mRNA and the accumulation of unspliced transcripts following SKIP/NCoA62 disruption indicate an additional role of SKIP/NCoA62 in the splicing process (Makarov et al., 2002; Zhang et al., 2003). Thus, a complex containing the VDR, other transcription factors like Pu.1, coactivators like SKIP/NCoA62 and CoAA, and splicing factors like SR proteins might provide the link between transcriptional and posttranscriptional regulation of 5-LO gene expression. In summary, our results with the 5-LO cds clearly support the concept that mRNA processing/splicing is involved in the regulation of gene expression by 1,25$(OH)_2D_3$/TGFβ. The competitive recruitment of coregulator proteins like CoAA and/or SKIP/NCoA62 required for the transcriptional activity of a promoter to VDR containing multi-protein complexes built up on the transcript might account for the low reporter gene activity of the 1,25$(OH)_2D_3$/TGFβ responsive 5-LO cds-containing plasmids (Auboeuf et al., 2004). On the other hand, formation of the 5-LO-luciferase fusion protein might be the reason for the attenuated basal reporter gene activity.

Since purified SR proteins have been reported to bind nonspecifically to RNA, it has been suggested that interaction with other nuclear proteins confers RNA-binding specificity to these splicing factors (Fu, 1995). The VDR or other transcription factors might be such binding proteins. The interface for RNA-binding probably is distinct from the DNA-binding interface (DBD). Moreover, 1,25$(OH)_2D_3$, RA and TGFβ have been described as stimuli of alternative splicing events. It has been hypothesized that nuclear hormones and their receptors influence alternative splicing by changing the conformation of the CTD of the largest subunit of RNA polymerase II, which directs

splicing, capping, and polyadenylation factors to pre-mRNA (Akker et al., 2001; Magnuson et al., 1991). The hyperphosphorylated form of RNA polymerase II CTD has been proposed to serve as loading platform for RNA processing factors. In contrast to the diffuse distribution of many basic transcription factors in the nucleus, polymerase II also colocalizes with splicing factors in their presumable storage compartment, the nuclear speckles. Upon activation of transcription, RNA polymerase II and splicing factors translocate to the periphery of the speckles, where active mRNA synthesis occurs. Phosphorylation of SR proteins is also involved in their release. It has been suggested that sequence-specific DNA binding proteins or transcriptional coactivators might be involved in the recruitment of distinct processing factors to promoter-bound RNA polymerase II (Hirose and Manley, 2000; Monsalve et al., 2000). However, the recruitment of splicing factors by 5-LO promoter-bound VDR seems rather unlikely due to the observed promoter-independent upregulation of reporter gene activity by $1,25(OH)_2D_3$ and TGFβ.

RT-PCR analysis of the transcripts generated in the transiently transfected HeLa cells was difficult to perform when the region between exon 8 and 13 was amplified. More cycles were necessary (paper II: tab. 1) and in some PCR reactions multiple products were detected. Although trying several sets of primers, it was not possible to amplify the whole cds, at least three definite bands that were smaller than expected were obtained. It cannot be ruled out that strong secondary structures of the mRNA in this region simply disturbed the RT-PCR reactions, though. Sequencing a 450 bp PCR product solely allowed to verify the last 100 bp of exon 14. The sequencing data further upstream could not be interpreted due to superimposing of different sequences. Taken together, it seems that in untreated HeLa cells, 5-LO mRNA processing occurs that removes parts of the cds. RT-PCR data suggest that $1,25(OH)_2D_3$/TGFβ induce the generation of more complete transcripts. On the other hand, the artificial construction of the intron-containing plasmid might be responsible for these observations. In the pN10-cdsInJM construct the “first” exon, containing exons 1-10, is extremely large. Consequently, exon recognition may be disturbed. Nevertheless, one previous publication reporting the occurence of differential 5-LO expression (Boado et al., 1992) and $1,25(OH)_2D_3$-induced changes in the alternative splicing of fibronectin pre-mRNA (Magnuson et al., 1991) indicate consistency with aberrant splicing of 5-LO pre-mRNA that is suppressed by $1,25(OH)_2D_3$/TGFβ.

Coding sequence deletion studies (paper II: figs. 7 and 8) revealed that the respective deletion of parts A to D reduced induction of reporter gene activity by $1,25(OH)_2D_3$/TGFβ (paper II: fig. 8). In constructs in which larger parts of the cds are deleted (pdcdsABC and pdcdsBCD), the $1,25(OH)_2D_3$-mediated induction of reporter gene activity was almost completely lost and only the minor TGFβ-mediated enhancement could be measured. The fact that deletion of large parts of the cds is required in order to achieve an almost complete loss of the response to $1,25(OH)_2D_3$/TGFβ suggests that the regulation of 5-LO mRNA processing by both agents involves multiple parts of the cds. Similarly, the regulation of the sex-specific alternative splicing of the *Drosophila transformer* (*tra*) gene involves multiple binding sequences for the negative regulator *Sex lethal* (*Sxl*) located both upstream and downstream of the respective exon (Inoue et al., 1995).

The attempt to identify functional binding sites for the VDR in the cds by deletion of putative VDREs found by sequence analysis failed (fig. 20). Together with the fact, that no distinct part of the cds could be shown to be essential for $1,25(OH)_2D_3$/TGFβ-mediated induction of 5-LO expression, this might lead to the suggestion, that interaction of the VDR to the cds involves a binding interface other than the DBD and sequence elements distinct from the classical VDREs. Alternatively, the VDR may bind to another DNA or RNA binding protein (possibly part of a multi-protein complex, see above).

In order to antagonize the $1,25(OH)_2D_3$-mediated effects, transiently transfected HeLa cells have been incubated with the $1,25(OH)_2D_3$ analogon ZK191732. Interestingly, the compound displayed clear antagonistic features on 5-LO activity in MM6 cells (Gaschott et al., 2001), whereas concerning endogenous 5-LO protein expression antagonistic as well as agonistic effects could be distinguished (fig. 23). Thus, like many $1,25(OH)_2D_3$ analoga ZK191732 is a typical selective VDR modulator (also see chapter 1.12.3). In the reporter gene assay system the intrinsic VDR agonistic activity of the compound seems to be selectively determined (fig. 22). Transfection of the positive control p(DR3)4tkluc as well as of pN10-cdsInJM led to the identification of ZK191732 as agonist, suggesting that the agonistic action is not specific for 5-LO and might also be mediated through classical VDREs. The increased potency and dose-dependency observed for pN10-cdsInJM compared to p(DR3)4tkluc again indicates differential signalling pathways, though.

In summary, the results provide clear evidence that the 5-LO coding region mediates the upregulation of 5-LO expression by 1,25$(OH)_2D_3$ and TGFβ. These effects do not seem to be mediated by classical VDREs in the cds. The here reported data suggest that both mediators regulate 5-LO mRNA processing and possibly involve interaction of the liganded VDR with coactivators like SKIP/NCoA62 and/or CoAA that in turn associate with spliceosome components, which represents a novel mechanism in the regulation of gene expression by 1,25$(OH)_2D_3$ and TGFβ.

5.3 Induction of 5-LO gene expression by the Smad pathway

The role of TGFβ and Smad signalling in 5-LO gene regulation with respect to a possible participation of sequences located in the cds was investigated. Coexpression of Smads, the downstream effectors in TGFβ signalling, strongly enhanced reporter gene activity in reporter gene constructs containing the 5-LO cds. Interestingly and consistent with the above described VDR/RXR and 1,25$(OH)_2D_3$ effects, this upregulation did not depend on the 5-LO promoter and was also observed with the respective pGL3-basic and pGL3-promoter plasmids pGL3-ba-cdsInJM and pGL3-prom-cdsInJM.

Furthermore, the strong coding sequence-dependent induction of reporter gene activity was only observed with Smads but not with TGFβ as stimulus, indicating that this effect mainly depends on Smads but not on TGFβ signalling in general. However, cell culture media have not been serum-depleted. Thus, TGFβ contained in FCS supplements might have attenuated the measured TGFβ-mediated effects.

Deletion of exon 10 to exon 14 (part C) of the 5-LO cds abolished the Smad effect suggesting that this part contains essential REs required for Smad signalling. Deletion of other parts of the cds also reduced inducibility by Smads3/4 which points to the existence of additional REs in the other parts of the cds.

In silico analysis of the 5-LO gene revealed putative SBEs within the promoter as well as in the 3'-UTR, the coding region and in particular in the region from exon 10 to 14. Interestingly, deletion of a Smad consensus sequence in exon 10 strongly reduced response to Smad3/4 coexpression. Additionally, reduction of the Smad effects was also observed when a short DNA stretch within intron M was deleted that contains 4 putative SBEs suggesting that these elements might be involved in the regulation of 5-LO

expression by the TGFβ/Smad pathway. Interestingly, coexpression of Smads did not significantly alter the response to TGFβ (paper III: figs. 4, 7A+B, 8C) and deletion of various parts of the cds did not significantly change the effects of TGFβ on reporter gene activity. Furthermore, compared to Smads, the TGFβ-mediated effect is rather low and does not differ between the different plasmids and related deletion constructs in the reporter gene assays. The data suggest, that at least under the experimental conditions of the reporter gene assays, the cellular availability and maybe the nuclear localization of Smad proteins seem to be the limiting and critical parameter in the upregulation of luciferase gene activity.

As found before for the regulation of cellular 5-LO mRNA expression in the human monocytic cell line MM6 (Härle et al., 1999) and in reporter gene assays (paper II: fig. 4), there is a synergistic effect between TGFβ and vitamin D signalling. In agreement, the highest reporter gene activity was obtained when VDR/RXR and Smads were cotransfected and TGFβ and $1,25(OH)_2D_3$ were added (paper III: fig. 5). The influence of $1,25(OH)_2D_3$ alone is attenuated and induction values are rather decreased, though, which is probably due to a near to maximal upregulation level. Interaction between the TGFβ and $1,25(OH)_2D_3$ signalling pathways has previously been described. The VDR has been shown to physically and functionally interact with Smad3 and Smad7 *in vitro* and *in vivo* (Aschenbrenner et al., 2001; Yanagi et al., 1999; Yang et al., 2001b). Since the affinity of Smads to SBEs is rather weak, probably additional DNA contacts via binding partners such as the VDR are required (more information in chapter 1.12.4). In another publication, multiple mechanisms responsible for TGFβ/$1,25(OH)_2D_3$ crosstalk have been reported: ligand-binding to the VDR induces TGFβ mRNA expression, the VDR enhances TβRII expression in certain cell types, Smad3 activates the VDR and the crosslink is dependent on the PI 3-kinase pathway (Yang et al., 2001b). The performed experiments suggest that 5-LO is another example for a gene where Smads and the VDR interact and activate transcription in concert. To measure the TGFβ-mediated upregulation a 24 h preincubation time was required, indicating that 5-LO is not an immediate response gene of this mediator. Thus, the above described hierarchical model of gene regulation by TGFβ (chapter 1.12.4) might be supported by 5-LO gene expression. TGFβ-mediated activation of Smad3 may lead to upregulation of the VDR, thereby amplifying 5-LO induction by $1,25(OH)_2D_3$.

Interestingly, the NR coactivator SKIP/NCoA62 that has been shown to interact with VDR and also with components of the splicing machinery (Zhang et al., 2003), in addition has been found to augment TGFβ-dependent transactivation by direct binding to Smad proteins (Leong et al., 2001). Thus, this coactivator might establish a functional link between the $1,25(OH)_2D_3$ and TGFβ pathway in 5-LO gene regulation. Moreover, the participation of mRNA processing reactions in the Smad-mediated induction of 5-LO expression is indicated. This suggestion is further supported by the identification of novel Smad binding proteins that are involved in translation initiation and splicing. One of these is the splicing factor SF3b2/SAP145. The multiprotein complex SF3b is an integral component of both the major RNA splicing complex U2 snRNP and the minor RNA splicing complex U11/U12 di-snRNP and is essential for spliceosome assembly and the accurate excision of introns from pre-mRNA (Golas et al., 2003; Martinez et al., 2001; Warner et al., 2003). Moreover, it is highly concentrated in nuclear speckles (Eilbracht and Schmidt-Zachmann, 2001) (also see chapter 5.3) and its subunit spliceosome-associated protein 130 (SAP130) interacts with the HAT-containing coactivator complex STAGA (SPT3-TAF_{II}31-GCN5L acetylase) (Martinez et al., 2001).

In summary, the presented results provide evidence for the participation of the Smad signalling pathway in 5-LO gene regulation. Furthermore, the involvement of the cds in Smad signalling was demonstrated and some SBEs located in exon 10 and intron M have been identified that seem to be relevant for the regulation of 5-LO expression by Smads. Further experiments will be required to prove binding of Smads to these sites.

In conclusion, the involvement of multi-protein complexes containing Smad proteins, VDR-RXR heterodimers, additional transcription factors, several coactivators, RNA polymerase II, and splicing factors in the regulation of 5-LO gene expression by $1,25(OH)_2D_3$/TGFβ is highly indicated. Thus, 5-LO gene transcription appears to be closely connected to mRNA processing events. Aberrant RNA splicing seems to be at least one mechanism to downregulate 5-LO in undifferentiated cells which is overcome by $1,25(OH)_2D_3$/TGFβ stimulation.

5.4 Histone acetylation: impact and crosstalk with other signalling pathways in the regulation of (5-)LO gene expression

Inhibition of HDAC activity affects the expression of only 2 % of mammalian genes (Van Lint et al., 1996). However, the butyrate-induced upregulation of 5-LO and 15-LO-1 mRNA (Kamitani et al., 2000; Wächtershäuser et al., 2000) and the valproate-induced increase of 5-LO protein (Yildirim et al., 2003) indicate the involvement of histone acetylation in the regulation of LOs. Recently, it was shown that the 5-LO promoter was heavily methylated in 5-LO negative cell lines such as U937 (Uhl et al., 2002). In this cell line, $1,25(OH)_2D_3$/TGFβ and demethylation by AdC show additive effects on 5-LO upregulation. Since methylated CpG sites are recognized by a variety of methyl-CpG-binding proteins that are associated either directly or indirectly with HDACs (Ballestar and Wolffe, 2001), chromatin condensation could be one regulatory mechanism of 5-LO gene transcription (Ponton et al., 1996). For these reasons and because the HDACi TsA leads to hypomethylation at specific CpGs, it was of interest to investigate the regulation of 15-LO by histone acetylation and $1,25(OH)_2D_3$/TGFβ and the possible interplay of both in U937 cells, in comparison. In fact, 15-LO mRNA expression is enhanced by TsA but not by $1,25(OH)_2D_3$/TGFβ, suggesting that the 15-LO promoter like the 5-LO promoter is regulated by the histone acetylation status. Interestingly, combination of $1,25(OH)_2D_3$/TGFβ with TsA seems to suppress TsA-mediated 15-LO mRNA induction.

5-LO expression is induced by TsA treatment as determined by RT-PCR in MM6 cells and by reporter gene assays with the 5-LO promoter reporter gene construct in HeLa and MM6 cells. This TsA-mediated increase in 5-LO promoter activity was independent of the presence of $1,25(OH)_2D_3$/TGFβ and VDR/RXR coexpression in reporter gene assays. In contrast, luciferase assays with the p(DR3)4tkluc construct indicated a synergistic mechanism of $1,25(OH)_2D_3$ signal transduction and histone deacetylation, which is in agreement with previous observations made with other targets (Chen et al., 1999; Massagué and Wotton, 2000; Rachez and Freedman, 2000; Shen et al., 2002) (chapter 1.12.3). 5-LO mRNA levels in MM6 cells were predominantly increased by $1,25(OH)_2D_3$/TGFβ treatment, and the combination of both agents with TsA suppressed the $1,25(OH)_2D_3$/TGFβ effect.

Reporter gene assays with cds-containing plasmids led to similar results (fig. 27). Here, TsA seemed to inhibit the cds-mediated of $1,25(OH)_2D_3$/TGFβ-induced 5-LO upregulation. Taken together, it is suggested, that TsA mediates transcriptional activation by leading to 5-LO promoter hyperacetylation. Presence of the cds (and introns) opposes this effect. Since $1,25(OH)_2D_3$ is able to induce histone hyperacetylation at the promoters of target genes through VDR and its associated coactivators (Chen et al., 1999), part of the $1,25(OH)_2D_3$/TGFβ-mediated induction of 5-LO gene expression may be exerted by 5-LO promoter hyperacetylation. However, the strong induction of the promoterless construct pGL3-ba-cdsInJM is in contrast to this suggestion. Furthermore, pN10 in contrast to the cds-containing constructs does not significantly respond to $1,25(OH)_2D_3$/TGFβ and treatment with $1,25(OH)_2D_3$/TGFβ/TsA does not differ from TsA alone. This observation supports the finding of $1,25(OH)_2D_3$/TGFβ effects on 5-LO mRNA processing which cannot occur using pN10.

It has been shown that transcription from Sp1-driven promoters can be repressed by a tight association of the transcription factor Sp1 with HDAC1 in a multi-protein complex (Doetzlhofer et al., 1999). However, with respect to 5-LO it is obvious that inhibition of HDACs by TsA and the subsequent induction of 5-LO promoter transcription does not depend on the presence of the five tandemized Sp1 and Egr-1 sites in the 5-LO proximal core promoter region, which were found to be essential for basal transcription in previous studies (Uhl et al., 2002). These results suggest that there are binding sites for transcription factors outside of the five tandemized GC-boxes, which can recruit HDACs to the 5-LO promoter. For the promoters of some other genes like the HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) synthase, the human adenine nucleotide translocase isoform 2 (ANT2), $p21^{Waf1/Cip1}$, and $G\alpha_{i2}$, HDACi-mediated stimulation of gene expression has been mapped to promoter proximal Sp1 sites located in similar positions relative to the transcriptional start site (between -120 to -30 bp) (Camarero et al., 2003; Hodny et al., 2000; Nakano et al., 1997; Yang et al., 2001a). Based on these reports, the two single promoter proximal putative Sp1 sites in the 5-LO promoter (fig. 7, chapter 1.10) are possible candidates for the HDACi-mediated 5-LO promoter activation. Consistently, the region containing both putative Sp1 sites (pN13) was sufficient for TsA-dependent upregulation of 5-LO promoter activity (paper IV: fig. 3).

Deletion and mutation studies concerning these two sites are currently performed in our laboratory.

Although Sp1 is required for basal 5-LO promoter activity (Uhl et al., 2002), cotransfection of Sp1, Sp3 or Sp4 does not elevate luciferase activity of the promoter construct pN10 (figs. 25, 28B+E+H). This might be due to the high endogenous Sp1 level of HeLa cells.

Transient transfection experiments with 5-LO promoter reporter gene constructs with or without the 5-LO cds plus introns J-M revealed synergistic effects for histone hyperacetylation and Sp1 or Sp4 that depend upon the presence of the cds and introns J-M (fig. 28). The response of the 5-LO promoter (pN10) to TsA is enhanced by Sp4 but not Sp1 or Sp3 overexpression. Sp3 that can inhibit Sp1-mediated transactivation (Suske, 1999) does not induce either plasmid. It contains an inhibitory domain that has been described to be essential for silencing of its transcriptional activity in cell culture experiments. Like many other transcription factors, Sp3 is regulated by acetylation (Braun et al., 2001). Previously, Sp1 has also been shown to be phosphorylated (Choi et al., 2002) and acetylated (Ryu et al., 2003) following HDACi treatment. The subsequently increased affinity of Sp1 to its binding sites might be the explanation for the enhanced induction by Sp1 when cells were also treated by TsA. Furthermore, the affinity of Sp1 to the SV 40 early promoter was prominently reduced in a nucleosomal context compared to naked DNA (Li et al., 1994), and the purified ATP-dependent chromatin remodeling SWI/SNF complex stimulated nucleosome-binding by Sp1 (Utley et al., 1997). Hence, the synergistic effects of TsA and Sp1 or Sp4 mediated by the cds plus introns J-M may be due to a facilitation of Sp binding by disruption of the nucleosomal structure. The dependency of this profound increase on the presence of the cds plus introns J-M further indicates the existance of Sp binding sites in this part of the 5-LO sequence. An alternative explanation would be a TsA-mediated upregulation of Sp1 and Sp4. However, Choi *et al.* did not observe changes in Sp1 protein levels following TsA incubation in Hep3B cells (Choi et al., 2002). In addition, Sp1 has been shown to interact with Sp3, HDAC1 and p300. TsA treatment led to the dissociation of Sp3 and HDAC1 from the multi-protein complex (Choi et al., 2002). Dissociation of HDAC1 from the complex led to a release of repression, enabling Sp1 to associate with other accessory proteins to affect transcription (Yang et al., 2001a). Thus, it is suggested, that TsA regulates multi-protein complexes that might also contain other transcription factors such as YY1 that interact with Sp1 and/or HDACs ((Doetzlhofer et

al., 1999) and references therein). Since only a small percentage of the large number of Sp1 responsive genes are also activated by HDACis, the involvement of additional factors in the targeting of HDACs to specific promoters is highly indicated (Hodny et al., 2000). In contrast to the above described data, butyrate did neither affect Sp1 phosphorylation nor binding of Sp1 or Sp3 to the WAF1/Cip1 promoter (Nakano et al., 1997), which might be due to different mechanisms of TsA and butyrate. In summary, 5-LO gene expression is synergistically induced by histone hyperacetylation and Sp1/Sp4 in a cds- and/or introns J-M-dependent manner. Due to data from other target genes, enhanced affinity of Sp transcription factors to remodeled chromatin, posttranslational modifications of Sp family members and the regulation of multi-protein complexes might contribute to this crosstalk. However, to elucidate the mechanism, additional experiments such as co-immunoprecipitation studies, EMSAs or ChIP assays will be required.

In transient transfection experiments with the 5-LO promoter reporter gene construct pN10, TsA treatment reduced methylation-dependent repression, indicating that recruitment of HDACs could be involved in gene silencing by DNA methylation. The association of MBDs with HDACs has already been reported (Ballestar and Wolffe, 2001; Ng et al., 1999). In addition, HDAC-independent mechanisms seem also to play a role, since the presence of TsA could not completely prevent the methylation-dependent suppression. Thus, the dominance of methylation versus TsA-sensitive histone deacetylation in silencing the 5-LO promoter is indicated, which results in a TsA-resistant repression (Boeke et al., 2000; Yu et al., 2000). This is similar to other reports, which showed that transcriptionally silenced genes in cancer cell lines can be partially reactivated in the presence of a Dnmt inhibitor, but complete reactivation was not achieved (Rice and Allis, 2001; Singal and Ginder, 1999).

6 Summary

Arachidonate 5-lipoxygenase (5-LO), the key enzyme in the biosynthesis of proinflammatory leukotrienes, catalyzes the conversion of arachidonic acid to (5S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HpETE) and further to leukotriene A_4 ((5S)-6-oxido-7,9,11-trans-14-cis-eicosatetraenoic acid).

The cell type- and differentiation-specifically expressed enzyme occurs in a variety of immune competent cells. 5-LO is regulated on transcriptional and posttranscriptional levels. Basal 5-LO promoter activity seems to be regulated by DNA methylation in a tissue-specific manner. Depending on the cell type, several cytokines like granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) have been shown to be inducers of the 5-LO pathway. 5-LO mRNA, protein expression, and activity are strongly increased during differentiation of myeloid cell lines such as Mono Mac 6 (MM6) and HL-60 cultured with transforming growth factor-β (TGFβ) and 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). In this study, a profound cytosolic and nuclear accumulation of mature 5-LO mRNA but not of pre-mRNA has been measured in MM6 cells, which suggests enhanced mRNA processing as one mechanism of 5-LO upregulation.

Sequence analysis of the 5-LO promoter revealed the presence of several putative transcription factor binding sites including vitamin D response elements (VDREs). Binding of the vitamin D receptor-retinoid X receptor (VDR-RXR) heterodimer to putative VDREs within the 5-LO core promoter region have been confirmed by DNAse I footprinting and electrophoretic mobility shift assay (EMSA) experiments. However, neither in transiently (luciferase assays with MM6, HeLa, RBL-1, HL-60, and U937 cells) nor in stably (RT-PCR with HL-60 cells) transfected cells 5-LO promoter reporter gene constructs were responsive to 1,25$(OH)_2D_3$/TGFβ. Since the stable integration of the sequence into the genome did not lead to inducibility, the simple lack of bridging factors or coactivators in transient transfections could be excluded. In contrast, a transiently transfected plasmid containing a 4-times concatemerized rat atrial natriuretic factor VDRE in front of the thymidine kinase promoter (p(DR3)4tkluc) was prominently activated by 1,25$(OH)_2D_3$/TGFβ when the expression vectors for the human receptors VDR and RXR were cotransfected. Taken together, it was strongly suggested that the effects of TGFβ/1,25$(OH)_2D_3$ on 5-LO gene expression are mediated

by regulatory elements located outside of the 5-LO promoter, which were absent in transient and stable reporter gene transfections.

Exons, introns or the 3'untranslated region (3'UTR) might contain sequences like mRNA processing signals, elements that affect mRNA stability, or transcription factor binding sites. Thus, the possible contribution of these sequences to the regulation of 5-LO gene expression has been investigated in transient transfection experiments predominantly in HeLa cells. Interestingly, inclusion of the coding sequence (cds) caused a 5-fold upregulation of reporter gene activity by TGFβ and $1,25(OH)_2D_3$ which was enhanced to 13-fold when the last four introns of the 5-LO gene were also included. This induction of reporter gene activity depended on the availability of sufficient amounts of both nuclear receptors VDR and RXR. There even seems to be a considerable ligand-independent activity of the VDR regarding the induction of reporter gene activity mediated by the 5-LO cds. Strikingly, the effect did not depend upon the presence of the 5-LO promoter but was also obtained with plasmids containing a CMV or even no promoter. RT-PCR analysis of the transcripts generated by the transfected cells implies that parts of the cds are removed during 5-LO mRNA processing leading to incomplete transcripts. Addition of TGFβ and $1,25(OH)_2D_3$ induced the appearance of exon 13 in the 5-LO transcripts which is reflected by an about 14-fold increase of the corresponding PCR product. The partial inhibition of the $1,25(OH)_2D_3$/TGFβ effects by cycloheximide suggests that the response to both agents requires at least in part protein biosynthesis. Thus, it is possible that $1,25(OH)_2D_3$/TGFβ induce the generation of proteins involved in 5-LO mRNA splicing and/or other processing reactions. Deletion of various parts of the 5-LO cds slightly reduced the inducibility by TGFβ and $1,25(OH)_2D_3$, whereas deletion of large parts of the cds was required for an almost complete loss of this response. This suggests that the regulation of 5-LO mRNA processing by TGFβ and $1,25(OH)_2D_3$ involves multiple parts of the cds.

Previously, the $1,25(OH)_2D_3$ analogon ZK191732 has been shown to display clear antagonistic features on 5-LO enzyme activity in MM6 cells. However, in this thesis the compound induced endogenous 5-LO protein expression but in combination with $1,25(OH)_2D_3$/TGFβ partly antagonized the marked 5-LO protein upregulation by these agents in MM6 cells. In contrast, the analogon prominently induced the reporter gene activity of both the VDRE control plasmid and the 5-LO promoter and cds plus introns-containing plasmid in transiently transfected HeLa cells. Nevertheless, activation of the

control plasmid was less profound and not dose-dependent, implying a mechanism distinct from activation through classical VDREs in the cds-containing construct.

In transient reporter gene assays Smads3/4, the downstream effectors in TGFβ signalling, mediated a prominent upregulation of reporter gene activity that strongly depended on the cds and to a lesser extent on the 3'-UTR and introns J to M. Since TGFβ did not contribute to the effect to a large extent, it seemed to be mainly mediated by Smads. Thus, at least under the experimental conditions of the reporter gene assays, the cellular availability and maybe the nuclear localization of Smad proteins seems to be the limiting and critical parameter in the upregulation of luciferase activity. The requirement for preincubation with TGFβ in order to measure any induction by this agent indicates a secondary response mechanism. Like the effect mediated by VDR/RXR/1,25$(OH)_2D_3$, the upregulation of luciferase activity by Smads3/4 did not depend upon the 5-LO promoter.

Deletion of various parts of the 5-LO cds reduced the inducibility by Smads3/4, the decrease being most profound when exons 10 to 14 were deleted. Deletion studies supported the existence of up to four functional Smad binding elements in intron M and another TGFβ responsive element in exon 10 that had been detected by sequence analysis before.

The highest reporter gene activity was obtained when VDR/RXR and Smads3/4 were cotransfected and both TGFβ and 1,25$(OH)_2D_3$ were added. This observation is in agreement with previous reports about VDR-Smad interactions and functional crosstalk between the TGFβ and 1,25$(OH)_2D_3$ signalling pathways. Reports about interactions of Smad proteins with key components of the spliceosome further support a role of mRNA processing events in the regulation of 5-LO gene expression.

In conclusion, 5-LO seems to be downregulated in undifferentiated cells by aberrant mRNA processing (splicing, mRNA maturation). Differentiation by 1,25$(OH)_2D_3$/TGFβ activates 5-LO gene expression by a mechanism possibly involving multi-protein complexes containing Smad proteins, VDR-RXR heterodimers, other transcription factors, several coactivators, RNA polymerase II, and splicing factors.

Several reports indicate the involvement of epigenetic mechanisms in the regulation of gene expression of lipoxygenases. In the 5-LO negative cell line U937 the 5-LO promoter is heavily methylated. Because of similarities in the gene expression of lipoxygenases it was of interest to investigate 15-LO expression in U937 cells in

comparison. The regulation of chromatin organization and DNA methylation are closely associated processes. In fact, 15-LO mRNA expression was enhanced by histonedeacetylase (HDAC) inhibition with trichostatin A (TsA) but not by $1,25(OH)_2D_3$/TGFβ, suggesting that 15-LO like 5-LO is regulated by histone acetylation status. In contrast, 5-LO mRNA was not upregulated by TsA treatment in U937 cells, which might be due to a domination of DNA methylation over histone acetylation. This same dominance of methylation has also been observed in transient transfection analyses with *Hpa II*-methylated 5-LO promoter reporter gene plasmids, where DNA methylation reduced the TsA-mediated activation of reporter gene activity. The recruitment of HDACs being part of the methylation-dependent repression is indicated. However, 5-LO expression is induced by TsA treatment as determined by RT-PCR in MM6 cells and in reporter gene assays with the 5-LO promoter reporter gene construct in HeLa and MM6 cells. The combination of TsA with $1,25(OH)_2D_3$/TGFβ suppressed the prominent increase of 5-LO mRNA induced by $1,25(OH)_2D_3$/TGFβ in MM6 cells. Consistently, in reporter gene assays with the $1,25(OH)_2D_3$/TGFβ-responsive cds-containing constructs TsA reduces the strong induction of reporter gene activity mediated by $1,25(OH)_2D_3$/TGFβ. In contrast, control experiments with the p(DR3)4tkluc plasmid indicated a synergistic mechanism of $1,25(OH)_2D_3$ signal transduction and histone acetylation. The profound TsA-mediated increase in 5-LO promoter activity was independent of $1,25(OH)_2D_3$/TGFβ and VDR/RXR coexpression, though. A synergistic activation was not expected since the 5-LO promoter plasmid was not responsive to $1,25(OH)_2D_3$/TGFβ and VDR/RXR, anyway. Taken together, it is suggested, that TsA induces transcriptional activation by leading to 5-LO promoter hyperacetylation. Presence of the cds (and introns) opposes this effect possibly by recruitment of HDACs by proteins like the VDR or Smads. These proteins presumably directly or indirectly interact with regulatory elements in the coding region in either 5-LO DNA or mRNA.

The basal activity of the 5-LO promoter strongly depends upon the presence of multiple Sp1-binding sites, five of which are in tandem. Deletion experiments revealed that the induction of 5-LO transcription does not depend on the presence of this GC-box. The region containing two single promoter proximal putative Sp1 sites was sufficient for TsA-dependent upregulation of 5-LO promoter activity. Since these Sp1 binding sites are in similar positions relative to the transcriptional start site like those in other gene

promoters to which the HDACi-mediated stimulation of gene expression has previously been mapped, they are likely to be the target of TsA action.

Transient transfection experiments with 5-LO promoter reporter gene constructs with or without the 5-LO cds plus introns J-M revealed synergistic effects for histone hyperacetylation and Sp1 or Sp4 that depended upon the presence of the coding region and introns J-M. Also, the response of the 5-LO promoter to TsA was enhanced by Sp4 overexpression. These synergistic effects may be due to a facilitation of Sp transcription factor binding by disruption of the nucleosomal structure. The results further suggest the existence of Sp binding sites in the 5-LO cds and/or introns J-M. Taken together, Sp1 and Sp4 act in concert with histone acetylation in a cds-dependent manner to activate 5-LO gene expression.

7 Zusammenfassung

Die 5-Lipoxygenase (5-LO) ist das Schlüsselenzym in der Biosynthese proinflammatorischer Leukotriene. Sie katalysiert sowohl den Einbau von molekularem Sauerstoff in Arachidonsäure als auch die weitere Umsetzung des Zwischenproduktes (5S)-Hydroperoxy-6-trans-8,11,14-cis-eicosatetraensäure zu Leukotrien A_4.

Die 5-LO wird zelltyp- und differenzierungsspezifisch exprimiert und kommt hauptsächlich in immunkompetenten Zellen vor. Die 5-LO-Expression wird auf transkriptioneller und posttranskriptioneller Ebene reguliert. Die gewebespezifische Expression der 5-LO beruht auf der Regulation der basalen Promotoraktivität durch DNA-Methylierung. Als Stimuli der 5-LO-Expression wurden Zytokine wie der Granulozyten/Makrophagen-Kolonie-stimulierende Faktor und Interleukin-3 identifiziert. Werden myeloide Zelllinien wie Mono Mac 6 (MM6) und HL-60 mit 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) und transformierendem Wachstumsfaktor β (TGFβ) differenziert, so kommt es zu einer gleichzeitigen starken Erhöhung der 5-LO-mRNA- und -Protein-Bildung sowie der zellulären Enzymaktivität. Im Rahmen dieser Arbeit konnte in MM6-Zellen mittels RT-PCR-Analyse gezeigt werden, dass durch $1,25(OH)_2D_3$ und TGFβ nur die reife, nicht jedoch die prä-mRNA der 5-LO im Zytosol und im Zellkern stark angereichert wird. Die Daten legen nahe, dass mRNA-Prozessierungsvorgänge an der Hochregulation der 5-LO in differenzierenden Zellen beteiligt sind.

Durch Analyse der 5-LO-Promotor-Sequenz wurden potentielle Bindungsstellen für Transkriptionsfaktoren wie den Vitamin-D-Rezeptor (VDR) identifiziert. Tatsächlich konnte in DNAse I-Footprinting- und EMSA-Studien die Bindung von VDR-Retinoid-X-Rezeptor (RXR)-Heterodimeren an einen bestimmten Bereich des 5-LO-Promotors (-307 bis –268 bp) gezeigt werden. Unerwarteterweise wurde jedoch weder ein 5-LO-Promotor-Luziferase-Konstrukt in transient transfizierten Mono Mac 6-, HeLa-, RBL-1-, HL-60- und U937-Zellen, noch ein 5-LO-Promotor-CAT-Konstrukt in stabil transfizierten HL-60-Zellen durch $1,25(OH)_2D_3$ und TGFβ aktiviert. Daher konnte ausgeschlossen werden, dass das Fehlen von Cofaktoren oder anderen Regulatoren beim nicht stabil ins Genom integrierten Promotor der Grund für die fehlende Induktion war. Die Eignung der eingesetzten Testsysteme zum Nachweis von $1,25(OH)_2D_3$/TGFβ-vermittelten Effekten konnte jedoch in Kontrollexperimenten gezeigt werden. Ein

transient transfiziertes Reportergen-Plasmid, das ein viermal konkatemerisiertes Vitamin D-Response Element (VDRE) des atrialen natriuretischen Faktors der Ratte vor dem Thymidinkinase-Promotor enthält (p(DR3)4tkluc), wurde durch 1,25$(OH)_2D_3$ und TGFβ stark aktiviert, wenn die Expressionsplasmide für die humanen Rezeptoren VDR und RXR cotransfiziert wurden. Die Daten legten nahe, dass die Induktion der 5-LO-Expression durch 1,25$(OH)_2D_3$ und TGFβ durch regulatorische Elemente vermittelt wird, die sich außerhalb des in transienten und stabilen Reportergen-Transfektionen untersuchten Promotor-Bereiches befinden.

Derartige Sequenzen wie Transkriptionsfaktor-Bindungsstellen, mRNA-Prozessierungssignale und Elemente, welche die mRNA-Stabilität beeinflussen, könnten sich in den Exons, den Introns oder auch der 3'-untranslatierten Region (3'-UTR) des 5-LO-Gens befinden. Aus diesem Grund wurden diese Sequenzbereiche in Reportergenkonstrukte kloniert und im Hinblick auf eine mögliche Beteiligung an der 1,25$(OH)_2D_3$/TGFβ-vermittelten Hochregulation der 5-LO mittels Reportergen-Assays untersucht. Interessanterweise wurde in transienten Transfektionen von HeLa-Zellen durch das Einfügen der kodierenden Sequenz der 5-LO in das 5-LO-Promotor-Luziferase-Plasmid eine 5-fache Induktion der Reportergen-Aktivität durch 1,25$(OH)_2D_3$/TGFβ erreicht. Durch zusätzliches Klonieren der letzten vier Introns der 5-LO an die entsprechenden Stellen zwischen den Exons ergab sich sogar eine 13-fache Hochregulation. Die auf diese Weise gemessenen Stimulationen hingen allerdings von der Cotransfektion der nukleären Rezeptoren VDR und RXR ab. Weiterhin stimuliert der VDR auch unabhängig von seinem Liganden 1,25$(OH)_2D_3$ die Reportergen-Aktivität von Plasmiden, welche die kodierende Sequenz enthalten. Diese Effekte waren erstaunlicherweise unabhängig vom 5-LO-Promotor, da sie auch mit entsprechenden Plasmiden beobachtet wurden, die einen Cytomegalie-Virus- oder keinen Promotor enthielten. Die RT-PCR-Analyse der in den transfizierten Zellen gebildeten Transkripte wies darauf hin, dass im Laufe der mRNA-Prozessierung Teile der kodierenden Sequenz entfernt werden, was zu unvollständigen Transkripten führt. Inkubation der Zellen mit 1,25$(OH)_2D_3$ und TGFβ führte zu einem verstärkten Auftreten des Exons 13 in der mRNA der 5-LO, was durch einen 14-fachen Anstieg des entsprechenden PCR-Produktes gezeigt wurde. Die Hemmung der Proteinbiosynthese durch Cycloheximid konnte diesen Anstieg teilweise verringern, was möglicherweise daran liegt, dass 1,25$(OH)_2D_3$/TGFβ die Bildung von Proteinen induzieren, die am

Splicing und/oder anderen Prozessierungsreaktionen der mRNA der 5-LO beteiligt sind. Abschnittsweise Deletionen der kodierenden Region der 5-LO verringerte die Induzierbarkeit durch $1,25(OH)_2D_3$ und TGFβ nur geringfügig und erst die Deletion größerer Teile dieser Sequenz hob die Stimulierbarkeit fast vollständig auf. Für die Regulation der Prozessierung der mRNA der 5-LO durch $1,25(OH)_2D_3$ und TGFβ scheinen also verschiedene Teile der kodierenden Sequenz von Bedeutung zu sein.

Um den Mechanismus der $1,25(OH)_2D_3$-induzierten 5-LO-Expression weiter zu charakterisieren, wurden zusätzlich Versuche mit dem Analogon ZK191732 durchgeführt. Diese Substanz zeigte in MM6-Zellen rein $1,25(OH)_2D_3$-antagonistische Effekte auf die Induktion der zellulären 5-LO-Aktivität. Im Gegensatz dazu wirkte ZK191732 partial agonistisch bezüglich der Induktion der Expression des 5-LO-Proteins. In Reportergen-Assays zeigte das Analogon eine deutliche agonistische Aktivität in transient transfizierten HeLa-Zellen sowohl mit p(DR3)4tkluc als Positivkontrolle als auch mit dem Konstrukt, das den 5-LO-Promoter, die kodierende Sequenz und die Introns J-K enthält. Allerdings wies die weniger starke Stimulierung von p(DR3)4tkluc darauf hin, dass sich der Mechanismus der Aktivierung durch die kodierende Sequenz von der Transkriptionsstimulation durch klassische VDREs unterscheidet.

Aufgrund der synergistischen Wirkung von $1,25(OH)_2D_3$ und TGFβ auf die Genexpression der 5-LO wurde außerdem die Beteiligung des TGFβ-Signaltransduktionsweges untersucht. Die Cotransfektion der Smad-Proteine 3 und 4, die als TGFβ-Effektoren bekannt sind, führte in Abhängigkeit von der kodierenden Sequenz und in geringerem Maße auch von der 3'-UTR und den Introns J-M zu einer starken Erhöhung der Reportergenaktivität. Dieser Effekt schien vorrangig durch Smads und nur geringfügig durch TGFβ vermittelt zu sein. Anscheinend stellen die Verfügbarkeit und vielleicht auch die nukleäre Lokalisation der Smad-Proteine zumindest unter den experimentellen Bedinungen der Reportergen-Assays den limitierenden und kritischen Faktor in der Regulation der 5-LO-Expression dar. Der durch TGFβ vermittelte geringe Effekt wurde erst nach Vorinkubation der Zellen mit diesem Agens vor der Transfektion beobachtet, was auf einen sekundären Effekt hinweist. Wie die Einflüsse von VDR/RXR und $1,25(OH)_2D_3$, ist auch die stimulierende Wirkung von Smads auf die Reportergen-Aktivität unabhängig vom 5-LO-Promotor.

Die Smad-Induzierbarkeit konnte durch die Deletion einzelner Teile der kodierenden Region verringert werden. Die stärksten Auswirkungen hatte hierbei die Deletion einer Sequenz von Exon 10 bis 14. Die Bedeutung einer durch Sequenzanalyse ermittelten Ansammlung von bis zu vier potentiellen Smad-Bindungsstellen in Intron M und einem weiteren TGFβ-responsiven Element in Exon 10 konnte durch zusätzliche gezielte Deletionen bestätigt werden.

In verschiedenen Veröffentlichungen wurden bereits Interaktionen zwischen VDR und Smads sowie ein Zusammenspiel der Signalkaskaden von $1,25(OH)_2D_3$ und TGFβ beschrieben. Daher war es nicht überraschend, dass auch in den hier durchgeführten transienten Transfektionen die Kombination der Cotransfektionen von VDR/RXR und Smads3/4 bei gleichzeitiger Inkubation mit $1,25(OH)_2D_3$ und TGFβ zu den höchsten absoluten Luziferaseaktivitäten führte.

Zusammenfassend läßt sich sagen, dass die 5-LO in den untersuchten Zellen vermutlich durch posttranskriptionelle Prozesse (Splicing) herunterreguliert wird. Die Differenzierung der Zellen durch $1,25(OH)_2D_3$ und TGFβ scheint die 5-LO-Expression durch eine Gegenregulation zu erhöhen, an der aktivierte VDR-RXR-Heterodimere und Smad-Proteine beteiligt sind.

Da bereits etliche Publikationen den Einfluss von epigenetischen Mechanismen auf die Genexpression von Lipoxygenasen belegen, wurde dies im zweiten Teil dieser Arbeit näher untersucht. Bereits früher wurde gezeigt, dass der Promotor in der 5-LO-negativen Zelllinie U937 stark methyliert ist. Da die LOs starke Übereinstimmungen in der Regulation ihrer Expression zeigen, wurde im Vergleich zur 5-LO die Expression der 15-LO in dieser Zelllinie untersucht. Aufgrund des bekannten Zusammenspiels von DNA-Methylierung und der Organisation der Chromatinstruktur, wurden die Zellen mit dem Histondeacetylase (HDAC)-Inhibitor Trichostatin A (TsA) inkubiert. In RT-PCR-Experimenten wurde ein starker Anstieg der 15-LO-mRNA-Menge durch Behandlung der Zellen mit TsA festgestellt, wohingegen $1,25(OH)_2D_3$/TGFβ keinen Effekt zeigten.

Die mRNA der 5-LO hingegen konnte in U937-Zellen durch TsA nicht erhöht werden, was an einer Dominanz der DNA-Methylierung gegenüber der Histonacetylierung liegen könnte. In ähnlicher Weise wurden nämlich durch *Hpa II* methylierte 5-LO-Promotor-Reportergen-Plasmide in transienten Transfektionen in geringerem Maße durch TsA stimuliert als die unmethylierten Vektoren. Die Rekrutierung von HDACs trägt wahrscheinlich zum repressiven Effekt der Methylierung bei. Auch die 5-LO-

mRNA wurde durch TsA in MM6-Zellen deutlich induziert. Allerdings reduzierte TsA in Kombination mit $1,25(OH)_2D_3$/TGFβ die durch $1,25(OH)_2D_3$/TGFβ erhaltene Steigerung der 5-LO-mRNA-Menge. In gleicher Weise wurde die starke $1,25(OH)_2D_3$/TGFβ-vermittelte Stimulation der Plasmide, die die kodierende Sequenz der 5-LO enthalten, durch TsA deutlich gehemmt. Im Gegensatz dazu wurde die Reportergenaktivität des VDRE-Kontrollplasmides durch $1,25(OH)_2D_3$ und Histonacetylierung synergistisch induziert. Die TsA-induzierte Hyperacetylierung des 5-LO-Promotors führt einerseits zu einer transkriptionellen Aktivierung, während die cds-vermittelte 5-LO-Induktion durch $1,25(OH)_2D_3$/TGFβ von TsA gehemmt wird. Dies legt nahe, dass die auf mRNA-Prozessierungsvorgängen beruhende 5-LO-Induktion durch Histonhyperacetylierung eher verringert wird.

Die basale 5-LO-Promotoraktivität wird durch multiple Sp1-Bindungsstellen im Promotor vermittelt. Es wurde gezeigt, dass eine Region des Promotors, die zwei proximale putative Sp1-Bindungsstellen umfasst, die TsA-induzierte Stimulation der 5-LO-Promotoraktivität vermittelt.

Bei einem Vergleich der 5-LO-Promotor-Konstrukte mit und ohne kodierender Sequenz plus Introns J-M in Reportergen-Assays stellte sich heraus, dass die kodierende Region plus Introns J-M zu einem deutlichen Synergieeffekt von Sp1 bzw. Sp4 und der Histonhyperacetylierung führt. Außerdem wurde auch die TsA-vermittelte Aktivierung des 5-LO-Promotors durch die Cotransfektion von Sp4 verstärkt. Die Auflockerung der Chromatinstruktur durch Histonacetylierung ist vermutlich an der Synergie beteiligt. Außerdem könnten die durch die kodierende Sequenz vermittelten Effekte auch auf zusätzliche Sp-Bindungsstellen in diesem Bereich des 5-LO-Gens hinweisen.

8 References

Abramovitz, M., E. Wong, M.E. Cox, C.D. Richardson, C. Li, and P.J. Vickers. 1993. 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *European Journal of Biochemistry*. 215:105-111.

Akker, S.A., P.J. Smith, and S.L. Chew. 2001. Nuclear post-transcriptional control of gene expression. *J. Mol. Endocrinol.* 27:123-131.

Albert, D., Zündorf, I., Dingermann, T., Müller, W., Steinhilber, D. and Werz, O. 2002. Hyperforin is a dual inhibitor of cyclooxygenase-1 and 5-lipoygenase. *Biochemical Pharmacology*. 64:1767-1775.

Altmann, A., D. Poeckel, L. Fischer, M. Schubert-Zsilavecz, D. Steinhilber, and O. Werz. 2004. Coupling of boswellic acid-induced Ca2+ mobilisation and MAPK activation to lipid metabolism and peroxide formation in human leucocytes. *Br. J. Pharmacol.* 141:223-232.

Ares, M., Jr., L. Grate, and M.H. Pauling. 1999. A handful of intron-containing genes produces the lion's share of yeast mRNA. *Rna*. 5:1138-1139.

Arthur, J.R. 2000. The glutathione peroxidases. *Cell. Mol. Life Sci.* 57:1825-1835.

Aschenbrenner, J.K., H.W. Sollinger, B.N. Becker, and D.A. Hullett. 2001. 1,25-(OH(2))D(3) alters the transforming growth factor beta signaling pathway in renal tissue. *J. Surg. Res.* 100:171-175.

Attwood, J.T., R.L. Yung, and B.C. Richardson. 2002. DNA methylation and the regulation of gene transcription. *Cell. Mol. Life Sci.* 59:241-257.

Auboeuf, D., D.H. Dowhan, X. Li, K. Larkin, L. Ko, S.M. Berget, and B.W. O'Malley. 2004. CoAA, a nuclear receptor coactivator protein at the interface of transcriptional coactivation and RNA splicing. *Mol. Cell. Biol.* 24:442-453.

Auboeuf, D., A. Hönig, S.M. Berget, and B.W. O'Malley. 2002. Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science*. 298:416-419.

Avis, I., S.H. Hong, A. Martinez, T. Moody, Y.H. Choi, J. Trepel, R. Das, M. Jett, and J.L. Mulshine. 2001. Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. *Faseb J.* 15:2007-2009.

Ballestar, E., and A.P. Wolffe. 2001. Methyl-CpG-binding proteins. Targeting specific gene repression. *Eur. J. Biochem.* 268:1-6.

Barry, J.B., G.M. Leong, W.B. Church, L.L. Issa, J.A. Eisman, and E.M. Gardiner. 2003. Interactions of SKIP/NCoA-62, TFIIB, and retinoid X receptor with vitamin D receptor helix H10 residues. *J. Biol. Chem.* 278:8224-8228.

Beato, M., R. Candau, S. Chavez, C. Mows, and M. Truss. 1996. Interaction of steroid hormone receptors with transcription factors involves chromatin remodelling. *J. Steroid. Biochem. Mol. Biol.* 56:47-59.

Bentley, D. 2002. The mRNA assembly line: transcription and processing machines in the same factory. *Curr. Opin. Cell Biol.* 14:336-342.

Berg, J.P., and E. Haug. 1999. Vitamin D: a hormonal regulator of the cAMP signaling pathway. *Crit. Rev. Biochem. Mol. Biol.* 34:315-323.

Berget, S.M. 1995. Exon recognition in vertebrate splicing. *J. Biol. Chem.* 270:2411-2414.

Bigby, T.D. 1999. Regulation of expression of the 5-lipoxygenase pathway. *Clin. Rev. Allergy Immunol.* 17:43-58.

Black, D.L. 1995. Finding splice sites within a wilderness of RNA. *RNA*. 1:763-771.

Boado, R.J., W.M. Pardridge, H.V. Vinters, and K.L. Black. 1992. Differential expression of arachidonate 5-lipoxygenase transcripts in human brain tumors: evidence for the expression of a multitranscript family. *Proc. Natl. Acad. Sci. U S A*. 89:9044-9048.

Boeke, J., O. Ammerpohl, S. Kegel, U. Moehren, and R. Renkawitz. 2000. The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A. *J. Biol. Chem.* 275:34963-34967.

Borgeat, P., M. Hamberg, and B. Samuelsson. 1976. Transformation of arachidonic acid and homo-γ-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. *J. Biol. Chem.* 251:7816-7820.

Brash, A.R. 1999. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *J. Biol. Chem.* 274:23679-23682.

Braun, H., R. Koop, A. Ertmer, S. Nacht, and G. Suske. 2001. Transcription factor Sp3 is regulated by acetylation. *Nucleic Acids Res.* 29:4994-5000.

Brodin, G., A. Åhgren, P. ten Dijke, C.H. Heldin, and R. Heuchel. 2000. Efficient TGF-beta induction of the Smad7 gene requires cooperation between AP-1, Sp1, and Smad proteins on the mouse Smad7 promoter. *J. Biol. Chem.* 275:29023-29030.

Brooks, C.D.W., and J.B. Summers. 1996. Modulators of leukotriene biosynthesis and receptor action. *J. Med. Chem.* 39:2629-2654.

Brungs, M., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1994. On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGF beta. *Biochem. Biophys. Res. Commun.* 205:1572-1580.

Brungs, M., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1995. Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25- dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U S A*. 92:107-111.

Camarero, N., A. Nadal, M.J. Barrero, D. Haro, and P.F. Marrero. 2003. Histone deacetylase inhibitors stimulate mitochondrial HMG-CoA synthase gene expression via a promoter proximal Sp1 site. *Nucleic Acids Res.* 31:1693-1703.

Carlberg, C. 1995. Mechanisms of nuclear signalling by vitamin D3. Interplay with retinoid and thyroid hormone signalling. *Eur. J. Biochem.* 231:517-527.

Carlberg, C. 1996. The concept of multiple vitamin D signaling pathways. *J. Investig. Dermatol. Symp. Proc.* 1:10-14.

Carlberg, C. 2003. Molecular basis of the selective activity of vitamin D analogues. *J. Cell. Biochem.* 88:274-281.

Carlberg, C., and P. Polly. 1998. Gene regulation by vitamin D3. *Crit. Rev. Eukaryot. Gene Expr.* 8:19-42.

Carlberg, C., and J.H. Saurat. 1996. Vitamin D-retinoid association: molecular basis and clinical applications. *J. Investig. Dermatol. Symp. Proc.* 1:82-86.

Chan, M.F., G. Liang, and P.A. Jones. 2000. Relationship between transcription and DNA methylation. *Curr. Top. Microbiol. Immunol.* 249:75-86.

Charng, M.J., D. Zhang, P. Kinnunen, and M.D. Schneider. 1998. A novel protein distinguishes between quiescent and activated forms of the type I transforming growth factor beta receptor. *J. Biol. Chem.* 273:9365-9368.

Chen, H., R.J. Lin, W. Xie, D. Wilpitz, and R.M. Evans. 1999. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell*. 98:675-686.

Chen, X., Zhang YY, Funk CD. 1998. Determinants of 5-lipoxygenase nuclear localization using green fluorescent protein/5-lipoxygenase fusion proteins. *J. Biol. Chem.* 273:31237-31244.

Chen, X.S., and C.D. Funk. 2001. The N-terminal "ß-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation. *J. Biol. Chem.* 276:811-818.

Choi, H.S., J.H. Lee, J.G. Park, and Y.I. Lee. 2002. Trichostatin A, a histone deacetylase inhibitor, activates the IGFBP-3 promoter by upregulating Sp1 activity in hepatoma cells: alteration of the Sp1/Sp3/HDAC1 multiprotein complex. *Biochem. Biophys. Res. Commun.* 296:1005-1012.

Claesson, H.E., and S.E. Dahlen. 1999. Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J. Intern. Med.* 245:205-227.

Claesson, H.E., P.J. Jakobsson, D. Steinhilber, B. Odlander, and B. Samuelsson. 1993. Expression of 5-lipoxygenase and biosynthesis of leukotriene B4 in human monomorphonuclear leukocytes. *J. Lipid. Mediat.* 6:15-22.

Coffey, M.J., S.M. Phare, and M. Peters-Golden. 2000. Prolonged exposure to lipopolysaccharide inhibits macrophage 5- lipoxygenase metabolism via induction of nitric oxide synthesis. *J. Immunol.* 165:3592-3598.

Davie, J.R. 2003. Inhibition of histone deacetylase activity by butyrate. *J. Nutr.* 133:2485S-2493S.

de Carvalho, M.G.S., Ashley L. McCormack, Eric Olson, Farideh Ghomashchi, Michael H. Gelb, John R. Yates III, and Christina C. Leslie. 1996. Identification of Phosphorylation Sites of Human 85-kDa Cytosolic Phospholipase A Expressed in Insect Cells and Present in Human Monocyte**s**. *J. Biol. Chem.* 271:6987-6997.

de Groot, J.J.V., G.A.; Vliegenthart, J.F.; Boldingh, J.; Wever, R. and van Gelder, B.F. 1975. Demonstration By EPR spectroscopy of the functional role of iron in soybean lipoxygenase-1. *Biochim. Biophys. Acta*. 377:71-79.

de Ruijter, A.J., A.H. van Gennip, H.N. Caron, S. Kemp, and A.B. van Kuilenburg. 2003. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* 370:737-749.

Devchand, P.R., A.K. Hihi, M. Perroud, W.D. Schleuning, B.M. Spiegelman, and W. Wahli. 1999. Chemical probes that differentially modulate peroxisome proliferator-activated receptor alpha and BLTR, nuclear and cell surface receptors for leukotriene B(4). *J. Biol. Chem.* 274:23341-23348.

Ding, X.Z., P. Iversen, M.W. Cluck, J.A. Knezetic, and T.E. Adrian. 1999. Lipoxygenase inhibitors abolish proliferation of human pancreatic cancer cells. *Biochem. Biophys. Res. Commun.* 261:218-223.

Dixon, D.A., C.D. Kaplan, T.M. McIntyre, G.A. Zimmerman, and S.M. Prescott. 2000. Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3'-untranslated region. *J. Biol. Chem.* 275:11750-11757.

Dixon, R.A., R.E. Jones, R.E. Diehl, C.D. Bennett, S. Kargman, and C.A. Rouzer. 1988. Cloning of the cDNA for human 5-lipoxygenase. *Proc. Natl. Acad. Sci. U S A*. 85:416-420.

Dixon, R.A.F., R.E. Diehl, E. Opas, E. Rands, P.J. Vickers, J.F. Evans, J.W. Gillard, and D.K. Miller. 1990. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature*. 343:282-284.

Doetzlhofer, A., H. Rotheneder, G. Lagger, M. Koranda, V. Kurtev, G. Brosch, E. Wintersberger, and C. Seiser. 1999. Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol. Cell. Biol.* 19:5504-5511.

Drazen, J.M., and E.S. Silverman. 1999. Genetic determinants of 5-lipoxygenase transcription. *Int. Arch. Allergy. Immunol.* 118:275-278.

Drazen, J.M., C.N. Yandava, L. Dube, N. Szczerback, R. Hippensteel, A. Pillari, E. Israel, N. Schork, E.S. Silverman, D.A. Katz, and J. Drajesk. 1999. Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nat. Genet.* 22:168-170.

Dwyer, J.H., H. Allayee, K.M. Dwyer, J. Fan, H. Wu, R. Mar, A.J. Lusis, and M. Mehrabian. 2004. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N. Engl. J. Med.* 350:29-37.

Eilbracht, J., and M.S. Schmidt-Zachmann. 2001. Identification of a sequence element directing a protein to nuclear speckles. *Proc. Natl. Acad. Sci. U S A*. 98:3849-3854.

Engel, M.E., M.A. McDonnell, B.K. Law, and H.L. Moses. 1999. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J. Biol. Chem.* 274:37413-37420.

Feng, X.H., X. Lin, and R. Derynck. 2000. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. *Embo J.* 19:5178-5193.

Flamand, N., S. Boudreault, S. Picard, M. Austin, M.E. Surette, H. Plante, E. Krump, M.J. Vallee, C. Gilbert, P. Naccache, M. Laviolette, and P. Borgeat. 2000. Adenosine, a potent natural suppressor of arachidonic acid release and leukotriene biosynthesis in human neutrophils. *Am. J. Respir. Crit. Care Med.* 161:S88-94.

Fong, Y.W., and Q. Zhou. 2001. Stimulatory effect of splicing factors on transcriptional elongation. *Nature*. 414:929-933.

Ford-Hutchinson, A.W. 1994. leukotriene C4 synthase and 5-lipoxygenase-activating protein. Regulators of the biosynthesis of sulfido-leukotrienes. *Annals of the New York Academy of Science*. 744:78-83.

Ford-Hutchinson, A.W., M. Gresser, and R.N. Young. 1994. 5-Lipoxygenase. *Annu. Rev. Biochem.* 63:383-417.

Freedman, L.P. 1999. Increasing the complexity of coactivation in nuclear receptor signaling. *Cell*. 97:5-8.

Fu, X.D. 1995. The superfamily of arginine/serine-rich splicing factors. *RNA*. 1:663-680.

Funk, C.D. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*. 294:1871-1875.

Funk, C.D.H., S.; Matsumoto, T.; Rådmark, O. and Samuelsson, B. 1989. Characterization of the human 5-lipoxygenase gene. *Proc. Natl. Acad. Sci. USA*. 86:2587-2591.

Gaschott, T., O. Werz, A. Steinmeyer, D. Steinhilber, and J. Stein. 2001. Butyrate-induced differentiation of Caco-2 cells is mediated by vitamin D receptor. *Biochem. Biophys. Res. Commun.* 288:690-696.

Ghosh, J.M., C. 1998. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl. Acad. Sci. USA*. 95.

Golas, M.M., B. Sander, C.L. Will, R. Luhrmann, and H. Stark. 2003. Molecular architecture of the multiprotein splicing factor SF3b. *Science*. 300:980-984.

González, E.A., S. Disthabanchong, R. Kowalewski, and K.J. Martin. 2002. Mechanisms of the regulation of EGF receptor gene expression by calcitriol and parathyroid hormone in UMR 106-01 cells. *Kidney Int.* 61:1627-1634.

Graveley, B.R. 2000. Sorting out the complexity of SR protein functions. *RNA*. 6:1197-1211.

Gupta, S., M. Srivastava, N. Ahmad, K. Sakamoto, D.G. Bostwick, and H. Mukhtar. 2001. Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. *Cancer*. 91:737-743.

Habenicht, A.J., M. Goerig, D.E. Rothe, E. Specht, R. Ziegler, J.A. Glomset, and T. Graf. 1989. Early reversible induction of leukotriene synthesis in chicken myelomonocytic cells transformed by a temperature-sensitive mutant of avian leukemia virus E26. *Proc. Natl. Acad. Sci. U S A*. 86:921-924.

Haeggström, J., Kull F, Rudberg PC, Tholander F, Thunnissen MM. 2002. Leukotriene A4 hydrolase. *Prostaglandins Other Lipid Mediat. 2002*. 68-69:495-510.

Hamberg, M.a.S., B. 1974. Prostaglandine endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. USA*. 71:3400-3404.

Hammarberg, T., S. Kuprin, O. Rådmark, and A. Holmgren. 2001. EPR investigation of the active site of recombinant human 5- lipoxygenase: inhibition by selenide. *Biochemistry*. 40:6371-6378.

Hammarberg, T., P. Provost, B. Persson, and O. Rådmark. 2000. The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *J. Biol. Chem.* 275:38787-38793.

Hammarberg, T., and O. Rådmark. 1999. 5-lipoxygenase binds calcium. *Biochemistry*. 38:4441-4447.

Hammarberg, T., Y.Y. Zhang, B. Lind, O. Rådmark, and B. Samuelsson. 1995. Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase. *Eur. J. Biochem.* 230:401-407.

Härle, D., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1998. Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. *Eur. J. Biochem.* 254:275-281.

Härle, D., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1999. Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Adv. Exp. Med. Biol.* 469:105-111.

Hatzelmann, A., and V. Ullrich. 1987. Regulation of 5-lipoxygenase activity by the glutathione status in human polymorphonuclear leukocytes. *Eur. J. Biochem.* 169:175-184.

Healy, A., Peters-Golden M, Yao JP, Brock TG. 1999. Identification of a bipartite nuclear localization sequence necessary for nuclear import of 5-lipoxygenase. *J. Biol. Chem.* 274:29812-29818.

Helgadottir, A., A. Manolescu, G. Thorleifsson, S. Gretarsdottir, H. Jonsdottir, U. Thorsteinsdottir, N.J. Samani, G. Gudmundsson, S.F. Grant, G. Thorgeirsson, S. Sveinbjornsdottir, E.M. Valdimarsson, S.E. Matthiasson, H. Johannsson, O. Gudmundsdottir, M.E. Gurney, J. Sainz, M. Thorhallsdottir, M. Andresdottir, M.L. Frigge, E.J. Topol, A. Kong, V. Gudnason, H. Hakonarson, J.R. Gulcher, and K. Stefansson. 2004. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat. Genet.* 36:233-239.

Hemak, J., Gale, D., Brock,T.,G. 2002. Stuctural characterization of the catalytic domain of the human 5-lipoxygenase enzyme. *Journal of molecular modeling*. 8:102-112.

Hirose, Y., and J.L. Manley. 2000. RNA polymerase II and the integration of nuclear events. *Genes Dev.* 14:1415-1429.

Hodny, Z., R. Li, P. Barath, and B.D. Nelson. 2000. Sp1 and chromatin environment are important contributors to the formation of repressive chromatin structures on the transfected human adenine nucleotide translocase-2 promoter. *Biochem. J.* 346 Pt 1:93-97.

Hoshiko, S.R., O.; Samuelsson, B. 1990. Characterization of the human 5-lipoxygenase gene promoter. *Proceedings of the National Academy of Science*. 87:9073-9077.

In, K.H., K. Asano, D. Beier, J. Grobholz, P.W. Finn, E.K. Silverman, E.S. Silverman, T. Collins, A.R. Fischer, T.P. Keith, K. Serino, S.W. Kim, G.T. De Sanctis, C. Yandava, A. Pillari, P. Rubin, J. Kemp, E. Israel, W. Busse, D. Ledford, J.J. Murray, A. Segal, D. Tinkleman, and J.M. Drazen. 1997. Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription. *J. Clin. Invest.* 99:1130-1137.

Inoue, K., M. Ohno, and Y. Shimura. 1995. Aspects of splice site selection in constitutive and alternative pre-mRNA splicing. *Gene Expr.* 4:177-182.

Issa, L.L., G.M. Leong, and J.A. Eisman. 1998. Molecular mechanism of vitamin D receptor action. *Inflamm. Res.* 47:451-475.

Itoh, S., J. Ericsson, J. Nishikawa, C.H. Heldin, and P. ten Dijke. 2000. The transcriptional co-activator P/CAF potentiates TGF-beta/Smad signaling. *Nucleic Acids Res.* 28:4291-4298.

Iwasaki, T., W.W. Chin, and L. Ko. 2001. Identification and characterization of RRM-containing coactivator activator (CoAA) as TRBP-interacting protein, and its splice variant as a coactivator modulator (CoAM). *J. Biol. Chem.* 276:33375-33383.

Izumi, T., T. Yokomizo, H. Obinata, H. Ogasawara, and T. Shimizu. 2002. Leukotriene receptors: classification, gene expression, and signal transduction. *J. Biochem. (Tokyo)*. 132:1-6.

Jakobsson, P.J., R. Morgenstern, J. Mancini, A. Ford-Hutchinson, and B. Persson. 2000. Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). A widespread protein superfamily. *Am. J. Respir. Crit. Care Med.* 161:S20-24.

Janssen-Timmen, U., P.J. Vickers, U. Wittig, W.D. Lehmann, H.J. Stark, N.E. Fusenig, T. Rosenbach, O. Rådmark, B. Samuelsson, and A.J.R. Habenicht. 1995. Expression of 5-lipoxygenase in differentiating human skin keratinocytes. *Proc. Natl. Acad. Sci. USA*. 92:6966-6970.

Jones, G., S.A. Strugnell, and H.F. DeLuca. 1998. Current understanding of the molecular actions of vitamin D. *Physiol. Rev.* 78:1193-1231.

Jones, S., Luo M, Peters-Golden M, Brock TG. 2003. Identification of two novel nuclear import sequences on the 5-lipoxygenase protein. *J. Biol. Chem.* 278:10257-10263.

Jones, S.M., M. Luo, A.M. Healy, M. Peters-Golden, and T.G. Brock. 2002. Structural and functional criteria reveal a new nuclear import sequence on the 5-lipoxygenase protein. *J. Biol. Chem.* 277:38550-38556.

Jung, M. 2001. Inhibitors of histone deacetylase as new anticancer agents. *Curr. Med. Chem.* 8:1505-1511.

Kamitani, H., H. Kameda, U.P. Kelavkar, and T.E. Eling. 2000. A GATA binding site is involved in the regulation of 15-lipoxygenase-1 expression in human colorectal carcinoma cell line, caco-2. *FEBS Lett*. 467:341-347.

Kargman, S., P. Rousseau, G.K. Reid, C.A. Rouzer, J.A. Mancini, E. Rands, R.A. Dixon, R.E. Diehl, C. Leveille, D. Nathaniel, and et al. 1993. Leukotriene synthesis in U937 cells expressing recombinant 5-lipoxygenase. *J. Lipid. Mediat.* 7:31-45.

Khorasanizadeh, S. 2004. The nucleosome: from genomic organization to genomic regulation. *Cell*. 116:259-272.

Kosugi, H., M. Towatari, S. Hatano, K. Kitamura, H. Kiyoi, T. Kinoshita, M. Tanimoto, T. Murate, K. Kawashima, H. Saito, and T. Naoe. 1999. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. *Leukemia*. 13:1316-1324.

Kracklauer, M.P., C. Schmidt, and G.M. Sclabas. 2003. TGFbeta1 signaling via alphaVbeta6 integrin. *Mol. Cancer*. 2:28.

Krump, E., S. Picard, J. Mancini, and P. Borgeat. 1997. Suppression of leukotriene B4 biosynthesis by endogenous adenosine in ligand-activated human neutrophils. *J. Exp. Med.* 186:1401-1406.

Lai, C.F., X. Feng, R. Nishimura, S.L. Teitelbaum, L.V. Avioli, F.P. Ross, and S.L. Cheng. 2000. Transforming growth factor-beta up-regulates the beta 5 integrin subunit expression via Sp1 and Smad signaling. *J. Biol. Chem.* 275:36400-36406.

Lammers, C.H., P. Schweitzer, P. Facchinetti, J.M. Arrang, S.G. Madamba, G.R. Siggins, and D. Piomelli. 1996. Arachidonate 5-lipoxygenase and its activating protein: prominent hippocampal expression and role in somatostatin signaling. *J. Neurochem.* 66:147-152.

Lemm, I., and J. Ross. 2002. Regulation of c-myc mRNA decay by translational pausing in a coding region instability determinant. *Mol. Cell. Biol.* 22:3959-3969.

Leong, G.M., N. Subramaniam, J. Figueroa, J.L. Flanagan, M.J. Hayman, J.A. Eisman, and A.P. Kouzmenko. 2001. Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-beta-dependent transcription. *J. Biol. Chem.* 276:18243-18248.

Leonhardt, H., and M.C. Cardoso. 2000. DNA methylation, nuclear structure, gene expression and cancer. *J. Cell. Biochem. Suppl.* Suppl 35:78-83.

Lepley, R.A., and F.A. Fitzpatrick. 1994. 5-lipoxygenase contains a functional Src homology 3- binding motif that interacts with the Src homology 3 domain of Grb2 and cytoskeletal proteins. *J. Biol. Chem.* 269:24163-24168.

Lepley, R.A., and F.A. Fitzpatrick. 1996. Inhibition of mitogen-activated protein kinase kinase blocks activation and redistribution of 5-lipoxygenase in HL-60 cells. *Arch. Biochem. Biophys.* 331:141-144.

Li, B., C.C. Adams, and J.L. Workman. 1994. Nucleosome binding by the constitutive transcription factor Sp1. *J. Biol. Chem.* 269:7756-7763.

López-Rovira, T., E. Chalaux, J.L. Rosa, R. Bartrons, and F. Ventura. 2000. Interaction and functional cooperation of NF-kappa B with Smads. Transcriptional regulation of the junB promoter. *J. Biol. Chem.* 275:28937-28946.

Maccarrone, M., M.T. Corasaniti, P. Guerrieri, G. Nistico, and A.F. Agro. 1996. Nitric oxide-donor compounds inhibit lipoxygenase activity. *Biochem. Biophys. Res. Commun.* 219:128-133.

Magnuson, V.L., M. Young, D.G. Schattenberg, M.A. Mancini, D.L. Chen, B. Steffensen, and R.J. Klebe. 1991. The alternative splicing of fibronectin pre-mRNA is altered during aging and in response to growth factors. *J. Biol. Chem.* 266:14654-14662.

Makarov, E.M., O.V. Makarova, H. Urlaub, M. Gentzel, C.L. Will, M. Wilm, and R. Lührmann. 2002. Small nuclear ribonucleoprotein remodeling during catalytic activation of the spliceosome. *Science*. 298:2205-2208.

Manev, H. 2000. 5-Lipoxygenase gene polymorphism and onset of Alzheimer's disease. *Med. Hypotheses*. 54:75-76.

Manev, H., and T. Uz. 2002. DNA hypomethylating agents 5-aza-2'-deoxycytidine and valproate increase neuronal 5-lipoxygenase mRNA. *Eur. J. Pharmacol.* 445:149-150.

Manev, H., T. Uz, K. Sugaya, and T. Qu. 2000. Putative role of neuronal 5-lipoxygenase in an aging brain. *Faseb J.* 14:1464-1469.

Martinez, E., V.B. Palhan, A. Tjernberg, E.S. Lymar, A.M. Gamper, T.K. Kundu, B.T. Chait, and R.G. Roeder. 2001. Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors in vivo. *Mol. Cell. Biol.* 21:6782-6795.

Massagué, J. 1998. TGF-beta signal transduction. *Annu. Rev. Biochem.* 67:753-791.

Massagué, J. 2000. How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* 1:169-178.

Massagué, J., S.W. Blain, and R.S. Lo. 2000. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell*. 103:295-309.

Massagué, J., and Y.G. Chen. 2000. Controlling TGF-beta signaling. *Genes Dev.* 14:627-644.

Massagué, J., and D. Wotton. 2000. Transcriptional control by the TGF-beta/Smad signaling system. *Embo J.* 19:1745-1754.

Masuyama, H., S.C. Jefcoat, Jr., and P.N. MacDonald. 1997. The N-terminal domain of transcription factor IIB is required for direct interaction with the vitamin D receptor and participates in vitamin D-mediated transcription. *Mol. Endocrinol.* 11:218-228.

Matsumoto, T., C.D. Funk, O. Rådmark, J.O. Hoog, H. Jornvall, and B. Samuelsson. 1988. Molecular cloning and amino acid sequence of human 5-lipoxygenase [published erratum appears in Proc Natl Acad Sci U S A 1988 May;85(10):3406]. *Proc. Natl. Acad. Sci. U S A*. 85:26-30.

McColl, S.R., E. Krump, P.H. Naccache, P.E. Poubelle, P. Braquet, M. Braquet, and P. Borgeat. 1991. Granulocyte-macrophage colony-stimulating factor increases the synthesis of leukotriene B4 by human neutrophils in response to platelet-activating factor. Enhancement of both arachidonic acid availability and 5-lipoxygenase activation. *J. Immunol.* 146:1204-1211.

Mehrabian, M., and H. Allayee. 2003. 5-lipoxygenase and atherosclerosis. *Curr. Opin. Lipidol.* 14:447-457.

Mehrabian, M.A., H.; Wong, J.; Shih, W.; Wand, X.; Shaposhnik, Z.; Funk, C.D. and Jusis, A. 2002. Identification of 5-Lipoxygenase as a Major Gene Contributing to Atherosclerosis Susceptibility in Mice. *Circulation Research*. 91:120-126.

Miller, D.K., J.W. Gillard, P.J. Vickers, S. Sadowski, C. Léveillé, J.A. Mancini, P. Charleson, R.A.F. Dixon, A.W. Ford-Hutchinson, R. Fortin, J.Y. Gauthier, J. Rodkey, R. Rosen, C. Rouzer, I.S. Sigal, C.D. Strader, and J.F. Evans. 1990. Identification and isolation of a membrane protein necessary for leukotriene production. *Nature*. 343:278-281.

Monsalve, M., Z. Wu, G. Adelmant, P. Puigserver, M. Fan, and B.M. Spiegelman. 2000. Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol. Cell*. 6:307-316.

Mount, S.M. 2000. Genomic sequence, splicing, and gene annotation. *Am. J. Hum. Genet.* 67:788-792.

Moustakas, A., and D. Kardassis. 1998. Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. *Proc. Natl. Acad. Sci. U S A*. 95:6733-6738.

Murakami, M., K.F. Austen, C.O. Bingham, 3rd, D.S. Friend, J.F. Penrose, and J.P. Arm. 1995. Interleukin-3 regulates development of the 5-lipoxygenase/leukotriene C4 synthase pathway in mouse mast cells. *J. Biol. Chem.* 270:22653-22656.

Nakano, K., T. Mizuno, Y. Sowa, T. Orita, T. Yoshino, Y. Okuyama, T. Fujita, N. Ohtani-Fujita, Y. Matsukawa, T. Tokino, H. Yamagishi, T. Oka, H. Nomura, and T. Sakai. 1997. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J. Biol. Chem*. 272:22199-22206.

Newell-Price, J., A.J. Clark, and P. King. 2000. DNA Methylation and Silencing of Gene Expression. *Trends Endocrinol Metab*. 11:142-148.

Ng, H.H., Y. Zhang, B. Hendrich, C.A. Johnson, B.M. Turner, H. Erdjument-Bromage, P. Tempst, D. Reinberg, and A. Bird. 1999. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat. Genet.* 23:58-61.

Noguchi, M., M. Miyano, and T. Matsumoto. 1996. Physicochemical characterization of ATP binding to human 5-lipoxygenase. *Lipids*. 31:367-371.

Öhd, J.W., K.; Sjölander, A. 2000. Leukotrienes induce cell-survival signaling in intestinal epithelial cells. *Gastroenterology*. 119:1007-1018.

O'Prey, J., J. Chester, B.J. Thiele, S. Janetzki, S. Prehn, J. Fleming, and P.R. Harrison. 1989. The promoter structure and complete sequence of the gene encoding the rabbit erythroid cell-specific 15-lipoxygenase. *Gene*. 84:493-499.

Pardali, K., A. Kurisaki, A. Moren, P. ten Dijke, D. Kardassis, and A. Moustakas. 2000. Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J. Biol. Chem.* 275:29244-29256.

Piek, E., C.H. Heldin, and P. Ten Dijke. 1999. Specificity, diversity, and regulation in TGF-beta superfamily signaling. *Faseb J.* 13:2105-2124.

Polly, P., M. Herdick, U. Moehren, A. Baniahmad, T. Heinzel, and C. Carlberg. 2000. VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. *Faseb J.* 14:1455-1463.

Ponton, A., J.P. Thirion, and P. Sirois. 1996. Changes in chromatin conformation regulate the 5-lipoxygenase gene expression during differentiation of HL60 cells. *Prostaglandins Leukot. Essent. Fatty Acids*. 55:139-143.

Powell, W.S., S. Gravel, and F. Halwani. 1999. 5-oxo-6,8,11,14-eicosatetraenoic acid is a potent stimulator of L-selectin shedding, surface expression of CD11b, actin polymerization, and calcium mobilization in human eosinophils. *Am. J. Respir. Cell. Mol. Biol.* 20:163-170.

Provost, P., Dishart D, Doucet J, Frendewey D, Samuelsson B, Rådmark O. 2002. Ribonuclease activity and RNA binding of recombinant human Dicer. *EMBO J.* 21:5864-5874.

Provost, P., J. Doucet, T. Hammarberg, G. Gerisch, B. Samuelsson, and O. Rådmark. 2001. 5-Lipoxygenase interacts with Coactosin-Like Protein. *J. Biol. Chem.* 276:16520-16527.

Provost, P., B. Samuelsson, and O. Rådmark. 1999. Interaction of 5-lipoxygenase with cellular proteins. *Proc. Natl. Acad. Sci. U S A*. 96:1881-1885.

Qiu, Z.H., M.A. Gijon, M.S. deCarvalho, D.M. Spencer, and C.C. Leslie. 1998. The role of calcium and phosphorylation of cytosolic phospholipase A(2) in regulating arachidonic acid release in macrophages. *J. Biol. Chem.* 273:8203-8211.

Quack, M., and C. Carlberg. 2000. The impact of functional vitamin D(3) receptor conformations on DNA-dependent vitamin D(3) signaling. *Mol. Pharmacol.* 57:375-384.

Rachez, C., and L.P. Freedman. 2000. Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. *Gene*. 246:9-21.

Rådmark, O. 2002. Arachidonate 5-lipoxygenase. *Prostaglandins Other Lipid Mediat.* 68-69:211-234.

Reddy, K.V., T. Hammarberg, and O. Rådmark. 2000. Mg2+ activates 5-lipoxygenase in vitro: dependency on concentrations of phosphatidylcholine and arachidonic acid. *Biochemistry*. 39:1840-1848.

Rice, J.C., and C.D. Allis. 2001. Histone methylation versus histone acetylation: new insights into epigenetic regulation. *Curr. Opin. Cell Biol.* 13:263-273.

Richards, E.J., and S.C. Elgin. 2002. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell*. 108:489-500.

Riendeau, D., D. Denis, L.Y. Choo, and D.J. Nathaniel. 1989. Stimulation of 5-lipoxygenase activity under conditions which promote lipid peroxidation. *Biochem. J.* 263:565-572.

Robertson, K.D. 2002. DNA methylation and chromatin - unraveling the tangled web. *Oncogene*. 21:5361-5379.

Rochel, N., J.M. Wurtz, A. Mitschler, B. Klaholz, and D. Moras. 2000. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol. Cell*. 5:173-179.

Romano, M., A. Catalano, M. Nutini, E. D'Urbano, C. Crescenzi, J. Claria, R. Libner, G. Davi, and A. Procopio. 2001. 5-lipoxygenase regulates malignant mesothelial cell survival: involvement of vascular endothelial growth factor. *Faseb J.* 15:2326-2336.

Rouzer, C.A., and S. Kargman. 1988. Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* 263:10980-10988.

Rouzer, C.A., and B. Samuelsson. 1985. On the nature of the 5-lipoxygenase reaction in human leukocytes: Enzyme purification and requirement for multiple stimulatory factors. *Proc. Natl. Acad. Sci. USA*. 82:6040-6044.

Rouzer, C.A., and B. Samuelsson. 1986. The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. *FEBS Lett.* 204:293-296.

Ryu, H., J. Lee, B.A. Olofsson, A. Mwidau, A. Deodeoglu, M. Escudero, E. Flemington, J. Azizkhan-Clifford, R.J. Ferrante, and R.R. Ratan. 2003. Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1-dependent pathway. *Proc. Natl. Acad. Sci. U S A*. 100:4281-4286.

Sailer, E.-R., S. Schweizer, S.E. Boden, H.P.T. Ammon, and H. Safayhi. 1998. Characterization of an acetyl-11-keto-ß-boswellic acid and arachidonate-binding regulatory site of 5-lipoxygenase using photoaffinity labeling. *Eur. J. Biochem.* 256:364-368.

Samuelsson, B. 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science.* 220:568-575.

Samuelsson, B., S.-E. Dahlén, J.-Å. Lindgren, C.A. Rouzer, and C.N. Serhan. 1987. Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science*. 237:1171-1176.

Sayers, I., S. Barton, S. Rorke, J. Sawyer, Q. Peng, B. Beghe, S. Ye, T. Keith, J.B. Clough, J.W. Holloway, A.P. Sampson, and S.T. Holgate. 2003. Promoter polymorphism in the 5-lipoxygenase (ALOX5) and 5-lipoxygenase-activating protein (ALOX5AP) genes and asthma susceptibility in a Caucasian population. *Clin. Exp. Allergy*. 33:1103-1110.

Schaal, T.D., and T. Maniatis. 1999. Multiple distinct splicing enhancers in the protein-coding sequences of a constitutively spliced pre-mRNA. *Mol. Cell. Biol.* 19:261-273.

Serhan, C.N., T. Takano, K. Gronert, N. Chiang, and C.B. Clish. 1999. Lipoxin and aspirin-triggered 15-epi-lipoxin cellular interactions anti-inflammatory lipid mediators. *Clin. Chem. Lab. Med.* 37:299-309.

Shen, J., M. Montecino, J.B. Lian, G.S. Stein, A.J. Van Wijnen, and J.L. Stein. 2002. Histone acetylation in vivo at the osteocalcin locus is functionally linked to vitamin D-dependent, bone tissue-specific transcription. *J. Biol. Chem.* 277:20284-20292.

Silverman, E.S., and J.M. Drazen. 1999. The biology of 5-lipoxygenase: function, structure, and regulatory mechanisms. *Proc. Assoc. Am. Physicians*. 111:525-536.

Silverman, E.S., and J.M. Drazen. 2000. Genetic variations in the 5-lipoxygenase core promoter. Description and functional implications. *Am. J. Respir. Crit. Care Med.* 161:S77-80.

Silverman, E.S., J. Du, G.T. De Sanctis, O. Rådmark, B. Samuelsson, J.M. Drazen, and T. Collins. 1998. Egr-1 and Sp1 interact functionally with the 5-lipoxygenase promoter and its naturally occurring mutants. *Am. J. Respir. Cell. Mol. Biol.* 19:316-323.

Silverman, E.S., L. Le, R.M. Baron, A. Hallock, J. Hjoberg, T. Shikanai, K. Storm van's Gravesande, P.E. Auron, and W. Lu. 2002. Cloning and functional analysis of the mouse 5-lipoxygenase promoter. *Am. J. Respir. Cell. Mol. Biol.* 26:475-483.

Singal, R., and G.D. Ginder. 1999. DNA methylation. *Blood*. 93:4059-70.

Song, C.Z., X. Tian, and T.D. Gelehrter. 1999. Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. *Proc. Natl. Acad. Sci. U S A*. 96:11776-11781.

Spanbroek, R., R. Grabner, K. Lotzer, M. Hildner, A. Urbach, K. Ruhling, M.P. Moos, B. Kaiser, T.U. Cohnert, T. Wahlers, A. Zieske, G. Plenz, H. Robenek, P. Salbach, H. Kuhn, O. Rådmark, B. Samuelsson, and A.J. Habenicht. 2003. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proc. Natl. Acad. Sci. U S A*. 100:1238-1243.

Spanbroek, R., H.J. Stark, U. Janssen-Timmen, S. Kraft, M. Hildner, T. Andl, F.X. Bosch, N.E. Fusenig, T. Bieber, O. Rådmark, B. Samuelsson, and A.J. Habenicht. 1998. 5-Lipoxygenase expression in Langerhans cells of normal human epidermis. *Proc. Natl. Acad. Sci. U S A*. 95:663-668.

Steinhilber, D. 1999. 5-Lipoxygenase: a target for antiinflammatory drugs revisited. *Curr. Med. Chem.* 6:71-85.

Steinhilber, D., M. Brungs, O. Werz, I. Wiesenberg, C. Danielsson, J.-P. Kahlen, S. Nayeri, M. Schräder, and C. Carlberg. 1995. The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. *J. Biol. Chem.* 270:7037-7040.

Steinhilber, D., O. Rådmark, and B. Samuelsson. 1993. Transforming growth factor beta upregulates 5-lipoxygenase activity during myeloid cell maturation. *Proc. Natl. Acad. Sci. USA*. 90:5984-5988.

Sterner, D.E., and S.L. Berger. 2000. Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* 64:435-459.

Straif, D., O. Werz, R. Kellner, U. Bahr, and D. Steinhilber. 2000. Glutathione peroxidase-1 but not 4 is involved in the regulation of cellular 5-lipoxygenase activity in monocytic cells. *Biochem. J.* 349:455-461.

Subramaniam, N., G.M. Leong, T.A. Cock, J.L. Flanagan, C. Fong, J.A. Eisman, and A.P. Kouzmenko. 2001. Cross-talk between 1,25-dihydroxyvitamin D3 and transforming growth factor-beta signaling requires binding of VDR and Smad3 proteins to their cognate DNA recognition elements. *J. Biol. Chem.* 276:15741-15746.

Sun, D.M., M.; Chen, X.S.; Lakkis, M.M.; Li, H.; Isaacs, S.N.; Elsea S.H.; Patel, P.I. and Funk, C.D. 1998. Human 12(R)-lipoxygenase and the mouse ortholog. Molecular cloning, expression and gene chromosomal assignment. *J. Biol. Chem.* 273:33540-33547.

Surette, M.E., E. Krump, S. Picard, and P. Borgeat. 1999. Activation of leukotriene synthesis in human neutrophils by exogenous arachidonic acid: inhibition by adenosine A(2a) receptor agonists and crucial role of autocrine activation by leukotriene B(4). *Mol. Pharmacol.* 56:1055-1062.

Suske, G. 1999. The Sp-family of transcription factors. *Gene*. 238:291-300.

Takeshita, A., K. Imai, S. Kato, S. Kitano, and S. Hanazawa. 1998. 1alpha,25-dehydroxyvitamin D3 synergism toward transforming growth factor-beta1-induced AP-1 transcriptional activity in mouse osteoblastic cells via its nuclear receptor. *J. Biol. Chem.* 273:14738-14744.

Tang, D.G.a.C., Y.Q. and Honn, K.V. 1999. 12(s)Hete in cancer metastasis. *Adv. in Exp. Med. & Biol.* 447:181-191.

Theorell, H., Holman, R.T. and Akeson, A. 1947. Crystalline Lipoxydase. *Acta Chemica Scandinavica*. 1:571-576.

Thompson, P.D., J.C. Hsieh, G.K. Whitfield, C.A. Haussler, P.W. Jurutka, M.A. Galligan, J.B. Tillman, S.R. Spindler, and M.R. Haussler. 1999. Vitamin D receptor displays DNA binding and transactivation as a heterodimer with the retinoid X receptor, but not with the thyroid hormone receptor. *J. Cell. Biochem.* 75:462-480.

Tierney, M.J., and R.L. Medcalf. 2001. Plasminogen activator inhibitor type 2 contains mRNA instability elements within exon 4 of the coding region. *J. Biol. Chem.* 276:13675-13684.

Turner, C.R., W.B. Smith, C.J. Andresen, J.F. Eggler, and J.W. Watson. 1996. The effect of 5-lipoxygenase inhibition on Ascaris antigen (Ag)-induced responses in atopic monkeys. *Inflamm. Res.* 45:42-49.

Turner, S.R., Tainer, J.A. and Lynn, W. S. 1975. Biogenesis of chemotactic molecules by rhe arachidonate lipoxygenase system of platelets. *Nature*. 257:680-681.

Tyler, J.K., and J.T. Kadonaga. 1999. The "dark side" of chromatin remodeling: repressive effects on transcription. *Cell*. 99:443-446.

Uhl, J., N. Klan, M. Rose, K.D. Entian, O. Werz, and D. Steinhilber. 2002. The 5-lipoxygenase promoter is regulated by DNA methylation. *J. Biol. Chem.* 277:4374-4379.

Umesono, K., K.K. Murakami, C.C. Thompson, and R.M. Evans. 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell*. 65:1255-1266.

Utley, R.T., J. Côté, T. Owen-Hughes, and J.L. Workman. 1997. SWI/SNF stimulates the formation of disparate activator-nucleosome complexes but is partially redundant with cooperative binding. *J. Biol. Chem.* 272:12642-12649.

Van Lint, C., S. Emiliani, and E. Verdin. 1996. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr.* 5:245-253.

Vanderhoek, J.Y.B., R.W.; Bailey, J.M. 1980. 15-hydroxy-5,8,11,13-eicosatetraenoic acid: A potent and selective inhibitor of platelet lipoxygenase. *J. Biol. Chem*. 255:5996-5998.

Wächtershäuser, A., D. Steinhilber, S.M. Loitsch, and J. Stein. 2000. Expression of 5-lipoxygenase by human colorectal carcinoma Caco-2 cells during butyrate-induced cell differentiation. *Biochem. Biophys. Res. Commun.* 268:778-783.

Warner, D.R., E.A. Roberts, R.M. Greene, and M.M. Pisano. 2003. Identification of novel Smad binding proteins. *Biochem. Biophys. Res. Commun.* 312:1185-1190.

Watanabe, M., and M. Whitman. 1999. The role of transcription factors involved in TGFbeta superfamily signaling during development. *Cell. Mol. Biol.* 45:537-543.

Weitzel, F., and A. Wendel. 1993. Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J. Biol. Chem.* 268:6288-6292.

Werz, O. 2002a. 5-Lipoxygenase: Cellular Biology and Molecular Pharmacology. *Current Drug Targets-Inflammation & Allergy*. 1:23-44.

Werz, O., E. Bürkert, L. Fischer, D. Szellas, D. Dishart, B. Samuelsson, O. Rådmark, and D. Steinhilber. 2002. Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *Faseb J.* 16:1441-1443.

Werz, O., Bürkert, E., Samuelsson, B., Rådmark, O., and Steinhilber, D. 2002b. Activation of 5-lipoxygenase by cell stress is calcium-independent in human polymorphonuclear leukocytes. *Blood*. 99:1044-1052.

Werz, O., D. Szellas, M. Henseler, and D. Steinhilber. 1998. Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. *Mol. Pharmacol.* 54:445-451.

Werz, O., H. Wiesinger, A. Steinmeyer, and D. Steinhilber. 2000. New vitamin D receptor agonists with decreased metabolic stability. *Biochem. Pharmacol.* 59:1597-1601.

Wolffe, A.P., and D. Guschin. 2000. Review: chromatin structural features and targets that regulate transcription. *J. Struct. Biol.* 129:102-122.

Wong, C., E.M. Rougier-Chapman, J.P. Frederick, M.B. Datto, N.T. Liberati, J.M. Li, and X.F. Wang. 1999. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. *Mol. Cell. Biol.* 19:1821-1830.

Wurthner, J.U., D.B. Frank, A. Felici, H.M. Green, Z. Cao, M.D. Schneider, J.G. McNally, R.J. Lechleider, and A.B. Roberts. 2001. Transforming growth factor-beta receptor-associated protein 1 is a Smad4 chaperone. *J. Biol. Chem.* 276:19495-19502.

Yamamoto, S., H. Suzuki, and N. Ueda. 1997. Arachidonate 12-lipoxygenases. *Prog. Lipid. Res.* 36:23-41.

Yanagi, Y., M. Suzawa, M. Kawabata, K. Miyazono, J. Yanagisawa, and S. Kato. 1999. Positive and negative modulation of vitamin D receptor function by transforming growth factor-beta signaling through smad proteins. *J. Biol. Chem.* 274:12971-12974.

Yanagisawa, J., Y. Yanagi, Y. Masuhiro, M. Suzawa, M. Watanabe, K. Kashiwagi, T. Toriyabe, M. Kawabata, K. Miyazono, and S. Kato. 1999. Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science*. 283:1317-1321.

Yang, J., Y. Kawai, R.W. Hanson, and I.J. Arinze. 2001a. Sodium butyrate induces transcription from the G alpha(i2) gene promoter through multiple Sp1 sites in the promoter and by activating the MEK-ERK signal transduction pathway. *J. Biol. Chem.* 276:25742-25752.

Yang, L., J. Yang, S. Venkateswarlu, T. Ko, and M.G. Brattain. 2001b. Autocrine TGFbeta signaling mediates vitamin D3 analog-induced growth inhibition in breast cells. *J. Cell. Physiol.* 188:383-393.

Yang, W.M., Y.L. Yao, J.M. Sun, J.R. Davie, and E. Seto. 1997. Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. *J. Biol. Chem.* 272:28001-28007.

Yang, X., M.R. Bani, S.J. Lu, S. Rowan, Y. Ben-David, and B. Chabot. 1994. The A1 and A1B proteins of heterogeneous nuclear ribonucleoparticles modulate 5' splice site selection in vivo. *Proc. Natl. Acad. Sci. U S A*. 91:6924-6928.

Yang, Y.C., E. Piek, J. Zavadil, D. Liang, D. Xie, J. Heyer, P. Pavlidis, R. Kucherlapati, A.B. Roberts, and E.P. Böttinger. 2003. Hierarchical model of gene regulation by transforming growth factor beta. *Proc. Natl. Acad. Sci. U S A*. 100:10269-10274.

Yeakley, J.M., J.P. Morfin, M.G. Rosenfeld, and X.D. Fu. 1996. A complex of nuclear proteins mediates SR protein binding to a purine-rich splicing enhancer. *Proc. Natl. Acad. Sci. U S A*. 93:7582-7587.

Yildirim, E., Z. Zhang, T. Uz, C.Q. Chen, R. Manev, and H. Manev. 2003. Valproate administration to mice increases histone acetylation and 5-lipoxygenase content in the hippocampus. *Neurosci. Lett.* 345:141-143.

Yoshimura, R., M. Matsuyama, M. Mitsuhashi, Y. Takemoto, K. Tsuchida, Y. Kawahito, H. Sano, and T. Nakatani. 2004. Relationship between lipoxygenase and human testicular cancer. *Int. J. Mol. Med.* 13:389-393.

Yoshimura, R., M. Matsuyama, K. Tsuchida, Y. Kawahito, H. Sano, and T. Nakatani. 2003. Expression of lipoxygenase in human bladder carcinoma and growth inhibition by its inhibitors. *J. Urol.* 170:1994-1999.

Yu, F., J. Thiesen, and W.H. Strätling. 2000. Histone deacetylase-independent transcriptional repression by methyl-CpG-binding protein 2. *Nucleic Acids Res.* 28:2201-2206.

Zhang, C., T.A. Baudino, D.R. Dowd, H. Tokumaru, W. Wang, and P.N. MacDonald. 2001. Ternary complexes and cooperative interplay between NCoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription. *J. Biol. Chem.* 276:40614-40620.

Zhang, C., D.R. Dowd, A. Staal, C. Gu, J.B. Lian, A.J. van Wijnen, G.S. Stein, and P.N. MacDonald. 2003. Nuclear coactivator-62 kDa/Ski-interacting protein is a nuclear matrix-associated coactivator that may couple vitamin D receptor-mediated transcription and RNA splicing. *J. Biol. Chem.* 278:35325-35336.

Zhang, W., Z.M. Lei, and C.V. Rao. 1999. Immortalized hippocampal cells contain functional luteinizing hormone/human chorionic gonadotropin receptors. *Life Sci.* 65:2083-2098.

Zhang, Y.Y., T. Hammarberg, O. Rådmark, B. Samuelsson, C.F. Ng, C.D. Funk, and J. Loscalzo. 2000. Analysis of a nucleotide-binding site of 5-lipoxygenase by affinity labelling: binding characteristics and amino acid sequences. *Biochem. J.* 351 Pt 3:697-707.

Zhang, Z., C.Q. Chen, and H. Manev. 2004. DNA methylation as an epigenetic regulator of neural 5-lipoxygenase expression: evidence in human NT2 and NT2-N cells. *J. Neurochem.* 88:1424-1430.

9 Appendix (papers I-IV)

Paper I

Vitamin D-responsive elements within the human 5-lipoxygenase gene promoter bind vitamin D receptor, but do not confer vitamin D-dependent transcriptional activation.

Paper II

The coding sequence mediates induction of 5-lipoxygenase expression by $1,25(OH)_2D_3$ and TGFβ.

Paper III

Significance of the TGFβ/Smad signalling system in coding sequence-dependent 5-lipoxygenase gene regulation.

Paper IV

Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity.

Vitamin D-responsive elements within the human 5-lipoxygenase gene promoter bind vitamin D receptor, but do not confer vitamin D-dependent transcriptional activation

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ABSTRACT: The role of the vitamin D receptor (VDR) in the regulation of the 5-lipoxygenase (5-LO) expression during cell differentiation by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and transforming growth factor beta (TGFβ) was investigated. Several putative VD-responsive elements (VDREs) were identified in the human 5-LO gene promoter sequence (-313 to -258 bp). DNase I footprinting assays and electrophoretic mobility shift assays revealed that the purified VDR binds to putative VDREs within the 5-LO promoter as a heterodimer together with retinoic X receptor (RXR). Nuclear proteins extracted from TGFβ/$1,25(OH)_2D_3$-differentiated Mono Mac 6 cells caused similar band shifts, and consistent with the VDR expression levels, protein binding from extracts of undifferentiated cells (no TGFβ/$1,25(OH)_2D_3$) was less pronounced.

In cells transiently (Mono Mac 6, HeLa, RBL-1, HL-60 and U937) or stably (HL-60) transfected with reporter gene plasmids containing various 5-LO promoter regions, stimulation with TGFβ/$1,25(OH)_2D_3$ caused no significant change in reporter gene expression, although the transcriptional activity of a reporter gene containing a 4-times concatemerized rat atrial natriuretic factor vitamin D response element was strongly upregulated by TGFβ/$1,25(OH)_2D_3$. Detailed analysis of the 5-LO promoter in Mono Mac 6 cells revealed a positive regulatory region (-778 to -229 bp). Deletion of this region led to a reduction of 5-LO promoter activity when the VDR and the RXR were coexpressed. In summary, the VDR/RXR complex binds to putative VDREs in the 5-LO promoter, but the regulatory sequences involved in the prominent induction of 5-LO mRNA expression by TGFβ and $1,25(OH)_2D_3$ seem to be located in other parts of the 5-LO gene.

Arachidonate 5-lipoxygenase (5-LO) catalyzes the first two steps in the conversion of arachidonic acid to leukotriene A_4 (LTA_4) and plays a critical role in the control of cellular leukotriene production (1,2). 5-LO expression is believed to be upregulated during normal myeloid cell differentiation and in the myeloid cell lines HL-60 and Mono Mac 6, TGFβ and $1,25(OH)_2D_3$ were identified as potent inducers of 5-LO gene expression (3-5). In Mono Mac 6 cells, treatment with TGFβ and $1,25(OH)_2D_3$ led to an up to 5-fold increase in primary transcripts, a 64-fold increase in 5-LO mRNA, a 128-fold increase in protein amount and a more than 500-fold increase in 5-LO activity compared to the untreated controls (6).

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Abbreviations: (5-HPETE), 5-hydroperoxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; LTA_4, leukotriene A_4; TGFβ, transforming growth factor β; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; DRIP, VDR interacting protein; rANF, rat atrial natriuretic factor; RXR, retinoid X receptor; RZR, retinoid Z receptor; VDRE, vitamin D responsive element.

Interestingly, addition of cycloheximide inhibited the TGFβ but not the calcitriol effects, suggesting that there is a direct stimulation of 5-LO mRNA expression by $1,25(OH)_2D_3$ in presence of TGFβ-induced proteins (7). Also, $1,25(OH)_2D_3$ upregulated 5-LO metabolism in blood monocytes differentiating to mature monocytes, and ionophore induced formation of LTB_4 and 5-HETE was reduced in alveolar macrophages from $1,25(OH)_2D_3$-deficient rats (8,9).

It has been shown that the transcription factors Egr-1 and/or Sp1 can stimulate transcription of 5-LO reporter gene constructs in transfected cells (10). Naturally occurring mutations were found in the 5-LO promoter consisting of the deletion of one or two, or the addition of one Sp1-binding site (11). These mutations only slightly alter 5-LO promoter activity in reporter gene assays but have a significant impact on the response of asthma patients to 5-LO inhibitors (12). Recently, the core part of the mouse 5-LO gene promoter, which contained only one Sp1/Sp3 binding site and displays only weak homology to the human promoter, mediated high basal transcription of reporter genes in the RAW 264.7 monocyte-macrophage cell line, but not in 3T3 cells (13).

Further analysis of the 5-LO promoter indicated the presence of putative response elements for TGFβ, the retinoid Z receptor (RZR) (14), and the vitamin D receptor (VDR) (15), which is the mediator of the

genomic actions of $1,25(OH)_2D_3$. Among the putative VDREs within the 5-LO promoter, the sequence located at positions -290 bp to -275 bp (AATTCA GGAG AGAACG) shows some homology to the DR4-type mouse phosphate intestinal transporter VDRE (AGTTCA TGAG AGTTCA) and the sequence –309 bp to –294 bp (AGGGCA AAG GGTGGA) was previously proposed for possible VDR binding (15).

In this study we addressed whether the VDR physically interacts with the putative VDREs within the 5-LO promoter and we determined the effects of TGFβ/$1,25(OH)_2D_3$ on receptor binding and VDR expression. To define the mechanisms involved in the regulation of 5-LO expression by TGFβ and $1,25(OH)_2D_3$ and to examine putative DNA responsive elements controlling expression of 5-LO, the 5-LO promoter was analyzed in 5-LO positive as well as in 5-LO negative cell lines.

MATERIALS AND METHODS

Reagents. Molecular biology reagents were from MBI Fermentas, Sigma, GIBCO, Promega or other sources as indicated in the text. Insulin was a gift from Aventis. Human TGFβ1 was purified from outdated platelets according to (16). Nucleospin Extract columns for direct purification of pDNA were from Macherey-Nagel (Düren, Germany).

Poly(dIdC)•poly(dIdC), poly(dAdT)•poly(dAdT) and [γ-^{32}P]dATP (3000 Ci/mmol) were from Amersham Pharmacia. Oligonucleotides were synthesized at Scandinavian Gene Synthesis AB (Köping, Sweden). Human recombinant VDR and RXRβ protein were purchased from Biomol (Plymouth Meeting, PA). The plasmid p(DR3)4tkluc containing the four times concatemerized rat atrial natriuretic factor (rANF) VDRE (AT GGGTCA TAT GGTTCA) in front of a thymidine kinase promoter driven luciferase reporter gene, the pSG5VDR and pSG5RXR expression plasmids for the human vitamin D receptor (VDR) and retinoid X receptor alpha (RXRα) have been described previously.

Cells and cell culture. HL-60 cells, U937 and RBL-1 cells were grown at 37°C in a humified atmosphere with 6 % CO_2 in RPMI 1640 medium supplemented with 10 % (vol/vol) fetal calf serum (FCS), streptomycin (100 µg/ml) and penicillin (100 U/ml). For cell culture of Mono Mac 6 cells, which were kindly provided by Dr. H.W.L. Ziegler-Heitbrock (Munich), the culture medium was supplemented with 1×nonessential amino acids, sodium pyruvate (1 mM), oxalacetate (1 mM) and insulin (10 µg/ml) (17). HeLa cells were obtained from Dr. W.E. Müller (Pharmacological Institute, Biocenter, Frankfurt) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (vol/vol) FCS, 100 µg/ml streptomycin and 100 units/ml penicillin.

RT-PCR analysis. HL-60 cells were seeded at 4×10^5 cells/ml and incubated for 24h with or without $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml). Cells were harvested by centrifugation at 1200×g for 10 min at room temperature (RT), total RNA was isolated from cells by the guanidinium thiocyanate method (18) and RT-PCR analysis was performed as decribed previously (6). The following PCR-primers were used at a concentration of 5 ng/µl:

β-actin (24 cycles) 5'GAGGAGCACCCCGTGCTGCTGA3' and
5'CTAGAAGCATTTGCTGTGGACGATGGAGGGGCC3';
5-LO (30 cycles)
5'ACCATTGAGCAGATCGTGGACACGC3' and
5'GCAGTCCTGCTCTGTGTAGAATGGG3', CAT (35 cycles) 5'CCTATAACCAGACCGTTCAGC3' and 5'CGCCAGCGGCATCAGCACC3'.

Signal intensities of ethidium bromide stained DNA bands were quantitated by densitometry (BioRad Gel Doc 1000 system) and analyzed with the Molecular Analyst program (BioRad). Results are expressed as relative changes in RNA amounts normalized with β-actin as internal standard.

Plasmid construction. The 5-LO promoter reporter gene deletion constructs were prepared using restriction enzymes and PCR methods. Starting with the plasmids K1 (provided by Dr Shigeru Hoshiko) and pGL3Basic (Promega), the 5-LO-promoter containing plasmid K1 was digested first by *BstE* II, the restriction site was blunted with T4 DNA polymerase, then digested with *Kpn* I and ligated into the promoterless luciferase reporter vector pGL3Basic, which was opened with *Kpn* I and *Sma* I. The intermediate construct obtained was then partially digested with *Cfr42* I, digested with *Xho* I and blunted with T4 DNA polymerase before final religation (pN0). The plasmids pN1-pN11 were constructed by digestion of pN0, which contains the promoter region from –6079 bp to +53 bp in relation to the transcription start (-6144 bp from the 5-LO ATG) using the restriction enzyme *Kpn* I and then *Afl* II (pN1), *Van*91I (pN2), *Nde* I (pN3), *Pvu* II (pN5), *Eco*147I (pN6), *Eco*R I (pN7), *Eco*RV (pN8), *Pme* I (pN9), *Pau* I (pN10) and *BstX* I (pN11). Overhangs were blunted by T4 DNA polymerase treatment and religated with T4 DNA ligase. Plasmids pN12, pN13 and pN14 were obtained by PCR deletion, using pN10 as the template, the reverse primer TATCGATAGAGAAATGTTCTGGCA, the forward primers CGCGTGAAGAGTGGGAGAGAAGTACTGCGG (pN12), CAGCCGGGAGCCTGGAGCCAGACC (pN13) or AGGGACCAGTGGTGGGAGGAGGCT (pN14), and *Pfu* I DNA polymerase. Following temperature cycling, the product was treated with *Dpn* I, plasmid ends were phosphorylated by T4 polynucleotide kinase and ligated by T4 DNA ligase. Plasmid sequences were confirmed by DNA sequencing.

Transfection of myeloid cell lines by electroporation. Cells (Mono Mac 6, HL-60, RBL-1 and U937) were seeded to 2×10^5 cells/ml and cultured at 37°C in a humified atmosphere with 6 % CO_2. After 48 hrs, cells were harvested by centrifugation (1200×g, 5 min, RT), washed twice with RPMI-1640, and resuspended at a density of 46×10^6cells/ml (Mono Mac 6 and U937), 24×10^6 cells/ml (RBL-1) and 20×10^6 cells/ml (HL-60), respectively in RPMI-1640 without glutamine. 300 µl (Mono Mac 6, U937) or 500 µl (HL-60, RBL-1) of the cell suspension were placed into a 0.4-cm electroporation cuvette. Forty micrograms of supercoiled plasmid DNA and 1 µg of internal standard (in 30 µl of water) were added to the cell suspension. For cotransfections, 5 µg of the expression vectors pSG5hVDR and pSG5hRXR were included. After 5 min incubation at RT (Mono Mac 6, RBL-1, HL-60) or 10 min on ice (U937), electroporation was performed using a Biorad Gene pulser II at 975 µF and 200 V (Mono Mac 6), 300 V (RBL-1) or 250 V (HL-60, U937). The cuvettes were immediately placed on ice for 20 min (Mono Mac 6, RBL-1, HL-60, U937) or for 5 min (U937). After another 5 min at RT (U937) or 20 min on ice the cells were transferred to 10 ml of cell culture medium.

Transfection of HeLa cells. Cells were plated into a 24-well tissue culture plate at a density of 6×10^4 cells per well, so that 60-80 % of the cells were confluent at the time of transfection (after about 24 hrs). Plasmid DNA (0.4 µg) and 0.01 µg pCMVSEAP as internal standard (diluted in 5 µl serum free DMEM) were precomplexed with 5 µl of PLUS reagent (Gibco) by incubation for 15 min at RT. Precomplexed

plasmid DNA was mixed with 25 µl of 1:50 diluted Lipofectin reagent and incubated for 30 min at RT. Then, the medium was replaced by 200 µl of fresh serum free DMEM and incubated 37 °C in 5 % CO_2 with the DNA-PLUS-Lipofectin reagent complexes. After 5 hrs, 1 ml of DMEM containing 15 % (vol/vol) FCS was added. 24 h after transfection, cells were washed once in phosphate-buffered saline pH 7.4 (PBS) and luciferase activity was determined as described below.

Stable transfections of HL-60 cells. HL-60 cells were stably transfected with the 5-LO promoter-CAT construct p5LO6079CAT containing the 5-LO promoter sequences -6079 bp to +81 bp (HL60-6079-5LO), the SV40-promoter containing plasmid pCATControl (HL60-SV40) and with the promoterless plasmid pCATBasic (HL60-Basic). Cells were cotransfected with pMC1NeopolyA+ and stably transfected cells were selected for neomycin resistance at 600 µg/ml.

Luciferase assays. 6 h (Mono Mac 6, U937, HL-60, RBL-1) and 24 h (HeLa) after transfection, cells were washed once in PBS containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and lysed in 100 µl lysis buffer (Luciferase Reporter Gene Assay constant light signal kit, Boehringer Mannheim, Germany). Luciferase activity was determined by monitoring light emission with a Microlumat Plus LB96V EG&G Berthold luminometer (19). The light emission signal was integrated for 5 seconds. Transfection efficiency was monitored and normalized by cotransfection with pCMVSEAP using the Phospha-Light™ kit (Tropix) to determine the secreted placental alkaline phosphatase (SEAP) activity (19-23).

Preparation of nuclear extracts. Mono Mac 6 cells were grown in absence or presence of TGFβ (2 ng/ml) plus $1,25(OH)_2D_3$ (50 nM) as described (5). After four days, cells ($0.5\text{-}1\times10^9$) were harvested and nuclear extracts were prepared according to the protocol of Shapiro et al. (24). Nuclear extracts (protein content: 2-8 µg/µl) were immediately frozen in aliquots and stored at –70°C for several months.

Electrophoretic mobility shift assays. The following oligonucleotides were used in the gel retardation assays: mouse osteopontin VDRE MO-23 mer: 5`-ACAAGGTTCACGAGGTTCACGTC-3´; 5LO-23 mer: 5´-AGGCAGGGCAAAGGGTGGAAGCA-3´ (-313 bp to -291 bp); 5LO-56 mer: 5´-AGGCAGGGCAAAGGGTGGAAGCAATTCAGGAGAG AACGAGTGAACGAATGGATGAG-3´ (-313 bp to -258 bp). To prepare double-stranded oligonucleotides, equal amounts of the complementary single-stranded DNA were combined in a solution containing 10 mM $MgCl_2$ and heated to 80°C for 5 min, and slowly cooled down to room temperature. The annealed oligonucleotides were endlabelled with [^{32}P]-γ-ATP using T4 DNA polynucleotide kinase (NEB, Beverly, MA). Radiolabelled double-stranded oligonucleotides were purified by Sephadex G-25 spin columns (Roche Molecular Biochemicals), and stored at -20°C.

The binding reaction was performed at RT for 25 min in binding buffer (Tris HCl 10 mM pH 7.5, glycerol 4 %, $MgCl_2$ 5 mM, EDTA 0.5 mM, DTT 0.5 mM, NaCl 50 mM, 0.05 mg/ml poly (dI-dC)•poly(dI-dC)), containing 50,000 cpm labeled probe and nuclear extracts (corresponding to 10 µg protein) or VDR and RXR (1 µg, each) in a total reaction volume of 12 µl. For competition studies, a 150-fold molar excess of unlabelled oligonucleotide was added to the reaction mixture prior to the addition of radiolabelled probe. EMSA reactions were resolved on 5 % or 10 % pre-run nondenaturing polyacrylamide TBE gels (BioRad, premade) which were electrophoresed at 100 V for 45-90 min. Gels were dried under vacuum and then exposed to Fuji Super RX film.

Western blot. Nuclear extracts (10 µg protein in 10 µl) were mixed with the same volume of 2 × SDS-b and boiled for 5 min at 95°C. Then, 4 µl of glycerol/0.1% bromphenolblue (1:1, vol/vol) were added and proteins were separated by SDS-PAGE using a Mini Protean system (Bio-Rad) on a 10 % gel. After electroblot to nitrocellulose membrane (Amersham Pharmacia), membranes were blocked with 5% non fat dry milk in 50 mM Tris/HCl, pH 7.4 and 100 mM NaCl (TBS) for 1 h at room temperature. Membranes were washed and then incubated with specific antibodies against the VDR (Santa Cruz Biotech, C-20, 1:1,000 dilution (Santa Cruz, CA)) for overnight at 4°C. The membranes were washed with TBS and incubated with 1:1,000 dilution of alkaline phosphatase-conjugated rabbit IgGs (Sigma) for 2 h at RT. After washing with TBS and TBS plus 0.1 % NP40, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in detection buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

DNase I footprinting. For DNase I footprinting, an oligonucleotide was obtained by restriction enzyme digestion of the plasmid 5LO931CAT (25). First, the plasmid was digested with Sac II. The smaller fragment was then purified by agarose gel electrophoresis and extracted with the QiaQuick gel extraction kit (Qiagen, Hilden, Germany). After digestion with Alu I, the DNA was treated with calf intestinal alkaline phoshatase (MBI Fermentas, Lithuania) and radiolabelled at the 5´ends using T4 polynucleotide kinase (NEB, Beverly, MA). The labelled DNA was digested with Sca I, and the subsequent ^{32}P-Alu I/Sca I fragment of the 5LO gene promoter was purified as after the first cleavage. 50,000 cpm (0.1-0.2 pmol) of ^{32}P-5´ labelled DNA (nucleotides -363 to -119) was incubated with various amounts (0.4-3 µg) of RXR and/or VDR (both from Biomol, Plymouth Meeting, PA) prior to digestion with DNase I. For DNase I footprinting reactions the SureTrack Footprinting Kit from Amersham Pharmacia was used, following the manufacturers intructions. The samples, resuspended in water/loading dye (4/6, v/v; loading dye is deionized formamide containing 10 mM EDTA, 0.3 % bromophenol blue and 0.3 % xylene cyanol), were heated at 85°C for 2 min and immediately loaded onto 8 % polyacrylamide sequencing gels. An A+G ladder covering the appropriate DNA sequence was prepared according to the sequencing method of Maxam & Gilbert and run alongside the samples.

RESULTS

VDR/RXR protect parts of the 5-LO promoter in the DNase I footprinting assay.

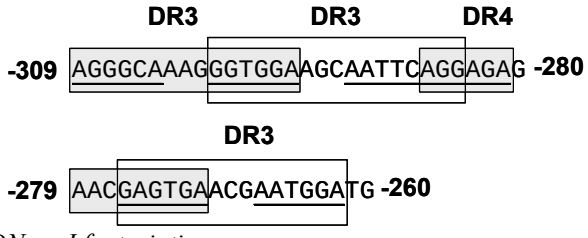

FIGURE 1. **Localization of putative VDREs within the human 5-LO promoter.**

Sequence analysis of the 5-LO promoter reveals the presence of several putative VDRE from –313 bp to -258 bp (fig. 1), numbered with the major trancription start site as zero. DNase I footprinting was performed

using a probe (245 bp long) containing the sequence between the *Alu* I site (-363 bp) and the Sca I site (-119 bp) of the 5-LO gene promoter. The fragment was labelled at the Alu I end. When RXR and VDR proteins were mixed and incubated with the probe, an apparent hypersensitive site appeared at –268 bp. The 96 bp of the probe upstream (5´) of this position was protected from DNase I, whereas the downstream part of the probe (149 bp) was accessible for DNase I.

One of the best protected parts of the sequence, giving a clear footprint, was the region between nucleotides -307 and -299 that matches the VDRE (-309 to –294 bp) proposed by Carlberg (15), (fig. 2). Protection against DNase I also comprised the DR4-type response element with homology to the mouse phosphate intestinal transporter VDRE and suddenly stopped at the hypersensitive site at –268 bp. The protection against DNase I was most prominent when RXR and VDR were combined. VDR alone gave slightly weaker footprints and RXR alone did not bind to the probe (data not shown). Purified SP1 protein (Promega, Madison, WI) alone gave no footprint in this part of the 5LO promoter, and in combination with VDR/RXR there was no additional protection against DNase I.

VDR and RXR bind to putative VDRE present in the 5-LO promoter; EMSA studies. In order to explore VDR/RXR binding to putative VDREs, EMSAs were performed using synthetic oligonucleotides corresponding to the sequence –313 to –291 bp (5LO-23) and –313 to –258 bp (5LO-56) of the 5-LO promoter, respectively. The well-documented VDRE from the mouse osteopontin promoter (MO-23) was used as a positive control. As depicted from fig. 3, VDR or RXR hardly bound to any of these oligonucleotides. However, the combination of both RXR and VDR caused retardation of 5LO-23 and 5LO-56 oligonucleotides, with comparable magnitudes to MO-23. Due to its larger size, the 5LO-56/VDR/RXR complex migrated slightly slower compared to the 23bp oligo/VDR/RXR complexes of 5LO-23 and MO-23. Purified SP1 protein alone caused no band shift and together with VDR/RXR there was no additional signal observed (not shown).

Next, we determined the ability of the oligonucleotides to bind endogenous proteins derived from nuclear extracts of Mono Mac 6 cells, cultured in presence or absence of 1 ng/ml TGFβ plus 50 nM $1,25(OH)_2D_3$. A considerable higher amount of oligonucleotides was retarded by nuclear proteins derived from differentiated (TGFβ plus $1,25(OH)_2D_3$) cells, as compared to untreated cells (fig. 4A). This correlated with the total amounts of VDR protein present in such extracts as determined by Western blot (fig. 4B). When EMSAs were performed using purified VDR and RXR proteins as well as nuclear extracts (obtained from TGFβ/$1,25(OH)_2D_3$-differentiated Mono Mac 6 cells) side by side, the migration pattern was apparently the same for 5LO-23 and MO-23 (fig. 3C). However, for 5LO-56 an additional more intense shift was observed after incubation with nuclear extracts from TGFβ/$1,25(OH)_2D_3$-differentiated cells (but not with extracts from untreated cells), implying the occurence of additional protein(s) induced by TGFβ/$1,25(OH)_2D_3$ that are capable to bind the VDR/RXR/5LO-56 complex.

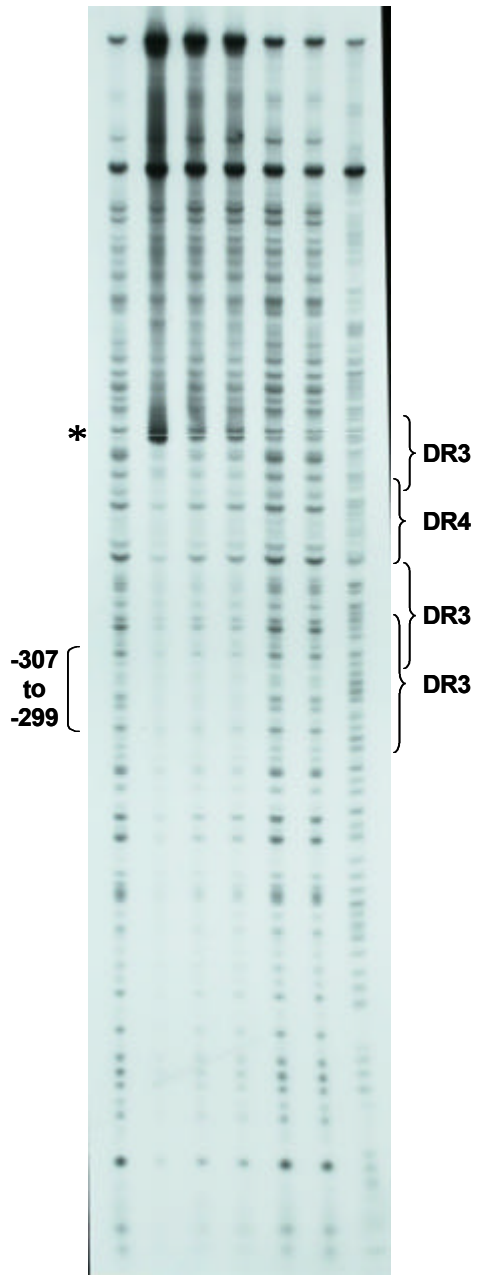

FIGURE 2. **Protection of regions in the 5-LO gene promoter by VDR and RXR**. DNase1 footprinting was performed with the *Alu* I (-363 bp) to *Sca* I (–119 bp) fragment of the promotor region of the 5-LO gene, radiolabelled at the *Alu* I end.

Lane 1: no addition (positive control). Lane 2: the probe was incubated with VDR protein (3 µg) and RXR protein (3 µg) prior to digestion with DNase I. Lane 3: VDR (1.5 µg) and RXR (1.5 µg). Lane 4: VDR (1.5 µg) and RXR (1.5 µg) and purified Sp1 (1 footprinting unit). Lane 5: only purified Sp1 protein (1 footprinting unit). Lane 6: no addition (positive control). Lane 7: Maxam-Gilbert A+G ladder. Electrophoresis was done with a 8 % polyacrylamide sequencing gel. Star indicates the hypersensitive site (-268 bp), and the heavily protected region -307 to –299 bp is indicated. Parentheses indicate parts of the sequence conforming to DR3 and DR4 motifs.

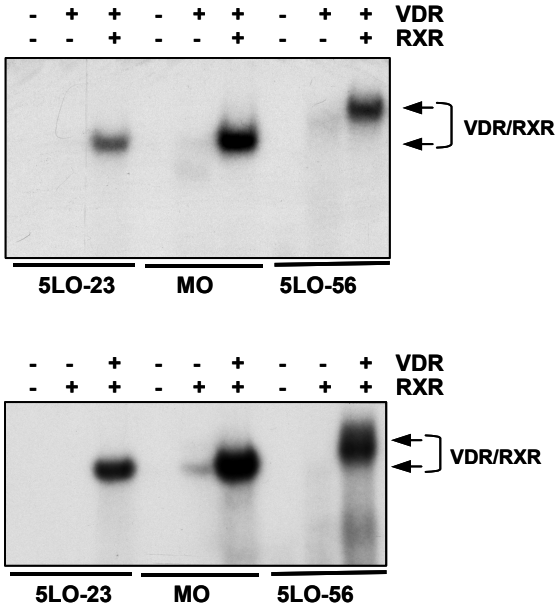

FIGURE 3. **The VDR and the RXR together bind to putative VDREs located within the 5-LO promoter**. Purified VDR and RXR (1 µg each) were incubated with ^{32}P-labelled (T4 DNA polynucleotide kinase) double-stranded oligonucleotides resembling the sequences of the 5-LO promoter -315 to -293 bp (5LO-23) and -315 to -259 bp (5LO-56), and the mouse osteopontin promoter (MO) as indicated. Protein-DNA complexes were resolved on 5 % pre-run nondenaturing polyacrylamide TBE gels and visualized by autoradiography. Arrows indicate the positions of the protein/DNA complexes. Similar results were obtained in at least three additional experiments.

Effects of 1,25(OH)$_2$D$_3$/TGFb on transcriptional activity of the 5-LO and the rANF promoter. In order to investigate effects of TGFβ/1,25(OH)$_2$D$_3$ on the 5-LO promoter activity, the myeloid cell lines RBL-1, Mono Mac 6, HL-60 and U937, as well as HeLa cells were transiently transfected with the plasmid pN10, composed of a 5-LO promoter sequence (-778 to +53 bp, containing at least four putative VDREs (see fig. 1)) in front of a luciferase gene (pGL3Basic). The luciferase reporter gene plasmid p(DR3)4tkluc, containing a 4-times concatemerized rANF vitamin D response element in front of the thymidine kinase promoter, was used as a positive control. To ensure the availability of sufficient amounts of nuclear receptor proteins, cells were cotransfected with expression vectors for VDR and RXR. After transfection, cells were incubated for 6 h (RBL-1, Mono Mac 6, HL-60 and U937) or 24 h (HeLa) in presence or absence of 50 nM 1,25(OH)$_2$D$_3$ and 1 ng/ml TGFβ respectively.
As shown in fig. 5A**,** the relative transcriptional activity of pN10 to pGL3Basic depends on the cell type. Compared to the promoterless vector pGL3Basic, only moderately higher (about 7- to 86-fold) 5-LO promoter activities were found in RBL-1, HeLa, HL-60 and U937 cells. However, in Mono Mac 6 cells, transcription from the 5-LO promoter was about 1200-fold higher than from pGL3Basic. Of particular interest, incubation of cells in presence of 1,25(OH)$_2$D$_3$ and TGFβ, that increased 5-LO mRNA expression in Mono Mac 6, HL-60 and U937 cells (not shown, compare (5,26)) and nuclear protein binding to 5LO-23 and 5-LO-56 (fig. 4), caused no enhancement of luciferase activity in either cell type (fig. 5A).

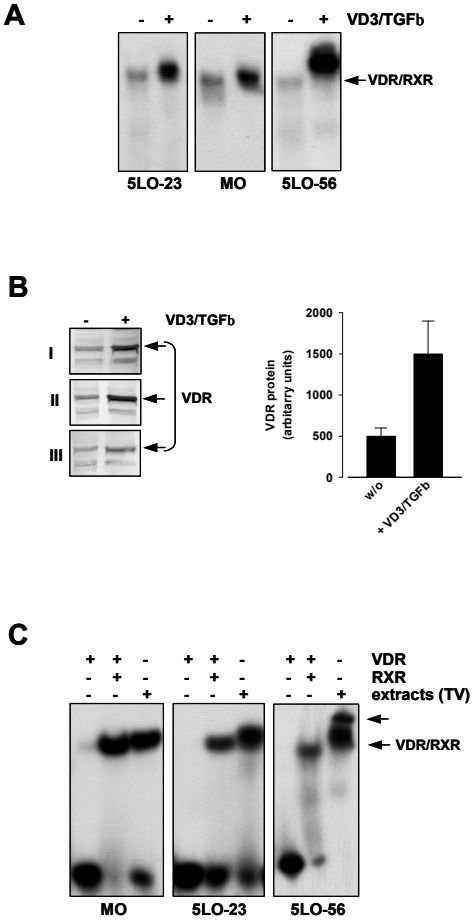

FIGURE 4. **Effects of TGFb and 1,25(OH)$_2$D$_3$ on the expression of VDR and nucleotide-binding capacity of the nuclear proteins from Mono Mac 6 cells.** Mono Mac 6 cells were grown with or without 1,25(OH)$_2$D$_3$ (50 nM) and TGFβ (1 ng/ml) for four days and nuclear extracts were prepared as described. (A) To determine effects of TGFβ and 1,25(OH)$_2$D$_3$ on nucleotide binding capacity, nuclear extracts (10 µg) from untreated or 1,25(OH)$_2$D$_3$/TGFβ-treated cells were incubated with ^{32}P-labelled 5LO-23, MO, and 5LO-56 as indicated. (B) To determine the expression of the VDR protein, nuclear extracts (10 µg) from untreated or 1,25(OH)$_2$D$_3$/TGFβ-treated cells were separated by SDS-PAGE and analyzed by Western blot. The results from three different experiments are shown (I, II, III; left panel), the corresponding relative intensities of blot bands (right panel) were determined by densitometry (arbitrary units) and are given as mean + S.E. (C) To compare the electrophoretic mobility of protein-DNA complexes, purified VDR and RXR proteins or nuclear proteins derived from Mono Mac 6 cells treated with TGFβ and 1,25(OH)$_2$D$_3$ were incubated with ^{32}P-labelled 5LO-23, MO, and 5LO-56 as indicated. Protein-DNA complexes were resolved on 5 % pre-run nondenaturing polyacrylamide TBE gels and visualized by autoradiography. Arrows indicate the positions of the protein/DNA complexes. Similar results were obtained in at least three additional experiments.

In analogy to pN10, also the basal expression of the plasmid p(DR3)4tkluc was cell type dependent in a similar fashion (fig. 5B). However, in sharp contrast to pN10, treatment of myeloid cells (but not HeLa cells) with $1,25(OH)_2D_3$ and TGFβ led to a considerable upregulation of the trancription of the p(DR3)4tkluc reporter gene construct (165-, 54-, 34- and 5.1-fold in HL-60, RBL-1, Mono Mac 6 and HeLa cells, respectively) which strongly depended on the coexpression of VDR/RXR. Without coexpression of VDR and RXR, TGFβ/$1,25(OH)_2D_3$ increased transcription from p(DR3)4tkluc by 9-, 2.3- and 1.6-fold in RBL-1, Mono Mac 6 and HeLa cells, respectively.

Taken together, unlike the VDREs in p(DR3)4tkluc, the VDREs of the 5-LO promoter appear to be unable to mediate an upregulation of transcriptional activity of pN10 in myeloid cells by $1,25(OH)_2D_3$ and TGFβ.

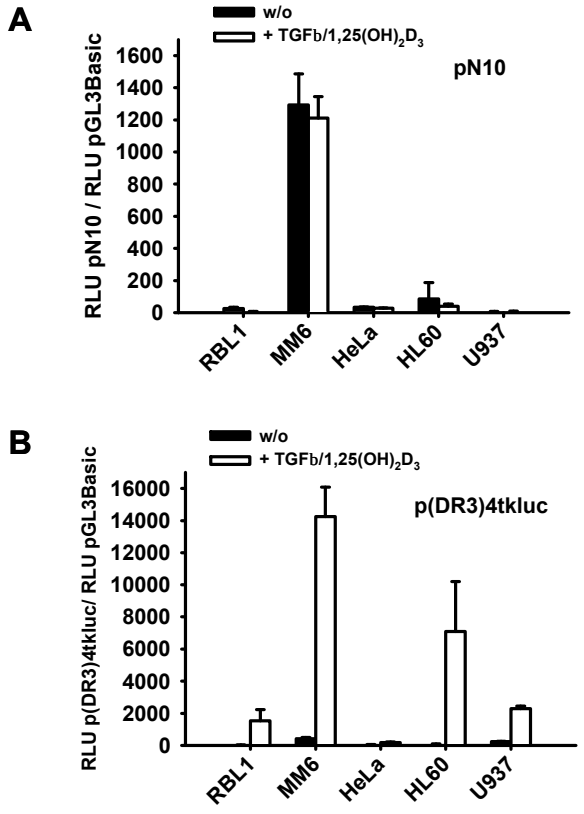

FIGURE 5. **Effects of TGFb and $1,25(OH)_2D_3$ on transcriptional activity of pN10 (A) and p(DR3)4tkluc (B) in various cell lines.** The constructs pN10 (A) and p(DR3)4tkluc (B) were transiently transfected into the indicated cell lines together with the expression vectors pSG5hRXR and pSG5hVDR. After transfection $1,25(OH)_2D_3$ and TGFβ (50 nM and 1 ng/ml, respectively) were added and reporter gene activity was determined after 6 h (RBL-1, HL-60, Mono Mac 6 and U937) or 24 h (HeLa). Results are presented as mean ± SE of triplicate transfections. Values were normalized for transfection efficiency by cotransfection of pCMVSEAP.

Identification of positive and negative regulatory regions of the 5-LO promoter. In order to identify positive and negative regulatory regions of the 5-LO promoter, Mono Mac 6 and HeLa cells were transiently transfected (6 h for Mono Mac 6, 24 h for HeLa) with plasmids encoding deletion constructs of the 5-LO promoter (-6079 to +53 bp) in front of the luciferase gene. To ensure the availability of sufficient amounts of nuclear receptor proteins, cells were cotransfected with expression vectors for VDR and RXR.

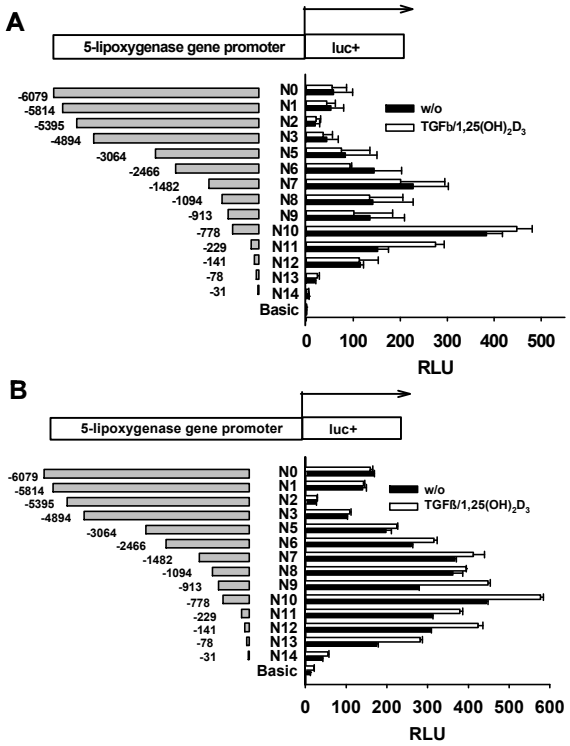

FIGURE 6. **5-LO promoter analysis in Mono Mac 6 (A) and HeLa cells (B).** The 5-LO promoter luciferase reporter gene construct pN0, processive deletion variants (pN1-pN14) or the promoterless vector pGL3Basic and the expression vectors pSG5hRXR and pSG5hVDR were transiently transfected into Mono Mac 6 and HeLa cells. After transfection, $1,25(OH)_2D_3$ and TGFβ (50 nM and 1 ng/ml, respectively) were added and reporter gene activity was determined after 6 h (Mono Mac 6) or 24 h (HeLa). Results are presented as mean ± SE of triplicate transfections, and were normalized for transfection efficiency by cotransfection of pCMVSEAP.

As shown in fig. 6A, the full length 5-LO promoter reporter gene construct (pN0, -6079 to +53 bp) significantly induced luciferase activity (by 57-fold) versus the promoterless vector in Mono Mac 6 cells. Systematic deletion of the 5-LO promoter indicates the presence of two negative (-5814 bp to –5395 bp and -913 bp to –778 bp) and two positive regulatory regions (–778 bp to –229 bp and –141 bp to –78 bp). The most prominent promoter activities were observed with the pN10 construct (-778 to +53 bp), which gave 1290-fold and 1210-fold higher activities compared to the promoterless vector. Again, except for the pN11 construct (-229 to +53 bp), there was no significant upregulation of the reporter gene activity in presence of TGFβ/$1,25(OH)_2D_3$ compared to untreated cells, indicating that TGFβ/$1,25(OH)_2D_3$ caused no transcriptional transactivation.

Also in HeLa cells, pN10 caused the most prominent upregulation of transcriptional activity among the reporter gene constructs (fig. 6B). In contrast to Mono Mac 6 cells, no positive regulatory region was found from –778 to –229 bp in HeLa cells. Similarly as found for Mono Mac 6 cells, the 5-LO promoter reporter gene constructs were not significantly responsive to TGFβ/1,25(OH)$_2$D$_3$ stimulation in HeLa cells.

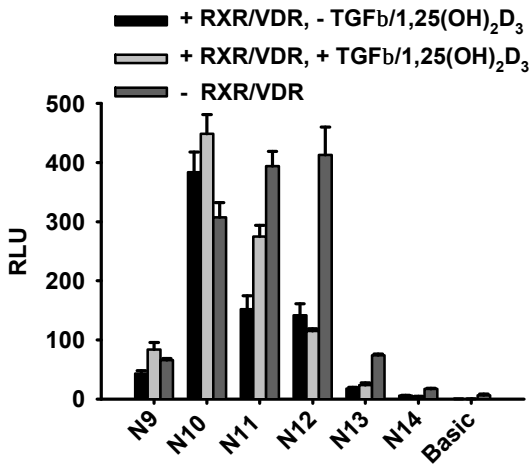

FIGURE 7. **Effects of VDR/RXR coexpression on the transcriptional activity of pN9-pN14 in transiently transfected Mono Mac 6 cells**. The 5-LO promoter reporter gene constructs pN9, processive deletion variants (pN10-pN14) or the promoterless vector pGL3Basic were transiently transfected into Mono Mac 6 cells with or without the expression vectors pSG5hRXR and pSG5hVDR. After transfection 1,25(OH)$_2$D$_3$ and TGFβ (50 nM and 1 ng/ml, respectively) were added as indicated and reporter gene activity was determined after 6 h. The luciferase activity obtained from pGL3Basic with coexpression of receptors was set to 1. Results are presented as mean ± SE of triplicate transfections, and were normalized for transfection efficiency by cotransfection of pCMVSEAP.

Effects of VDR/RXR coexpression on 5-LO promoter activity.

In order to check the effects of VDR/RXR coexpression on 5-LO promoter activity, Mono Mac 6 cells were transiently transfected with the 5-LO promoter reporter gene constructs pN9 – pN14 or pGL3Basic in presence or absence of plasmids encoding the VDR (pSG5hVDR) and the RXR (pSG5hRXR). Compared to pN10, the pN9 construct gave 4 to 5-fold lower transcriptional activity (fig. 7). Also, the presence of TGFβ and 1,25(OH)$_2$D$_3$ did not significantly increase expression of the pN9 and pN10 reporter gene constructs, and coexpression of the VDR/RXR caused no change in the promoter activity.

However, coexpression of VDR/RXR in Mono Mac 6 cells resulted in a 2 to 3-fold reduction of reporter gene activity for pN11, pN12, pN13 and pN14 which all lack the sequences from -778 bp to –229 bp (containing the putative VDREs). The expression of these truncated reporter constructs was not altered by treatment of cells with TGFβ and 1,25(OH)$_2$D$_3$.

Thus, coexpression of the receptors VDR and RXR does not significantly affect 5-LO promoter activity when the VDREs are present (plasmids pN9, pN10) but reduces 5-LO promoter activity in reporter gene constructs (pN11-pN14) that lack the DNA sequence from –778 bp to –229 bp, which contains the putative VDREs. Obviously, these sequences are required for maximal promoter activity when expression of VDR/RXR is high.

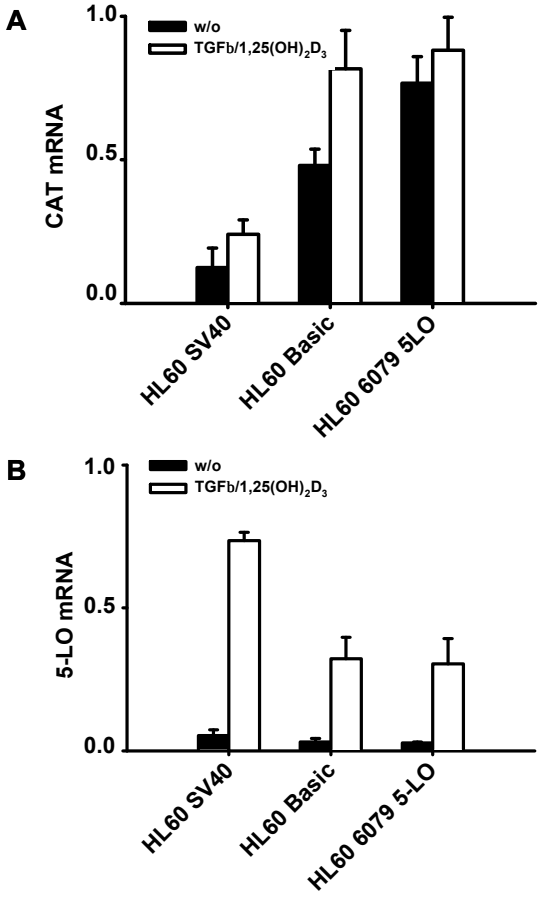

FIGURE 8. **Effects of TGFb/1,25(OH)$_2$D$_3$ on transcription of stably transfected CAT reporter gene constructs (A) and on endogenous 5-LO mRNA expression (B).** HL-60 cells were stably transfected with a 5-LO promoter construct comprising sequences from –6079 bp to +81 bp of the 5-LO promoter (HL60-6079-5LO), the SV40 promoter (HL60-SV40) or a promoterless plasmid (HL60-Basic) in front of the CAT reporter gene. After 24 h incubation with or without 1,25(OH)$_2$D$_3$ (50 nM) and TGFβ (1 ng/ml), cells were harvested and CAT mRNA transcripts were determined (A). Results are presented as mean ± SE of three independent experiments. (B) RT-PCR analysis of endogenous 5-LO mRNA expression in the stably transfected HL-60 cells. Mature 5-LO mRNA was analysed by RT-PCR and quantified as described under "Materials and Methods". Values are given as mean ± SE of at least three independent experiments.

Transcription of the stably transfected 5-LO promoter CAT reporter gene in HL-60 cells is independent of TGFb/1,25(OH)$_2$D$_3$. In order to investigate whether integration of the 5-LO promoter into the genome is required for the upregulatory effects of TGFβ and

$1,25(OH)_2D_3$, HL-60 cells were stably transfected with 5-LO promoter-CAT constructs and cultured with or without 1 ng/ml TGFβ and 50 nM $1,25(OH)_2D_3$. After 24 h, CAT and endogenous 5-LO mRNA expression was determined by RT-PCR. TGFβ and $1,25(OH)_2D_3$ did not significantly affect CAT expression in HL60-6079-5LO cells which contain a stably integrated CAT gene under the control of the 5-LO promoter (-6079 to +81 bp). TGFβ and $1,25(OH)_2D_3$ slightly stimulated (1.9- and 1.7-fold) CAT mRNA expression in HL60-SV40 (where the CAT reporter gene is under the control of an SV40 promoter) and in HL60-Basic cells, respectively (fig. 8A). In contrast, when the expression of endogenous 5-LO mRNA was determined, TGFβ and $1,25(OH)_2D_3$ caused a 13.8-fold, 11.1-fold and 7.1-fold increased gene expression in HL60-SV40, HL60-Basic, and HL60-6079-5LO, respectively (fig. 8B). Thus, induction of 5-LO mRNA expression by TGFβ and $1,25(OH)_2D_3$ seems to be mediated by regulatory elements located outside of the 5-LO promoter.

DISCUSSION

Previous reports have established a prominent role for $1,25(OH)_2D_3$ and TGFβ in the upregulation of 5-LO expression in myeloid cells during maturation (5). It was found that the about 42- to 64-fold induction of 5-LO mRNA in Mono Mac 6 cells cultured with TGFβ and $1,25(OH)_2D_3$ was due to both increased 5-LO gene transcription (about 5-fold) and to stimulated transcript elongation and maturation (7). Both TGFβ and $1,25(OH)_2D_3$ were required for these effects.

Genomic actions of $1,25(OH)_2D_3$ are generally mediated via ligation of the VDR that in turn transactivates VDREs (for review, see (26)). Since the 5-LO promoter contains several putative VDREs (within –313 bp to -258 bp), it appeared reasonable that the upregulative effects of $1,25(OH)_2D_3$ on 5-LO mRNA expression are due to ligand-dependent transactivation of the VDRE by VDR. In fact, we demonstrate such binding of VDR together with RXR to 5-LO promoter fragments spanning the putative VDREs in vitro which was similar to the well-documented VDRE of the mouse osteopontin promoter (figs. 2, 3). Also, differentiation of Mono Mac 6 cells by $1,25(OH)_2D_3$ and TGFβ caused markedly enhanced protein binding to VDREs from nuclear extracts, as compared to untreated cells (fig. 4). However, luciferase reporter gene assays failed to demonstrate significant transcriptional activation of the 5-LO promoter upon stimulation with $1,25(OH)_2D_3$ (plus TGFβ) in various myeloid cell lines (fig. 5A), although expression of a reporter construct containing a 4-times concatemerized rANF vitamin D response element in front of the thymidine kinase promoter (p(DR3)4tkluc) was strongly increased after treatment of these cell lines with $1,25(OH)_2D_3$ and TGFβ (fig. 5B). Moreover, in stably transfected HL-60 cells endogenous 5-LO mRNA was strongly upregulated by $1,25(OH)_2D_3$ and TGFβ, whereas the CAT reporter gene, representing the entire 5-LO promoter fragment (-6079 to +81 bp), was not responsive (fig. 8). Together, we conclude that the strong induction of 5-LO mRNA expression by $1,25(OH)_2D_3$ and TGFβ in myeloid cells seems to be mediated by regulatory elements located outside of the 5-LO promoter, and apparently does not involve the VDR/RXR interaction with the VDREs located within the 5-LO promoter.

The human 5-LO gene promoter was first characterized by Funk *et al.* (27) and Hoshiko *et al.* (25). Several features of the promoter region like the lack of TATAA or CCAAT boxes and repeated G+C-rich elements are characteristic for so called housekeeping genes. A major transcriptional start site was found 65 bp upstream of the translation start codon (ATG) and sequence analysis indicated the presence of putative cis-acting control elements such as multiple Sp1 sites, tandem repeat sites, several putative AP-2 binding sites and putative response elements, such as for the transcription factor NFκB. Five tandem Egr-1/Sp1 consensus binding sites comprising the sequences from –147 bp to –23 bp in relation to the transcriptional start site were required for functional interaction and activation of the promoter in Egr-1/Sp-1 cotransfection studies (10). Recent results underlined the importance of the 5-LO core promoter region from –193 bp to –31 bp and revealed a strong involvement of DNA methylation in the regulation of cell-type specific 5-LO gene expression (28). Moreover, TsA treatment of Mono Mac 6 and HeLa cells, which were transiently transfected with the 5-LO promoter reporter gene construct pN10, indicated a strong involvement of HDAC in the regulation of 5-LO promoter activity (29). However, the data indicated, that DNA methylation or histone acetylation were not related to the prominent effects of TGFβ/$1,25(OH)_2D_3$ on the increase in 5-LO mRNA observed in Mono Mac 6 and HL-60 cells (6).

By means of reporter gene assays using HeLa and Mono Mac 6 cells, we could confirm previous findings that the promoter region containing the five tandemized Sp1-sites is essential for basal promoter activity. For both cell lines we could show that there is a positive regulatory region located between –5814 and –5395 bp. Hoshiko et al. identified a positive regulatory region (-6079 to –3635 bp) which comprises our sequence. On the other hand, in HeLa cells we did not observe the presence of a negative regulatory region at –662 to -227 bp that was reported before. The reason for this discrepancy is unknown but could be due to different transfection protocols, different deletion constructs or experimental conditions. Interestingly, we could identify a new inhibitory region (from –5395 to -4894 bp) which contains two putative binding sites for the transcription factor p53 that match the consensus sequence (GAACATGTCC) to more than 80 %.

Using Mono Mac 6 cells we found a positive regulatory region (-778 to –229 bp) that is not functional in the 5-LO negative HeLa cell line. Interestingly, putative VDREs are located in that region, but as stated above, in reporter gene experiments TGFβ/$1,25(OH)_2D_3$ did not alter 5-LO promoter activity in any cell type investigated. These findings are in agreement with results from nuclear run-off assays (6,7) and previous

transfections (28), where increased transcription of the 5-LO gene could not be demonstrated.

Of interest, the coexpression of the VDR and RXR was found to be essential for high level induction of reporter gene activity of p(DR3)4tkluc, since low expression of VDR and RXR (no expression vectors added), caused only marginal effects of TGFβ/1,25$(OH)_2D_3$, except for RBL-1 cells, which could be due to a high expression level of VDR and/or VDR related coactivators.

Reporter gene activities of the 5-LO promoter constructs pN9-pN14 were also compared in experiments conducted with or without the cotransfection of the receptor expression vectors for VDR/RXR. Interestingly, coexpression of the receptors VDR and RXR does not significantly affect 5-LO promoter activity when the VDREs are present (plasmids pN9, pN10) but reduces 5-LO promoter activity in reporter gene constructs (pN11-pN14) that lack the DNA sequence from -778 to –229 bp, which contains the putative VDREs. One possible explanation could be that VDR/RXR recruit coactivators that are required for high 5-LO promoter activity. However, with 5-LO promoter constructs that lack the VDREs, the VDR does not bind anymore and instead sequesters coactivators from the 5-LO promoter. This conclusion is supported by DNA footprinting and EMSA analyses where a strong interaction of VDR/RXR with the VDREs at –309 to –294 bp and at –290 to –275 bp with homology to the VDRE of the mouse phosphate intestinal transporter could be demonstrated.

Generally, the amount of VDR and the differentiation of leukemic cells do not strictly correlate (30-32) and other factors, for example cell line-specific nuclear transcription factors, e.g. coactivators could play a role. Some of these factors, acting in large complexes, have histone modifying activities (33-39). However, the simple lack of bridging factors or coactivators in transient transfections could be excluded by experiments with the stably transfected cell line HL-60, where the endogenous 5-LO mRNA expression was strongly induced in presence of TGFβ/1,25$(OH)_2D_3$, but the CAT reporter gene mRNA and β-actin mRNA levels were not altered (fig. 8).

Collectively our data show that although VDR/RXR are capable to bind to putative VDREs in the 5-LO promoter, the regulatory sequences involved in the prominent induction of 5-LO mRNA expression by TGFβ and 1,25$(OH)_2D_3$ in the human myeloid cell lines HL-60 and Mono Mac 6 seem to be located outside this part of the 5-LO promoter.

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REFERENCES

1. Ford-Hutchinson, A. W., Gresser, M., and Young, R. N. (1994) *Annu. Rev. Biochem.* **63**, 383-417
2. Samuelsson, B., Dahlén, S.-E., Lindgren, J.-Å., Rouzer, C. A., and Serhan, C. N. (1987) *Science* **237**, 1171-1176
3. Kargman, S., and Rouzer, C. A. (1989) *J. Biol. Chem.* **264**, 13313-13320
4. Brungs, M., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1994) *Biochem. Biophys. Res. Commun.* **205**, 1572-1580
5. Brungs, M., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1995) *Proc. Natl. Acad. Sci. U S A* **92**, 107-111
6. Härle, D., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1999) *Adv. Exp. Med. Biol.* **469**, 105-111
7. Härle, D., Rådmark, O., Samuelsson, B., and Steinhilber, D. (1998) *Eur. J. Biochem.* **254**, 275-281
8. Coffey, M. J., Gyetko, M., and Peters-Golden, M. (1993) *J. Lipid Med.* **6**, 43-51
9. Coffey, M. J., Wilcoxen, S. E., Phare, S. M., Simpson, R. U., Gyetko, M. R., and Peters-Golden, M. (1994) *Prostaglandins* **48**, 313-329
10. Silverman, E. S., Du, J., De Sanctis, G. T., Rådmark, O., Samuelsson, B., Drazen, J. M., and Collins, T. (1998) *Am. J. Respir. Cell. Mol. Biol.* **19**, 316-323
11. In, K. H., Asano, K., Beier, D., Grobholz, J., Finn, P. W., Silverman, E. K., Silverman, E. S., Collins, T., Fischer, A. R., Keith, T. P., Serino, K., Kim, S. W., De Sanctis, G. T., Yandava, C., Pillari, A., Rubin, P., Kemp, J., Israel, E., Busse, W., Ledford, D., Murray, J. J., Segal, A., Tinkleman, D., and Drazen, J. M. (1997) *J. Clin. Invest.* **99**, 1130-1137
12. Drazen, J. M., Yandava, C. N., Dube, L., Szczerback, N., Hippensteel, R., Pillari, A., Israel, E., Schork, N., Silverman, E. S., Katz, D. A., and Drajesk, J. (1999) *Nat. Genet.* **22**, 168-170
13. Silverman, E. S., Le, L., Baron, R. M., Hallock, A., Hjoberg, J., Shikanai, T., Storm van's Gravesande, K., Auron, P. E., and Lu, W. (2002) *Am. J. Respir. Cell. Mol. Biol.* **26**, 475-483
14. Steinhilber, D., Brungs, M., Werz, O., Wiesenberg, I., Danielsson, C., Kahlen, J.-P., Nayeri, S., Schräder, M., and Carlberg, C. (1995) *J. Biol. Chem.* **270**, 7037-7040
15. Carlberg, C. (1995) *Eur. J. Biochem.* **231**, 517-527.
16. Werz, O., Brungs, M., and Steinhilber, D. (1996) *Pharmazie* **51**, 893-896
17. Ziegler-Heitbrock, H. W., Thiel, E., Futterer, A., Herzog, V., Wirtz, A., and Riethmuller, G. (1988) *Int. J. Cancer* **41**, 456-461
18. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156-159

19. Martin, C. S., Wight, P. A., Dobretsova, A., and Bronstein, I. (1996) *Biotechniques* **21**, 520-524
20. Himes, S. R., and Shannon, M. F. (2000) *Methods Mol. Biol.* **130**, 165-174.
21. Fulton, R., and Van Ness, B. (1993) *Biotechniques* **14**, 762-763.
22. Roelant, C. H., Burns, D. A., and Scheirer, W. (1996) *Biotechniques* **20**, 914-917
23. Yang, T. T., Sinai, P., Kitts, P. A., and Kain, S. R. (1997) *Biotechniques* **23**, 1110-1114
24. Shapiro, D. J., Sharp, P. A., Wahli, W. W., and Keller, M. J. (1988) *DNA* **7**, 47-55
25. Hoshiko, S. R., O.; Samuelsson, B. (1990) *Proceedings of the National Academy of Science* **87**, 9073-9077
26. Carlberg, C. (1996) *Endocrine* **4**, 91-105
27. Funk, C. D. H., S.; Matsumoto, T.; Rådmark, O. and Samuelsson, B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2587-2591
28. Uhl, J., Klan, N., Rose, M., Entian, K. D., Werz, O., and Steinhilber, D. (2002) *J. Biol. Chem.* **277**, 4374-4379
29. Klan, N., Seuter, S., Schnur, N., Jung, M., and Steinhilber, D. (2003) *Biol. Chem.* **384**, 777-785.
30. Ratnam, A. V., Bikle, D. D., Su, M. J., and Pillai, S. (1996) *J Invest Dermatol* **106**, 522-525.
31. Hayashi, Y., Honma, Y., Niitsu, N., Taki, T., Bessho, F., Sako, M., Mori, T., Yanagisawa, M., Tsuji, K., and Nakahata, T. (2000) *Cancer Research* **60**, 1139-1145
32. Heberden, C., Denis, I., Pointillart, A., and Mercier, T. (1998) *Gen Pharmacol* **30**, 145-151.
33. Yang, W., and Freedman, L. P. (1999) *J. Biol. Chem.* **274**, 16838-16845
34. Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K., and Kato, S. (1999) *Science* **283**, 1317-1321
35. Yanagi, Y., Suzawa, M., Kawabata, M., Miyazono, K., Yanagisawa, J., and Kato, S. (1999) *J. Biol. Chem.* **274**, 12971-12974
36. Subramaniam, N., Leong, G. M., Cock, T. A., Flanagan, J. L., Fong, C., Eisman, J. A., and Kouzmenko, A. P. (2001) *J. Biol. Chem.* **276**, 15741-15746
37. Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., and Carlberg, C. (2000) *Faseb J.* **14**, 1455-1463
38. Jurutka, P. W., Remus, L. S., Whitfield, G. K., Galligan, M. A., Haussler, C. A., and Haussler, M. R. (2000) *Biochem Biophys Res Commun* **267**, 813-819.
39. Gill, R. K., and Bell, N. H. (2000) *Calcif Tissue Int* **66**, 370-374.

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The Coding Sequence Mediates Induction of 5-Lipoxygenase Expression by 1,25-Dihydroxyvitamin D_3 and Transforming Growth Factor-b

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5-Lipoxygenase expression is strongly induced by transforming growth factor-b (TGFb) and 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) in the human myeloid cell line Mono Mac 6. In this study the mechanism of the TGFb and 1,25$(OH)_2D_3$ mediated upregulation of 5-lipoxygenase gene expression was investigated. RT-PCR analysis of 5-lipoxygenase RNA expression in Mono Mac 6 cells revealed a profound cytosolic and nuclear accumulation of mature 5-lipoxygenase mRNA but not of pre-mRNA. In reporter gene assays where HeLa cells were transiently transfected with plasmids containing the 5-lipoxygenase core promoter plus different parts of the 5-lipoxygenase gene we found that the promoter failed to mediate transcriptional activation by TGFb and 1,25$(OH)_2D_3$. However, inclusion of the coding sequence caused a 5-fold upregulation of reporter gene activity by TGFb and 1,25$(OH)_2D_3$ which was enhanced to 13-fold when the last four introns of the 5-lipoxygenase gene were also included. RT-PCR analysis of the transcripts generated by the transfected cells implies that parts of the coding sequence are removed during 5-lipoxygenase mRNA processing leading to incomplete transcripts. Addition of TGFb and 1,25$(OH)_2D_3$ induced the appearance of exon 13 in the 5-lipoxygenase transcripts which is reflected by an about 14-fold increase of the corresponding PCR product. Deletion of various parts of the 5-lipoxygenase coding sequence slightly reduced inducibility by TGFb and 1,25$(OH)_2D_3$ whereas deletion of large parts of the coding sequence was required for an almost complete loss of the response. This suggests that the regulation of 5-lipoxygenase mRNA processing by TGFb and 1,25$(OH)_2D_3$ involves multiple parts of the coding sequence.

Key words: coding sequence, mRNA processing, HeLa cell, reporter gene assay

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The enzyme 5-lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid to (5S)-hydroperoxy-6-trans-6,11,14-cis-eicosatetraenoic acid (5-HPETE) and further to leukotriene A_4 ((5S)-6-oxido-7,9,11,trans-14-cis-eicosatetraenoic acid) (Lewis et al., 1990; Samuelsson et al., 1987). 5-LO is expressed in a variety of immune competent cells including B-lymphocytes, granulocytes, monocytes, mast cells and dendritic cells (Steinhilber, 1999). Leukotrienes have been shown to be involved in inflammatory reactions and recent data suggest that the 5-lipoxygenase pathway plays a role in the development of cardiovascular diseases (Helgadottir et al., 2004; Mehrabian and Allayee, 2003).

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[1]The abbreviations used are: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; cds, coding sequence; 5-LO, 5-lipoxygenase; luc, luciferase; RE, responsive element; RXR, retinoid X receptor; SBE, Smad binding element; SEAP, secreted alkaline phosphatase; TGFβ, transforming growth factor β; TRE, TGFβ responsive element; UTR, untranslated region; VDR, vitamin D receptor; VDRE, vitamin D responsive element.

Expression of 5-LO, the key enzyme in the biosynthesis of proinflammatory leukotrienes, is regulated on transcriptional and posttranscriptional levels. Basal 5-LO promoter activity seems to be regulated by DNA methylation. It has been shown that DNA methylation of the GC-rich core region strongly suppresses 5-LO promoter activity. Furthermore, the 5-LO promoter was found to be methylated in 5-LO negative cell lines whereas it is completely demethylated in 5-LO positive cells (Uhl et al., 2002).

Depending on the cell type, several cytokines have been shown to be inducers of the 5-LO pathway. In granulocytes, 5-LO expression is stimulated by GM-CSF (McColl et al., 1991) whereas IL-3 regulates the development of the 5-LO pathway in mouse mast cells (Murakami et al., 1995). 5-LO activity, protein expression and mRNA are strongly increased during differentiation of myeloid cell lines such as Mono Mac 6 and HL-60. Transforming growth factor-β (TGFβ) and 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) were identified as potent inducers of 5-LO gene expression (Brungs et al., 1994; Brungs et al., 1995). In Mono Mac 6 cells, treatment with TGFβ and 1,25$(OH)_2D_3$ led to an up to 5-fold increase in primary transcripts, a 64-fold increase in mature 5-LO mRNA, a 128-fold increase in protein expression and a more than 500-fold increase in 5-LO activity (Härle et al., 1999). The upregulation of 5-LO mRNA expression only partially depended on protein synthesis which indicated that 5-LO is a primary and secondary vitamin D response gene.

Interestingly, this strong increase in 5-LO mRNA expression does not seem to be mediated by the promoter since TGFβ and 1,25$(OH)_2D_3$ had no effect on 5-LO transcription in nuclear run-off assays using nuclear extracts from Mono Mac 6 cells (Härle et al., 1999) and on 5-LO promoter activity in reporter gene assays in different cell types (Uhl et al., 2002). These data suggested that induction of of 5-LO mRNA expression by TGFβ and 1,25$(OH)_2D_3$ is mediated by response elements that are located outside of the 5-LO promoter region. Thus, the contribution of the coding region, the 3'-untranslated region (3'-UTR) and introns J, K, L and M to the induction of 5-LO gene expression was investigated and we found that the coding sequence mediates the induction of 5-LO mRNA expression by TGFβ and 1,25$(OH)_2D_3$ and that both agents affect 5-LO mRNA processing.

MATERIALS AND METHODS

Reagents. Molecular biology reagents were from MBI Fermentas, Sigma, Invitrogen, Promega or other sources as indicated in the text. Insulin was a gift from Aventis. Human TGFβ1 was purified from outdated platelets according to (Werz et al., 1996). Nucleospin Extract columns for direct purification of pDNA were from Macherey-Nagel (Düren, Germany). The plasmid p(DR3)4tkluc and the pSG5-VDR and pSG5-RXR expression plasmids for the human vitamin D receptor (VDR) and retinoid X receptor alpha (RXRα), respectively, were obtained from Dr. Carsten Carlberg (Kuopio, Finland).

Cells and cell culture. Mono Mac 6 cells, which were kindly provided by Dr. H.W.L. Ziegler-Heitbrock (Munich), were grown at 37 °C in a humified atmosphere with 6 % CO_2 in RPMI 1640 medium supplemented with 10 % (vol/vol) fetal calf serum (FCS), streptomycin (100 µg/ml), penicillin (100 U/ml), 1× nonessential amino acids, sodium pyruvate (1 mM), oxalacetate (1 mM) and insulin (10 µg/ml) (Ziegler-Heitbrock et al., 1988). HeLa cells were obtained from Dr. W.E. Müller (Pharmacological Institute, Biocenter, Frankfurt) and grown in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10 % (vol/vol) FCS, 100 µg/ml streptomycin and 100 units/ml penicillin.

Isolation of cytosolic and nuclear RNA and RT-PCR analysis. Mono Mac 6 cells were seeded at 4×10^5 cells/ml and incubated for 24 h with or without $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml). Cells were harvested by centrifugation at 1200×g for 10 min at room temperature (RT). Cytosolic RNA was isolated as described in (Sambrook, 1989). The pelleted nuclei were subjected to an isolation of total RNA by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RT-PCR analysis of nuclear and cytosolic RNA fractions was performed as described previously (Härle et al., 1999).

Transiently transfected HeLa cells were preincubated with or without TGFβ (1 ng/ml) for 24 h. After transfection cells were grown with or without $1,25(OH)_2D_3$ (50 nM), TGFβ (1 ng/ml) and cycloheximide (10 µM) for 8 h. Cells were harvested by scraping and centrifugation at 1200×g for 10 min at RT. Total RNA was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Prior to reverse transcription, RNA was incubated with DNAse I (1 unit/µg RNA) to remove plasmid DNA. RT-PCR analysis was performed as described previously (Härle et al., 1999). PCR-primers (Tab. 1) were used at a concentration of 50 pmol/µl.

Signal intensities of ethidium bromide stained DNA bands were quantified by densitometry (BioRad Gel Doc 1000 system) and analyzed with the Molecular Analyst program (BioRad). Results are expressed as x-fold induction of mRNA amounts, with respect to untreated cells and normalized with β-actin as internal standard.

Plasmid constructs. 5-LO promoter reporter gene contructs were prepared using restriction enzymes and PCR methods.

The 3'-UTR of the 5-LO gene was obtained by PCR amplification using random hexamer-primed cDNA from Mono Mac 6 cells as a template and the primers

5'-GTATTCTAGATGAGCACACTGCCAGTCTCA-3' and

5'-CGGCTCTAGACTGATTTATTTTTATGGCAAC-3' and *Pfu I* DNA polymerase. Following digestion of the PCR fragment and the plasmid pN10 (Klan et al., 2003) with *Xba I,* insert and pDNA were ligated using T4 DNA ligase (pN10-3UTR).

5-LO coding sequence (cds) was amplified using the plasmid pT3-5LO (Zhang et al., 1992) as a template and the primers

5'-CCCTCCTACACGGTCACCGTG-3' and

5'-ATGGCCACACTGTTCGGAATCC-3' and *Pfu I* DNA polymerase. pN10-3UTR was digested with *Nco I,* blunted using T4 DNA polymerase and ligated with the PCR fragment using T4 DNA ligase (pN10-cds3UTR).

The part of the 5-LO coding sequence containing the last four introns (introns J, K, L and M) was obtained by PCR amplification using genomic DNA from Mono Mac 6 cells as a template and the primers

5'-TATGCCTCCCTGTGCTTTCCCGA-3' and

5'-AGGTTCTTGCGGAATCGGGCCA-3' and *Pfu I* DNA polymerase. The fragment was digested with *Sfi I* and partially with *Nco I* (besides the *Nco I* site used for cloning there is an additional one in intron M). pN10-cds3UTR was digested with *Sfi I* and *Nco I*. Insert and pDNA were ligated using T4 DNA ligase (pN10-cdsInJM3UTR).

Contructs pN10-cds and pN10-cdsInJM were obtained by deleting the 3'-UTR via digestion of pN10-cds3UTR and pN10-cdsInJM3UTR, respectively, with *Xba I* and religation.

Plasmid pN10-cdsInJM-inv was obtained by cloning of the *Xho I*-framed fragment cdsInJM from pN10-cdsInJM into the *Sal I* site of pN10.

Contructs pGl3-ba-cdsInJM and pGL3-prom-cdsInJM were prepared using plasmids pGL3-Basic and pGL3-Promoter (both from Promega), respectively, and restriction fragments obtained from pN10-cdsInJM using *Xba I* and *Bgl II* or *Xba I* and *Hind III* (partial).

Sequential deletion of the coding sequence divided in four parts lead to the pN10-cds deletion constructs pdcdsA, pdcdsB, pdcdsC, pdcdsD, pdcdsAB, pdcdsBC, pdcdsCD, pdcdsABC and pdcdsBCD. Deletions were performed using PCR mutation and restriction enzymes.

Plasmid sequences were confirmed by DNA sequencing.

Transient transfections. 24 h prior to transfection, HeLa cells were plated into a 24-well tissue culture plate at a density of 5×10^4 cells per well. TGFâ was added for a 24 h-preincubation. Transient transfections were performed with the standard calcium phosphate method, adding 800 ng of reporter plasmid, 100 ng of different expression plasmids and 20 ng pCMV-SEAP as internal standard per well. After 16 h the calcium phosphate-DNA precipitate was removed, new cell culture medium added and incubated for another 8 h.

Luciferase assays. Cells were transfected as indicated, harvested and lysed in 100 µl lysis buffer (Steady-Glo^{TM} Luciferase Assay System, Promega). Luciferase activity was determined by monitoring light emission with a Microlumat Plus LB96V EG&G Berthold luminometer (Martin et al., 1996). The light emission signal was integrated for 5 seconds. Transfection efficiency was monitored and normalised by cotransfection of pCMV-SEAP using the Phospha-Light TM kit (Tropix) to determine the secreted placental alkaline phosphatase (SEAP) activity (Yang et al., 1997).

RESULTS

$1,25(OH)_2D_3$ and TGFb induce accumulation of mature 5-LO mRNA. The 5-LO positive human myeloid cell line Mono Mac 6 was incubated with $1,25(OH)_2D_3$ and TGFβ for 24 hours. Subsequently, nuclear and cytosolic RNA was isolated and subjected to RT-PCR analysis, using either oligo$(dT)_{12-18}$ or random hexamer primers for cDNA synthesis for the determination of mature and pre-mRNA, respectively. The time course experiment revealed a prominent increase of mature 5-LO mRNA in the nucleus as well as in the cytosol following incubation with $1,25(OH)_2D_3$ and TGFβ, whereas pre-mRNA levels were not significantly elevated (fig. 1).

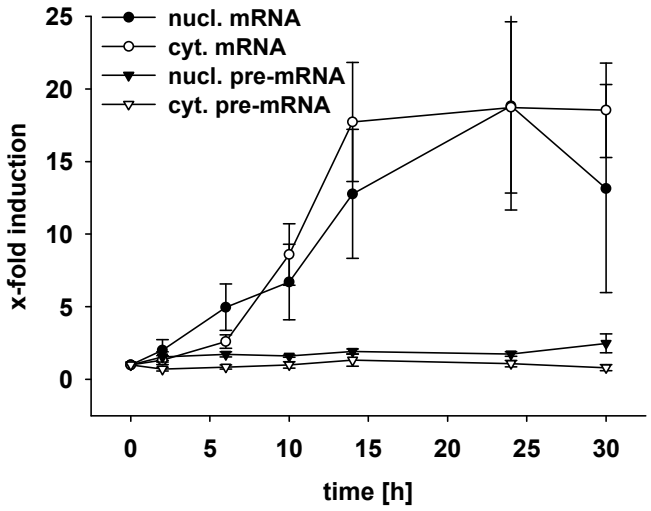

FIG. 1. **Effects of $1,25(OH)_2D_3$ and TGFb on nucleic and cytosolic 5-LO mRNA in Mono Mac 6 cells.** Mono Mac 6 cells were grown with or without $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml) for 24 h. Then, cytosolic and nucleic RNA was isolated as described and RT-PCR analysis was performed. Signal intensities of ethidium bromide stained DNA bands were quantified by densitometry. Results are expressed as fold induction in mRNA amounts in relation to untreated cells and normalized with β-actin as internal standard. Results are displayed as mean ± SE of at least three independent experiments.

Cotransfection of VDR and RXR induces reporter gene activity when the 5-LO coding sequence is present. In order to study the effects of VDR/RXR coexpression and to identify regulatory sequences located outside of the 5-LO promoter, reporter gene assays with plasmid constructs containing the 3'-untranslated region, the coding sequence (cds) and the last four introns (introns J, K, L and M) were performed in the presence and absence of pSG5-VDR and pSG5-RXR. For that purpose, the 3'-untranslated region, the coding sequence and the last four introns were cloned into the construct pN10 and in the pGL3 vectors (Promega). The constructs were cloned in such a way that the 5-LO coding sequence was in frame to the luciferase sequence. Two sets of luciferase reporter gene plasmids were constructed, the first containing the 5-LO core promoter (–778 to +53 bp) in the plasmid pGL3-Basic plus different parts of the 5-LO gene and the second containing the 5-LO cds plus the last four introns in front of the luciferase gene using the pGL3-Basic, pGL3-Promoter or pGL3-Control vector, respectively (fig. 2).

HeLa cells were transiently transfected with these reporter gene plasmids in the presence or absence of VDR and RXR expression constructs. As shown in fig. 3, reporter gene activity of constructs containing the 5-LO promoter alone or in combination with the 3'-UTR was not upregulated by cotransfection of RXR/VDR which was comparable to controls pGL3-Basic, -Promoter and -Control. However, inclusion of the 5-LO coding sequence led to an about 3-fold induction of luciferase activity by nuclear receptor overexpression and addition of introns J-M even led to an about 6-fold induction. This upregulation seemed to be independent from the 5-LO promoter since it was also observed with plasmids containing no promoter, an SV40-promoter or an SV40-promoter

plus -enhancer (pGL3-ba-cdsInJM, pGL3-prom-cdsInJM and pGL3-ctrl-cdsInJM, respectively).

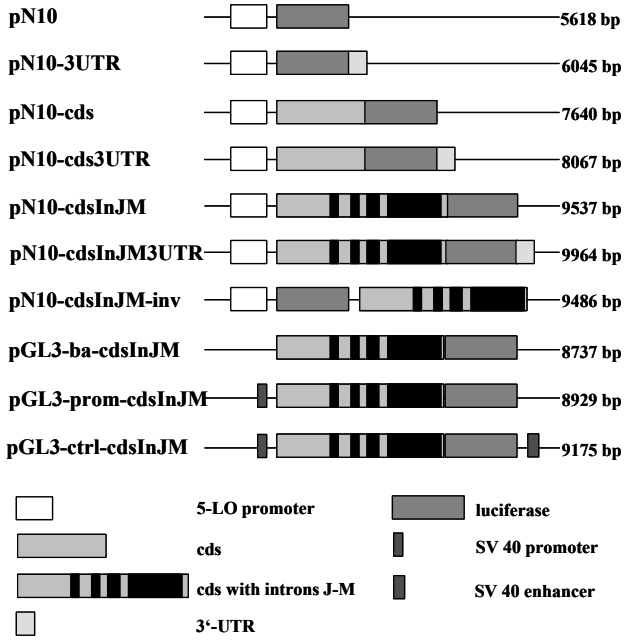

FIG. 2. **Overview of luciferase reporter gene plasmids.**

RT-PCR was used to confirm these data on mRNA level. HeLa cells were transiently transfected with pN10-cdsInJM in the presence or absence of expression plasmids for the nuclear receptors VDR and RXR and RT-PCR analysis was performed, using either oligo$(dT)_{18\text{-}20}$ or random hexamer primers for cDNA synthesis and different sets of primers for PCR reactions. VDR/RXR mediated induction of 5-LO pre-mRNA and mature mRNA was 2.2- (±0.4) and 4.6- (±1.8) fold, respectively. Taken together, the data show that the 5-LO coding sequence mediates induction by VDR/RXR. Interestingly, no effect of VDR/RXR overexpression was observed with the p(DR3)4tkluc plasmid that contains a 4-times concatemerized VDRE.

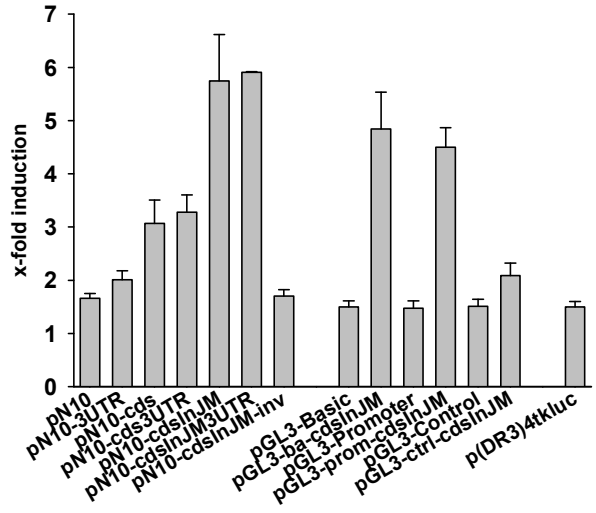

FIG. 3. **Effects of VDR/RXR on the transcriptional activity of luciferase reporter gene constructs in transiently transfected HeLa cells.** The indicated plasmids were transiently transfected into HeLa cells with or without the expression vectors pSG5-hVDR and pSG5-hRXR. p(DR3)4tkluc, containing a four times concatemerized DR3-type vitamin D response element in front of the thymidine kinase promoter was used as a positive control. Reporter gene activity was measured 24 hours after transfection. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed in respect to pSG5-cotransfected cells.

Induction of reporter gene activity by 1,25(OH)$_2$D$_3$ and TGFb is also mediated by the 5-LO coding sequence. In Mono Mac 6 cells, 5-LO mRNA expression is strongly enhanced by 1,25(OH)$_2$D$_3$ and TGFβ, but previous reporter gene assays with the 5-LO core promoter construct pN10 failed to show a transcriptional upregulation following incubation with 1,25(OH)$_2$D$_3$ and TGFβ (Uhl et al., 2002). In order to investigate whether the effect of these agents also depends on sequences located outside of the promoter like the coding region and 3'-UTR, reporter gene assays with the corresponding plasmid constructs (fig. 2) were performed. HeLa cells were incubated with 1 ng/ml TGFβ for 24 hours before transfection. Transfection was done as described above and after exchange of medium, cells were incubated with 1 ng/ml TGFβ and/or 50 nM of 1,25(OH)$_2$D$_3$ for another 8 hours before reporter gene activity was determined. TGFβ alone led to an only slight increase in reporter gene activity but the combination of both agents revealed a significant synergistic effect with reporter gene constructs that contain the coding sequence (fig. 4). In agreement to the well-known fact that VDR binds to DNA predominantly as heterodimer with RXR, overexpression of both nuclear receptors was required for upregulation of 5-LO expression by TGFβ and 1,25(OH)$_2$D$_3$ (data not shown). The upregulation by 1,25(OH)$_2$D$_3$ and TGFβ depended on the used constructs. With pN10 and pN10-3UTR, induction by TGFβ and 1,25(OH)$_2$D$_3$ did not differ from the negative control pGL3-Basic but presence of the 5-LO coding sequence (pN10-cds) led to a 5.1-fold induction and inclusion of the coding sequence plus introns J-M (pN10-cdsInJM) to an 12.4-fold upregulation. The reporter gene activity of the promoterless plasmid pGL3-ba-cdsInJM was also induced (about 16-fold) and the positive control plasmid p(DR3)4tkluc containing a 4-times concatemerized VDRE gave a 9-fold induction. Interestingly, introduction of the coding sequence plus the introns J-M downstream of the translational stop codon of the luciferase gene (pN10-cdsInJM-inv) led to a significant reduction of inducibility (3.6-fold).

Taken together, the data show that the 5-LO coding sequence mediates induction of reporter gene activity by 1,25(OH)$_2$D$_3$ and TGFβ.

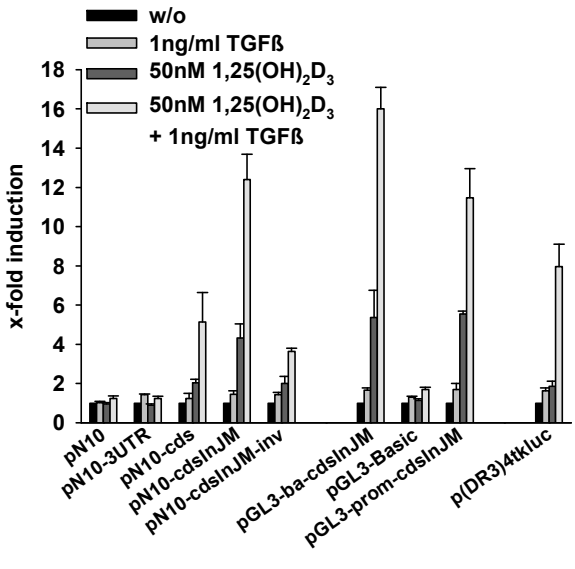

FIG. 4. **Effects of 1,25(OH)$_2$D$_3$ and TGFb on the transcriptional activity of luciferase reporter gene constructs in transiently transfected HeLa cells.** The indicated plasmids and the expession vectors pSG5-hVDR and pSG5-hRXR were transiently transfected into HeLa cells. p(DR3)4tkluc was used as positive control. Before transfection, cells were preincubated with TGFβ (1ng/ml) for 24 h and 16 h after transfection 1,25(OH)$_2$D$_3$ (50 nM) and TGFβ (1 ng/ml) were added for another 8 h before reporter gene activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to untreated cells.

1,25(OH)$_2$D$_3$ and TGFb regulate 5-LO mRNA processing. In order to check whether the effects of 1,25(OH)$_2$D$_3$/TGFβ occur at the mRNA level, HeLa cells were transiently transfected with pN10-cdsInJM and pSG5-VDR and pSG5-RXR and the formed mRNA transcripts were analyzed by RT-PCR. Cells were preincubated with TGFβ for 24 h and after transfection, 1,25(OH)$_2$D$_3$/TGFβ were added together with or without cycloheximide (CHX) for 8 h. After extraction of total RNA (Chomczynski and Sacchi, 1987), RT-PCR analysis was performed, using either oligo(dT)$_{18\text{-}20}$ or random hexamer priming for cDNA synthesis and different sets of primers for PCR reactions (tab. 1, fig. 5). With primer pairs that are located in the beginning or the end of the coding sequence, 28 PCR cycles were sufficient to obtain defined PCR products of correct size whereas 35 cycles were required when the respective primer pairs were located between exons 8 and 13 indicating that central parts of the coding sequence are removed during mRNA processing. Interestingly, when we used a primer pair (cds_start/Asurev) that spans the complete coding region, we got multiple PCR products indicating that mRNA processing seems to occur at several sites. The 1,25(OH)$_2$D$_3$/TGFβ mediated induction of transcripts varied significantly (given as fold induction, fig. 5) depending on the RNA position that was analyzed by PCR. The most prominent increase in 5-LO mRNA which roughly corresponds to the increase in luciferase activity was observed when primers that span intron M like 5-LO1/Asurev (14.6-fold) and InJM_5/Asurev (9.8-fold) or primers that are located within intron M (7.8-fold) were used. Interestingly, with these primer pairs, the increase was significantly inhibited when cycloheximide was added together with 1,25(OH)$_2$D$_3$ and TGFβ, indicating that protein synthesis was required (figs. 6B and D). Strikingly, using primers cds_start/1883_rev or Ncofor1/Asurev that amplify exons 1 to 8 or parts of exon 14 and the luciferase gene, respectively, (fig. 5) an only weak increase in the respective PCR signals (6.0-fold or 2.5-fold, respectively) was obtained that was not inhibited by cycloheximide (figs. 6A and C). Interestingly, these parts of the mRNA were prominently expressed since 28 cycles were sufficient to obtain clear PCR signals of correct size.

Taken together, characterization of the transcribed RNA species by RT-PCR suggests that central parts of the 5-LO coding sequence are removed during RNA processing and that 1,25(OH)$_2$D$_3$ and TGFβ induce the inclusion of exon 13 into the mature mRNA.

Upregulation of 5-LO mRNA processing by 1,25(OH)$_2$D$_3$/TGFb does not depend on a distinct part of the coding sequence. In order to identify sequences in the coding region that participate in the 1,25(OH)$_2$D$_3$/TGFβ-mediated regulation of 5-LO expression, deletion constructs of the vector pN10-cds were cloned and analyzed in reporter gene assays. The coding region was divided into four parts which were subsequently deleted (fig. 7). Domain A ranges from exon 1 to 4, domain B from exon 4 to 10, domain C from exon 10 to 14 and domain D represents the last part of exon 14 (fig. 5).

The respective deletion of parts A to D reduced induction of reporter gene activity by 1,25(OH)$_2$D$_3$/TGFβ (figs. 8A and B). A significant decrease in 5-LO upregulation by 1,25(OH)$_2$D$_3$/TGFβ could be seen when parts B+C, A+B+C or B+C+D were deleted (from 5.1-fold to 2.6-, 2.0- and 2.2-fold, respectively). In constructs in which large parts of the coding sequence are deleted (pdcdsABC and pdcdsBCD), the 1,25(OH)$_2$D$_3$-mediated induction of reporter gene activity was completely lost and only the minor TGFβ-mediated enhancement could be measured.

Taken together, the deletion analysis demonstrates that for an almost complete loss of the response to 1,25(OH)$_2$D$_3$/TGFβ, removal of large parts of the coding sequence is required. This suggests that the regulation of the 5-LO RNA processing by TGFβ and 1,25(OH)$_2$D$_3$ involves multiple parts of the coding sequence.

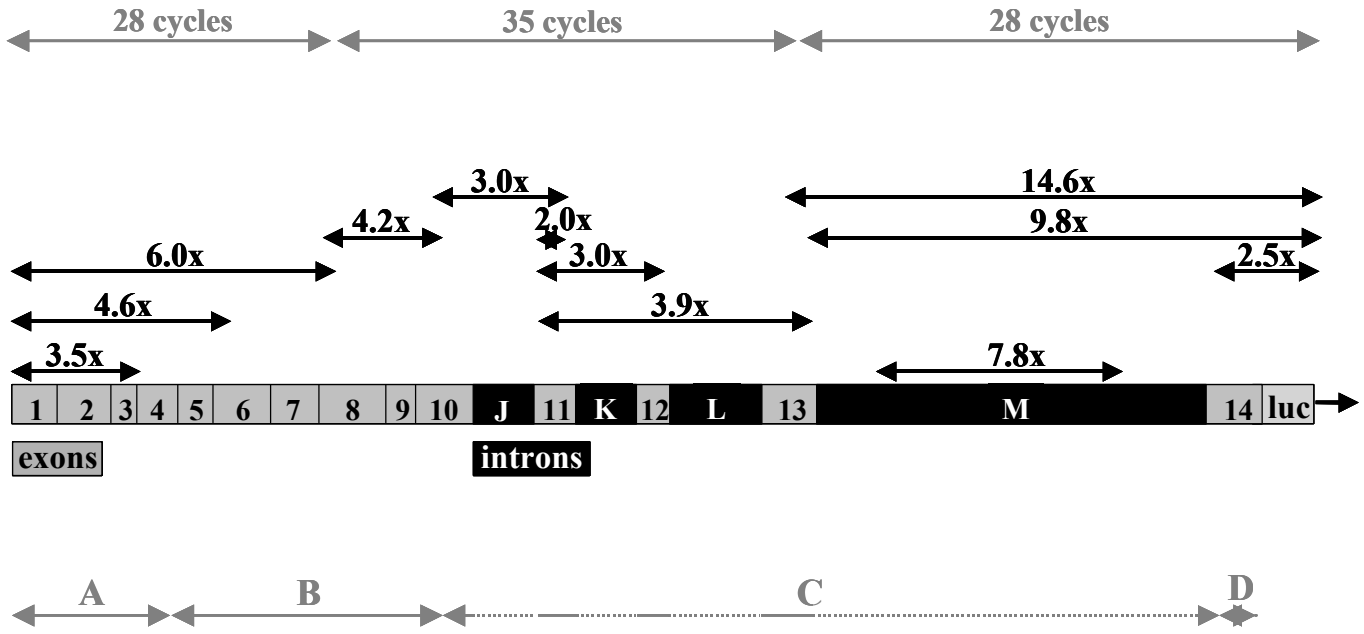

FIG. 5. **Effects of 1,25(OH)$_2$D$_3$/TGFb on mRNA transcripts generated from the transiently transfected luciferase reporter gene construct pN10-cdsInJM.** The reporter gene construct pN10-cdsInJM and the expession vectors pSG5-hVDR and pSG5-hRXR were transiently transfected into HeLa cells, which were preincubated with TGFβ (1 ng/ml) for 24 h. 16 h after transfection, 1,25(OH)$_2$D$_3$ (50 nM) and TGFβ (1 ng/ml) were added for another 8 h. Then, RT-PCR analysis was performed as described in "Materials and Methods". Different pairs of primers were used to scan the coding region. Localization of amplified fragments and the relative induction of the respective PCR products by 1,25(OH)$_2$D$_3$ (50 nM)/TGFβ (1ng/ml) are given. Values are expressed as mean ± SE of at least three independent experiments. The localizations of domains A-D of the coding sequence deletion constructs are indicated below and the numbers of PCR cycles are given above.

Primer pair	Locations	Primer sequence	T_m [°C]	Cycles
β-actin 1/2 (Clontech)		5‘-GAGGAGCACCCCGTGCTGCTGA-3‘ 5‘-CTAGAAGCATTTGCTGTGGACGATGGAGGGGCC-3‘	60	24
5-LO 1/2	exon 13/14	5‘-ACCATTGAGCAGATCGTGGACACGC-3‘ 5‘-GCAGTCCTGCTCTGTGTAGAATGGG-3’	60	28
intron A 1/2	intron A	5’-AGATGAGTCATGCTCACAGAACT-3’ 5’-AGATCAGCATTAGGAACTAGCTT-3’	60	35
cds_start ex3rev	exon 1/3	5’-ATGCCCTCCTACACGGTCAC-3’ 5’-CAGTTCTTTACGTCGGTGTTGCTTGAG-3’	56	28
cds_start ex6rev	exon 1/6	5’-ATGCCCTCCTACACGGTCAC-3’ 5’-CAGCTTCCTGCCAGTGATTCATG-3’	56	28
cds_start 1883_rev	exon 1/8	5’-ATGCCCTCCTACACGGTCAC-3’ 5’- GAGGAAAATAGGGTTCTCATCTCCC-3’	56	28
1883_for 2260_rev	exon 8/10	5’-GGGAGATGAGAACCCTATTTTCCTC-3’ 5’-GGGCCTTGATGGCCTC-3’	56	35
SfiI_for Exon11B	exon 10/11	5’-GACCTGACCTATGCCTCCCTGTGCTTTC-3’ 5’-CATGCCGTACACGTAGACATCGTT-3’	60	35
Exon11A Exon11B	exon 11	5’-GAGGTGGTAGACATCTACTAC-3’ 5’-CATGCCGTACACGTAGACATCGTT-3’	60	35
Exon11A Exon12B	exon 11/12	5’-GAGGTGGTAGACATCTACTAC-3’ 5’-TGGCCGAAGTTGACCGCGGCGT-3’	60	35
Exon11A Exon13B	exon 11/13	5’-GAGGTGGTAGACATCTACTAC-3’ 5’-CTCGTTTTCCTGGAACTGGCTCA-3’	60	35
5-LO1 Asurev	exon 13/luc	5’-ACCATTGAGCAGATCGTGGACACGC-3‘ 5’-GCCAACCGAACGGACATTTC-3’	56	28
InJM_5 Asurev	exon 13/luc	5’-CTGAGCCAGTTCCAGGAAAACGAG-3‘ 5’-GCCAACCGAACGGACATTTC-3’	56	28
Ncofor1 Asurev	exon 14/luc	5’-GAAGCCTGTGAAGGAAGCCATG-3’ 5’- GCCAACCGAACGGACATTTC-3’	56	28
intron M 1/2	intron M	5’-AGGGTGTGCAGGGCAGGAGA-3’ 5’-GGTTACGAGATGATGCAGATGAAGCTG-3’	60	28

TAB. 1. **PCR primer sequences.** Primers used for reverse transcription (RT-) analysis. T_m, annealing temperature.

DISCUSSION

Previous reports have established a prominent role for $1,25(OH)_2D_3$ and TGFβ in the upregulation of 5-LO gene expression in myeloid cells during maturation (Brungs et al., 1995). It was found that the about 40- to 60-fold induction of 5-LO mRNA in Mono Mac 6 cells cultured with TGFβ and $1,25(OH)_2D_3$ was mainly to due posttranscriptional events (Härle et al., 1998). Both TGFβ and $1,25(OH)_2D_3$ were required for these effects. Nuclear $1,25(OH)_2D_3$ signalling is generally mediated via binding of the $1,25(OH)_2D_3$/VDR/RXR complex to VDREs in gene promoters (for review, see (Carlberg, 2003; Carlberg and Polly, 1998)).

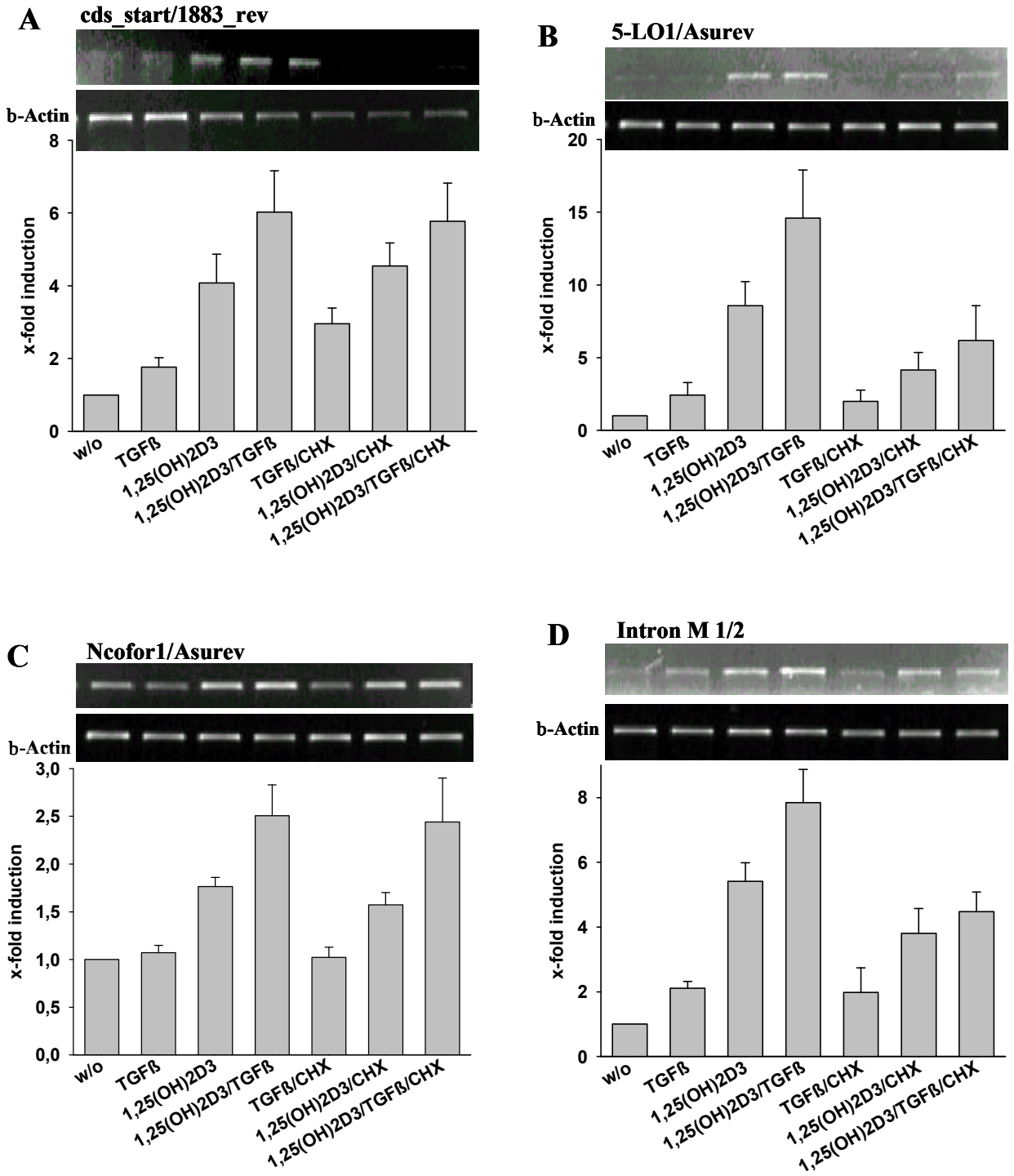

FIG. 6**. Effects of cycloheximide on the induction of mRNA transcripts by 1,25(OH)$_2$D$_3$ and TGFb.** Hela cells were transiently transfected with the luciferase reporter gene construct pN10-cdsInJM and preincubated as described in the legend to fig. 5. After transfection, cyloheximide (10 µM) was added together with 1,25(OH)$_2$D$_3$ (50 nM) and/or TGFβ (1 ng/ml). Then, RT-PCR analysis was performed as described in "Materials and Methods" with the indicated primer pairs. Values are expressed as mean ± SE of at least three independent experiments.

However, in reporter gene assays no significant transcriptional activation of the 5-LO promoter (pN10) by 1,25(OH)$_2$D$_3$ and TGFβ was observed. From these results we concluded that the strong induction of 5-LO mRNA expression by 1,25(OH)$_2$D$_3$ and TGFβ might be mediated by regulatory elements located outside of the 5-LO promoter. In fact, we could demonstrate with reporter gene assays, that induction of reporter gene activity by 1,25(OH)$_2$D$_3$ and TGFβ depends on the 5-LO coding sequence (fig. 4). This upregulation was independent of the promoter (5-LO, CMV, no promoter) indicating that at least under the in-vitro conditions of reporter gene assays, the 5-LO promoter is not required for the 1,25(OH)$_2$D$_3$ and TGFβ effect.

On the other hand, the induction of reporter gene activity mediated by the coding sequence depended on the coexpression of VDR/RXR which indicates that the vitamin D receptor is involved in signalling. Coexpression of VDR/RXR was also required for vitamin D signalling in our control experiments, i.e. the stimulation of reporter gene activity of the p(DR3)4tkluc plasmid that contains a four times concatemerized DR3 vitamin D response element in front of the thymidine kinase promoter. However, there was a significant difference in the reporter gene assays between classical vitamin D signalling and the induction of reporter gene activity mediated by the coding sequence. Whereas coexpression of VDR/RXR did not affect reporter gene activity of the p(DR3)4tkluc plasmid in the absence of 1,25(OH)$_2$D$_3$ and TGFβ (fig. 3), it led to an up to 6-fold stimulation of luciferase activity of vector constructs containing the 5-LO coding sequence. This suggests that there is a

considerable ligand-independent activity of the vitamin D receptor regarding the induction of reporter gene activity mediated by the 5-LO coding sequence. Inclusion of the last four introns (J, K, L and M) even enhanced the inducibility of the reporter gene constructs.

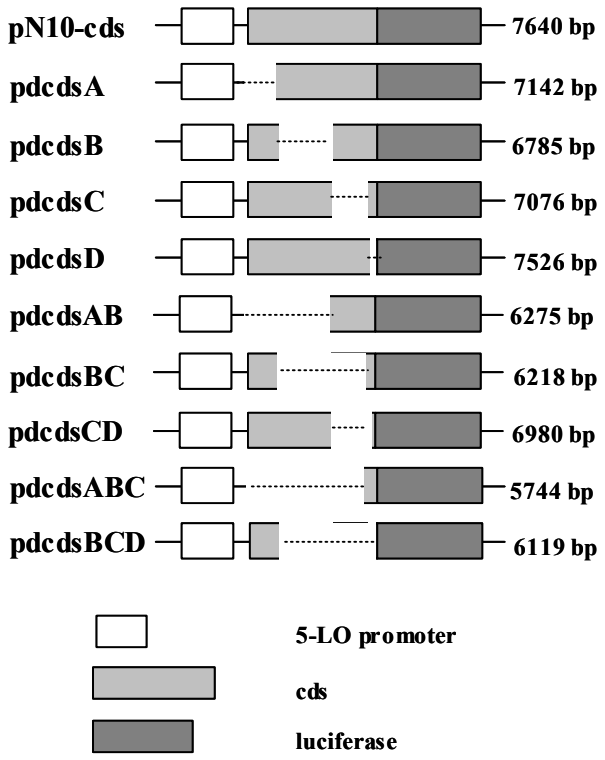

FIG. 7**. Overview of coding sequence deletion plasmids.**

As it was found before in Mono Mac 6 cells, $1,25(OH)_2D_3$ and TGFβ induce 5-LO mRNA expression mainly by posttranscriptional mechanisms (Härle et al., 1998). RT-PCR data obtained with Mono Mac 6 cells (fig. 1) gave a prominent induction of mature 5-LO mRNA but not pre-mRNA by $1,25(OH)_2D_3$/TGFβ, which suggests enhanced mRNA processing as mechanism of 5-LO upregulation. This conclusion is also supported by the fact that in reporter gene assays, there is an enhanced inducibility of plasmids containing intronic sequences by $1,25(OH)_2D_3$/TGFβ suggesting that splicing processes are involved. Another possible mechanism could be transcriptional arrest mediated by the coding sequence that is overcome by $1,25(OH)_2D_3$ and TGFβ. However, such a mechanism is rather unlikely because RT-PCR data show that there is only low expression of the middle part of the 5-LO coding sequence but not of the 3'-end. Furthermore, inducibility by $1,25(OH)_2D_3$/TGFβ was rather low with the pN10-cdsInJM-inv plasmid which contains the 5-LO coding sequence after the luciferase reporter gene. However, a transcriptional stop site should also be operative under these conditions.

It can only be speculated, if the first (very long) introns of the 5-LO gene also participate in its regulation. It was not possible to include these introns in the reporter gene experiments because of their huge size. Thus, it is very well possible that these introns also participate in the regulation of the expression of the 5-LO gene and that there are differences in the mechanisms that contribute to the observed $1,25(OH)_2D_3$/TGFβ effects on 5-LO expression in Mono Mac 6 cells and in our reporter gene assays.

The partial inhibition of the $1,25(OH)_2D_3$/TGFβ effects by cycloheximide suggest that the response to both agents requires at least in part protein biosynthesis. Thus, it is possible that $1,25(OH)_2D_3$/TGFβ induce the biosynthesis of proteins involved in 5-LO mRNA splicing and/or processing. This would explain the observed induction of luciferase activity without concomitant increases on the mRNA level in the reporter gene assays. In our constructs, the 5-LO and luciferase coding sequences are merged in frame so that translation of the transcribed RNA yields a 5-LO-luciferase fusion protein. Thus, aberrant RNA processing that leads to a frame shift in the 5-LO coding region prevents correct translation of the luciferase. Consequently, induction of correct 5-LO RNA processing by $1,25(OH)_2D_3$/TGFβ generates correctly translated luciferase protein and subsequent activity.

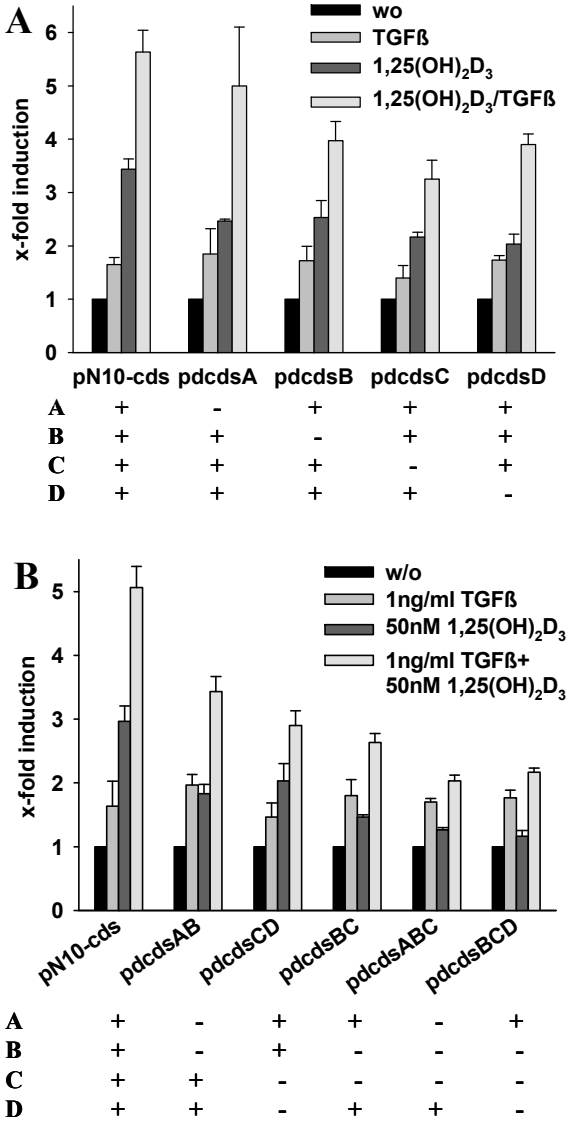

FIG. 8**. Effects of $1,25(OH)_2D_3$ and TGFb on the transcriptional activity of coding sequence deletion constructs in transiently transfected HeLa cells.** The indicated pN10-cds deletion constructs were transiently transfected into HeLa cells together with the expession vectors pSG5-hVDR and pSG5-hRXR. Before transfection, cells were preincubated with TGFβ (1 ng/ml) for 24 h. 16 h after transfection, $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml) were added for another 8 h and reporter gene activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to untreated cells.

Interestingly, nuclear receptors such as VDR have recently been shown to be involved in coupling of transcription to splicing (Zhang et al., 2003) (for review, see (Fong and Zhou, 2001; Maniatis and Reed, 2002)). Generally, the presence of introns in the transcription unit was shown to activate gene expression in yeast and mice (Fong and Zhou, 2001). Moreover, $1,25(OH)_2D_3$, retinoic acid and TGFβ have been described as stimuli of alternative splicing events (Magnuson et al., 1991) (for review, see (Akker et al., 2001)). Thus, our results with the 5-LO coding sequence clearly support the concept that mRNA processing/splicing is involved in the regulation of gene expression by $1,25(OH)_2D_3$/TGFβ.

RT-PCR analysis of the transcripts generated during the reporter gene assays was difficult to perform when the region between exon 8 and 13 was amplified. More cycles were necessary (Tab. 1) and in some PCR reactions multiple products were detected. It was not

possible to amplify the whole coding sequence, at least three definite bands that were smaller than expected where obtained. Sequencing a 450 bp PCR product solely allowed to verify the last 100 bp of exon 14. The sequencing data further upstream could not be interpreted due to superimposing of different sequences.

Taken together, it seems that in untreated Hela cells, 5-LO mRNA processing occurs that removes parts of the coding sequence. Our data suggest that $1,25(OH)_2D_3$/TGFβ induce the generation of more complete transcripts. Coding sequence deletion studies (figs. 7 and 8) revealed that the respective deletion of parts A to D reduced induction of reporter gene activity by $1,25(OH)_2D_3$/TGFβ (fig. 8). In constructs in which larger parts of the coding sequence are deleted (pdcdsABC and pdcdsBCD), the $1,25(OH)_2D_3$-mediated induction of reporter gene activity was almost completely lost and only the minor TGFβ-mediated enhancement could be measured. The fact that deletion of large parts of the coding sequence is required in order to achieve an almost complete loss of the response to $1,25(OH)_2D_3$/TGFβ suggests that the regulation of the 5-LO mRNA processing by both agents involves multiple parts of the coding sequence.

Taken together, our results provide clear evidence that the 5-LO coding region mediates the upregulation of 5-LO expression by $1,25(OH)_2D_3$ and TGFβ. These effects do not seem to be mediated by classical VDREs in the coding sequence. Our data suggest that both mediators regulate 5-LO mRNA processing which represents a novel mechanism of regulation of gene expression by $1,25(OH)_2D_3$ and TGFβ.

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REFERENCES

Akker, S.A., P.J. Smith, and S.L. Chew. 2001. Nuclear post-transcriptional control of gene expression. *J. Mol. Endocrinol.* 27:123-131.

Brungs, M., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1994. On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGF beta. *Biochem. Biophys. Res. Commun.* 205:1572-1580.

Brungs, M., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1995. Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25- dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U S A*. 92:107-111.

Carlberg, C. 2003. Molecular basis of the selective activity of vitamin D analogues. *J. Cell. Biochem.* 88:274-281.

Carlberg, C., and P. Polly. 1998. Gene regulation by vitamin D3. *Crit. Rev. Eukaryot. Gene Expr.* 8:19-42.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Fong, Y.W., and Q. Zhou. 2001. Stimulatory effect of splicing factors on transcriptional elongation. *Nature*. 414:929-933.

Härle, D., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1998. Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. *Eur. J. Biochem.* 254:275-281.

Härle, D., O. Rådmark, B. Samuelsson, and D. Steinhilber. 1999. Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Adv. Exp. Med. Biol.* 469:105-111.

Helgadottir, A., A. Manolescu, G. Thorleifsson, S. Gretarsdottir, H. Jonsdottir, U. Thorsteinsdottir, N.J. Samani, G. Gudmundsson, S.F. Grant, G. Thorgeirsson, S. Sveinbjornsdottir, E.M. Valdimarsson, S.E. Matthiasson, H. Johannsson, O. Gudmundsdottir, M.E. Gurney, J. Sainz, M. Thorhallsdottir, M. Andresdottir, M.L. Frigge, E.J. Topol, A. Kong, V. Gudnason, H. Hakonarson, J.R. Gulcher, and K. Stefansson. 2004. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat. Genet.* 36:233-239.

Klan, N., S. Seuter, N. Schnur, M. Jung, and D. Steinhilber. 2003. Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity. *Biol. Chem.* 384:777-785.

Lewis, R.A., K.F. Austen, and R.J. Soberman. 1990. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N. Engl. J. Med.* 323:645-655.

Magnuson, V.L., M. Young, D.G. Schattenberg, M.A. Mancini, D.L. Chen, B. Steffensen, and R.J. Klebe. 1991. The alternative splicing of fibronectin pre-mRNA is altered during aging and in response to growth factors. *J. Biol. Chem.* 266:14654-14662.

Maniatis, T., and R. Reed. 2002. An extensive network of coupling among gene expression machines. *Nature*. 416:499-506.

Martin, C.S., P.A. Wight, A. Dobretsova, and I. Bronstein. 1996. Dual luminescence-based reporter gene assay for luciferase and beta-galactosidase. *Biotechniques*. 21:520-524.

McColl, S.R., E. Krump, P.H. Naccache, P.E. Poubelle, P. Braquet, M. Braquet, and P. Borgeat. 1991. Granulocyte-macrophage colony-stimulating factor increases the synthesis of leukotriene B4 by human neutrophils in response to platelet-activating factor. Enhancement of both arachidonic acid availability and 5-lipoxygenase activation. *J. Immunol.* 146:1204-1211.

Mehrabian, M., and H. Allayee. 2003. 5-lipoxygenase and atherosclerosis. *Curr. Opin. Lipidol.* 14:447-457.

Murakami, M., K.F. Austen, C.O. Bingham, 3rd, D.S. Friend, J.F. Penrose, and J.P. Arm. 1995. Interleukin-3 regulates development of the 5-lipoxygenase/leukotriene C4 synthase pathway in mouse mast cells. *J. Biol. Chem.* 270:22653-22656.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Samuelsson, B., S.-E. Dahlén, J.-Å. Lindgren, C.A. Rouzer, and C.N. Serhan. 1987. Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science*. 237:1171-1176.

Steinhilber, D. 1999. 5-Lipoxygenase: a target for antiinflammatory drugs revisited. *Curr. Med. Chem.* 6:71-85.

Uhl, J., N. Klan, M. Rose, K.D. Entian, O. Werz, and D. Steinhilber. 2002. The 5-lipoxygenase promoter is regulated by DNA methylation. *J. Biol. Chem.* 277:4374-4379.

Werz, O., M. Brungs, and D. Steinhilber. 1996. Purification of transforming growth factor beta 1 from human platelets. *Pharmazie*. 51:893-896.

Yang, T.T., P. Sinai, P.A. Kitts, and S.R. Kain. 1997. Quantification of gene expression with a secreted alkaline phosphatase reporter system. *Biotechniques*. 23:1110-1114.

Zhang, C., D.R. Dowd, A. Staal, C. Gu, J.B. Lian, A.J. van Wijnen, G.S. Stein, and P.N. MacDonald. 2003. Nuclear coactivator-62 kDa/Ski-interacting protein is a nuclear matrix-associated coactivator that may couple vitamin D receptor-mediated transcription and RNA splicing. *J. Biol. Chem.* 278:35325-35336.

Zhang, Y.Y., O. Rådmark, and B. Samuelsson. 1992. Mutagenesis of some conserved residues in human 5-lipoxygenase: effects on enzyme activity. *Proc. Natl. Acad. Sci. U S A*. 89:485-489.

Ziegler-Heitbrock, H.W., E. Thiel, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller. 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer*. 41:456-461.

The coding sequence mediates induction of 5-lipoxygenase expression by Smads3/4

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5-Lipoxygenase (5-LO) expression is strongly induced by transforming growth factor-b (TGFb) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in Mono Mac 6 cells. Since Smad proteins have been described as downstream effectors of TGFb, we have investigated the role of the TGFb/Smad signalling system in the regulation of 5-LO gene expression. In transient reporter gene assays with plasmids containing the 5-LO promoter plus different parts of the gene Smads3/4 mediated a prominent upregulation of reporter activity that strongly depended on the coding sequence and to a lesser extent on the 3'-UTR and introns J to M.
Deletion of various parts of the 5-LO coding sequence reduced inducibility by Smads3/4, the decrease being most profound when exons 10 to 14 were deleted. Deletion studies indicated the existence of up to four functional Smad response elements in intron M and a TGFb responsive element in exon 10, that had been detected by sequence analysis.

Key Words: 5-lipoxygenase; coding sequence; transforming growth factor β; Smad3/4; gene expression; reporter gene assay.

Arachidonate 5-lipoxygenase (5-LO)[1], the key enzyme in the biosynthesis of proinflammatory leukotrienes, is expressed in a variety of immune competent cells including B-lymphocytes, granulocytes, monocytes, mast cells and dendritic cells [1]. Leukotrienes have been shown to be involved in inflammatory reactions and recent data suggest that the 5-lipoxygenase pathway plays a role in the development of cardiovascular diseases [2, 3]. Expression of 5-LO is regulated on transcriptional and posttranscriptional levels. Basal 5-LO promoter activity seems to be regulated by DNA methylation and it has been shown that DNA methylation of the GC-rich core region strongly suppresses 5-LO promoter activity. Furthermore, the 5-LO promoter was found to be methylated in 5-LO negative cell lines whereas it is completely demethylated in 5-LO positive cells [4].

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[1]The abbreviations used are: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; cds, coding sequence; 5-LO, 5-lipoxygenase; luc, luciferase; RE, responsive element; RXR, retinoid X receptor; SBE, Smad binding element; SEAP, secreted alkaline phosphatase; TGFβ, transforming growth factor β; TRE, TGFβ responsive element; UTR, untranslated region; VDR, vitamin D receptor; VDRE, vitamin D responsive element.

Depending on the cell type, several cytokines have been shown to be inducers of the 5-LO pathway. In granulocytes, 5-LO expression is stimulated by GM-CSF [5] whereas IL-3 regulates the development of the 5-LO pathway in mouse mast cells [6]. 5-LO activity, protein expression and mRNA are dramatically increased during differentiation of myeloid cell lines such as Mono Mac 6 and HL-60. Transforming growth factor-beta (TGFβ) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) were identified as potent inducers of 5-LO gene expression [7, 8]. In Mono Mac 6 cells, treatment with TGFβ and $1,25(OH)_2D_3$ led to an up to 5-fold increase in primary transcripts, a 64-fold increase in mature 5-LO mRNA, a 128-fold increase in protein expression and a more than 500-fold increase in 5-LO activity [9]. The upregulation of 5-LO mRNA expression partially depended on protein synthesis. This strong increase in 5-LO mRNA expression does not seem to be mediated by the promoter since TGFβ and $1,25(OH)_2D_3$ had no effect on 5-LO transcription in nuclear run-off assays using nuclear extracts from Mono Mac 6 cells [9] and on 5-LO promoter activity in reporter gene assays in different cell types [4].
Since Smad proteins have been described as downstream effectors of TGFβ, we further investigated the role of TGFβ/Smad signalling in 5-LO gene regulation by reporter gene analyses. Sequence analyses and deletion studies led to the identification of several functional TGFβ and Smad3/4 response elements within the 5-LO gene.

MATERIALS AND METHODS

Reagents. Molecular biology reagents were from MBI Fermentas, Sigma, Invitrogen, Promega or other sources as indicated in the text. Insulin was a gift from Aventis. Human TGFβ1 was purified from outdated platelets according to [10]. Nucleospin Extract columns for direct purification of pDNA were from Macherey-Nagel (Düren, Germany). The plasmid p(DR3)4tkluc and the pSG5-hVDR and pSG5-hRXR expression plasmids for the human vitamin D receptor (VDR) and retinoid X receptor alpha (RXRα), were obtained from Dr. Carsten Carlberg (Kuopio, Finland). The vector p3TP-Lux was provided by Dr. Joan Massagué (New York, USA). The pCGN-Smad3 and pCGN-Smad4 expression plasmids were obtained from Dr. X.F. Wang (Durham, USA).

Cells and cell culture. HeLa cells were obtained from Dr. W.E. Müller (Pharmacological Institute, Biocenter, Frankfurt) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (vol/vol) FCS, 100 µg/ml streptomycin and 100 units/ml penicillin. Mono Mac 6 cells, which were kindly provided by Dr. H.W.L. Ziegler-Heitbrock (Munich), were grown at 37 °C in a humified atmosphere with 6 % CO_2 in RPMI 1640 medium supplemented with 10 % (vol/vol) fetal calf serum (FCS), streptomycin (100 µg/ml), penicillin (100 U/ml), 1× nonessential amino acids, sodium pyruvate (1 mM), oxalacetate (1 mM) and insulin (10 µg/ml) [11].

Plasmid constructs. 5-LO promoter reporter gene contructs were prepared using restriction enzymes and PCR methods.
The 3'-UTR of the 5-LO gene was obtained by PCR amplification using random hexamer-primed cDNA from Mono Mac 6 cells as a template and the primers
5'-GTATTCTAGATGAGCACACTGCCAGTCTCA-3' and 5'-CGGCTCTAGACTGATTTATTTTATGGCAAC-3' and *Pfu I* DNA polymerase. Following digestion of the PCR fragment and the plasmid

pN10 (-778 to +53 bp) [12] with *Xba I,* insert and pDNA were ligated using T4 DNA ligase (pN10-3UTR).
5-LO coding sequence (cds) was amplified using the plasmid pT3-5LO [13] as a template and the primers 5'-CCCTCCTACACGGTCACCGTG-3' and 5'-ATGGCCACACTGTTCGGAATCC-3' and *Pfu I* DNA polymerase. pN10-3UTR was digested with *Nco I*, blunted using T4 DNA polymerase and ligated with the PCR fragment using T4 DNA ligase (pN10-cds3UTR).
The part of the 5-LO coding sequence containing the last four introns (introns J, K, L and M) was obtained by PCR amplification using genomic DNA from Mono Mac 6 cells as a template and the primers 5'-TATGCCTCCCTGTGCTTTCCCGA-3' and
5'-AGGTTCTTGCGGAATCGGGCCA-3' and *Pfu I* DNA polymerase. The fragment was digested with *Sfi I* and partially with *Nco I* (besides the *Nco I* site used for cloning there is an additional one in intron M). pN10-cds3UTR was digested with *Sfi I* and *Nco I*. Insert and pDNA were ligated using T4 DNA ligase (pN10-cdsInJM3UTR).
Contructs pN10-cds and pN10-cdsInJM were obtained by deleting the 3'-UTR via digestion of pN10-cds3UTR and pN10-cdsInJM3UTR, respectively, with *Xba I* and religation.
Plasmid pN10-cdsInJM-inv was obtained by cloning of the *Xho I*-framed fragment cdsInJM from pN10-cdsInJM into the *Sal I* site of pN10.
Contructs pGl3-ba-cdsInJM and pGL3-prom-cdsInJM were prepared using plasmids pGL3-Basic and pGL3-Promoter (both from Promega), respectively, and restriction fragments obtained from pN10-cdsInJM using *Xba I* and *Bgl II* or *Xba I* and *Hind III* (partial).
Sequential deletion of the coding sequence divided in four parts lead to the pN10-cds deletion constructs pdcdsA, pdcdsB, pdcdsC, pdcdsD, pdcdsAB, pdcdsBC, pdcdsCD, pdcdsABC and pdcdsBCD. Deletions were performed using PCR mutation and restriction enzymes. Deletions of putative response elements leading to plasmids pdTGFRE1, pdTGFRE2, pdTGFRE1+2 and pdSBE2 were performed by substitution of mutated PCR fragments with the native sequences.
Plasmid sequences were confirmed by DNA sequencing.

Transient transfections. 24 h prior to transfection, HeLa cells were plated into a 24-well tissue culture plate at a density of 5×10^4 cells per well. TGFâ was added for a 24 h-preincubation. Transient transfections were performed with the standard calcium phosphate method, adding 800 ng of reporter plasmid, 100 ng of different expression plasmids and 20 ng pCMV-SEAP as internal standard per well. After 16 h the calcium phosphate-DNA precipitate was removed, new cell culture medium added and incubated for another 8 h.

Luciferase assays. Cells were transfected as indicated, harvested and lysed in 100 µl lysis buffer (Steady-Glo^{TM} Luciferase Assay System, Promega). Luciferase activity was determined by monitoring light emission with a Microlumat Plus LB96V EG&G Berthold luminometer [14]. The light emission signal was integrated for 5 seconds. Transfection efficiency was monitored and normalised by cotransfection of pCMV-SEAP using the Phospha-Light TM kit (Tropix) to determine the secreted placental alkaline phosphatase (SEAP) activity [15].

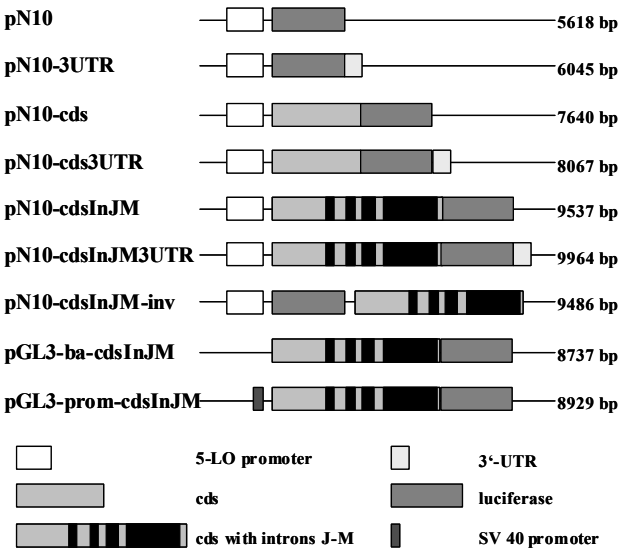

FIG. 1. **Overview of luciferase reporter gene plasmids.**

RESULTS

Effects of TGFb on 5-LO reporter gene activity. HeLa cells were transiently transfected with luciferase reporter gene constructs (for overview see fig. 1) in the absence of VDR/RXR and Smad3/Smad4 expression constructs. The constructs were cloned in such a way that the 5-LO coding sequence was in frame to the luciferase sequence. Cells were preincubated with or without TGFâ (1 ng/ml) for 24 h. After transfection, cells were grown with or without $1,25(OH)_2D_3$ (50 nM), TGFβ (1 ng/ml) for another 8 h. TGFβ led to an about 1.5-fold induction of reporter gene activity with the control pGL3-Basic (fig. 2). A similar induction was observed with pN10-3UTR and pN10-cdsINJM-inv. Interestingly, slightly increased effects (about 2-fold induction) were observed with plasmids that contain the 5-LO coding sequence (pN10cds, pN10-cdsInJM, pGL3-ba-cdsInJM and pGL3-prom-cdsInJM). With all plasmids tested, $1,25(OH)_2D_3$ alone did not significantly enhance transcriptional activity under these experimental conditions, i.e. in the absence of cotransfected VDR/RXR. A slight induction of transcriptional activity was obtained with the p(DR3)4tkluc plasmid when $1,25(OH)_2D_3$ and TGFβ were combined.

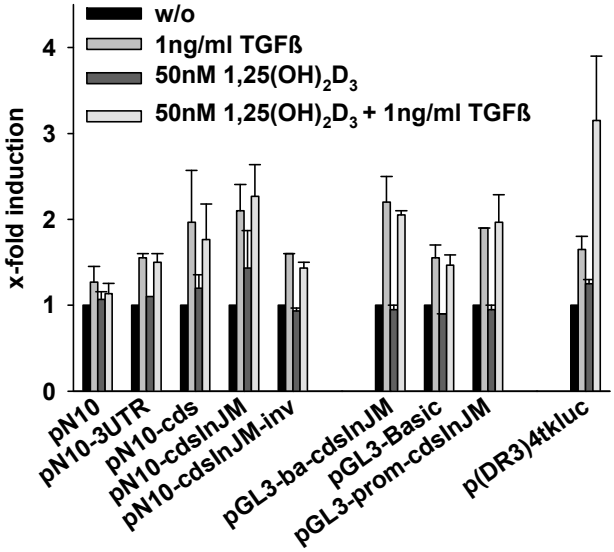

FIG. 2. **Effects of TGFb /1,25(OH)$_2$D$_3$ on the transcriptional activity of luciferase reporter gene constructs in transiently transfected HeLa cells.** The indicated plasmids were transiently transfected into HeLa cells. p(DR3)4tkluc containing a four times concatemerized DR3-type vitamin D response element in front of the thymidine kinase promoter was used as a positive control. Before transfection, cells were preincubated with TGFβ (1 ng/ml) for 24 h. 16 h after transfection, $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml) were added for another 8 h before reporter gene activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to untreated cells.

Smad3/Smad4 strongly induce reporter gene activity when the 5-LO coding sequence is present. HeLa cells were transiently transfected with luciferase reporter gene constructs (fig. 1) and cotransfected with either pCGN-Smad3 and pCGN-Smad4 or an empty cytomegalovirus (CMV) promoter construct as control. Coexpression of Smads increased reporter gene activity obtained with pN10 (containing the 5-LO core promoter, –778 to +53 bp) by 3.7-fold. Interestingly, the TGFβ-responsive plasmid p3TP-Lux, carrying three TGFβ tetradecanoyl phorbol acetate response elements from the PAI-1 gene, was only induced by 1.6-fold which was comparable to the negative control pGL3-Basic (2.2-fold). The effects of the Smad proteins on 5-LO gene expression was strongly enhanced by the presence of the coding sequence (fig. 3). Induction of pN10-cds and of pN10-cdsInJM (containing also introns J-M) was 17.5-fold and 24.0-fold, respectively. Interestingly, the upregulation of the 3'-UTR-containing plasmid pN10-3UTR was significantly higher than with pN10 (8.8-fold versus 3.7-fold) but inclusion of the 3'-UTR did not further enhance the induction of the constructs containing the coding sequence (pN10-cds3UTR and pN10-cdsInJM3UTR). When the 5-LO coding and intronic sequences were inserted behind the luciferase stop codon (pN10-

cdsInJM-inv) there was a strong reduction of the upregulation by cotransfection of Smad3/4 to pN10 levels (3.1-fold). Induction of reporter gene activity by Smad3/Smad4 also occurred with pGL3-ba-cdsInJM and pGL3-prom-InJM containing no and a SV 40 promoter, respectively, which suggests that the Smad3/4 effects do not depend on the 5-LO promoter.

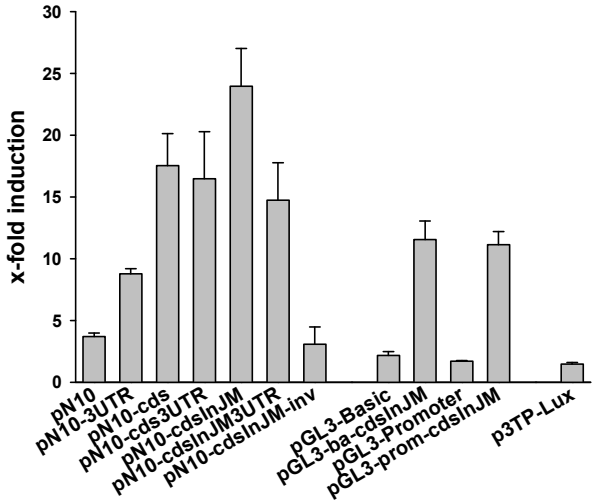

FIG. 3. **Effects of Smads3/4 on the transcriptional activity of luciferase reporter gene constructs in transiently transfected HeLa cells.** The indicated reporter gene plasmids were transiently transfected into HeLa cells with or without the expression vectors pCGN-Smad3 and pCGN-Smad4. Reporter gene activity was measured 24 h after transfection. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to pCMV-cotransfected cells.

TGFb effects are only marginally enhanced by Smad3/Smad4. In the absence of coexpressed Smads, TGFβ enhanced reporter gene activity by 1.5- to 2.7-fold for 5-LO promoter constructs and up to 4.1-fold for the positive control p3TP-Lux (fig. 4). Interestingly, in cells that were cotransfected with Smad3 and Smad4 there was no significantly altered response to TGFβ indicating that the TGFβ effects are independent of Smad overexpression.

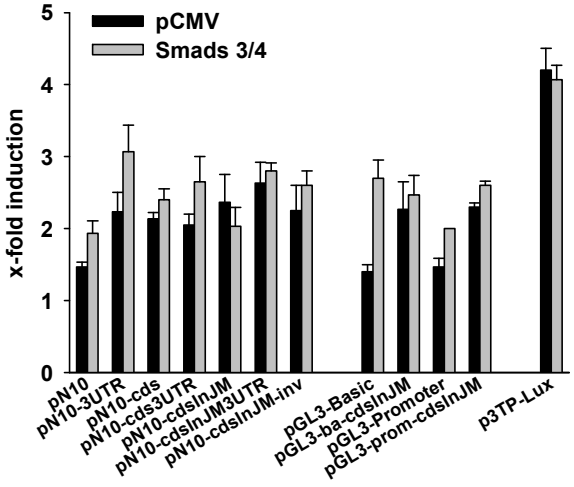

FIG. 4. **Effects of TGFb on the transcriptional activity of luciferase reporter gene constructs in transiently transfected HeLa cells.** The indicated reporter gene plasmids and the TGFβ-responsive construct p3TP-Lux were transiently transfected into HeLa cells with or without the expression vectors pCGN-Smad3 and pCGN-Smad4. Before transfection, cells were preincubated with TGFβ (1 ng/ml) for 24 h. 16 h after transfection, TGFβ (1 ng/ml) was added for another 8 h before luciferase activity was measured. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments and were normalized for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to uninduced reporter activity.

Synergism between RXR/VDR and Smad3/Smad4. Cotransfection of expression plasmids for VDR/RXR and/or Smad proteins 3/4 profoundly enhanced absolute reporter gene activity (fig. 5). In cells cotransfected with VDR/RXR, addition of TGFβ or $1,25(OH)_2D_3$ upregulated reporter gene activity and both ligands displayed a synergistic effect. In cells cotransfected with Smads only TGFβ enhanced reporter gene activity whereas $1,25(OH)_2D_3$ was without effect. In cells cotransfected with Smads and VDR/RXR, reporter gene activity was higher than in the cells transfected with either Smads or VDR/RXR alone. As expected, addition of either $1,25(OH)_2D_3$ or TGFβ increased reporter gene activity and combination of both agents had a synergistic effect (fig. 5). Interestingly, coexpression of Smads and VDR/RXR increased reporter gene activity by 12.6-fold, whereas the ligand-mediated inductions by $1,25(OH)_2D_3$ or TGFβ and the combination of both agents were 2.4-, 2.6- and 5.9-fold, respectively, suggesting a remarkable constitutive activity of Smads and VDR/RXR, respectively.

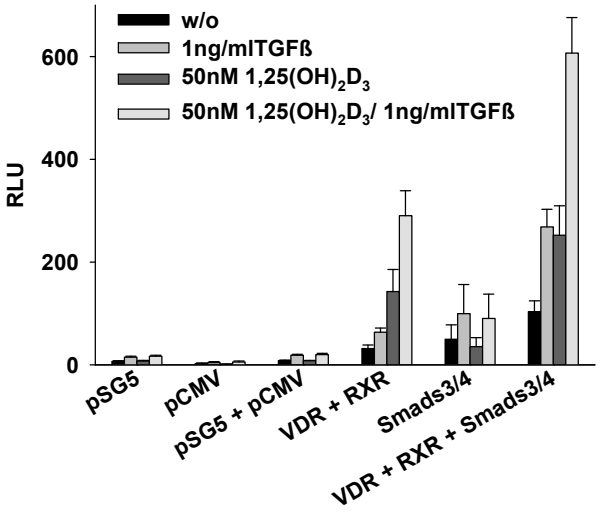

FIG. 5. **Simultaneous cotransfection of VDR/RXR and Smads3/4 in transiently transfected HeLa cells.** The 5-LO promoter reporter gene construct pN10-cdsInJM was transiently transfected into HeLa cells with or without the expression vectors pSG5-hVDR/pSG5-hRXR and pCGN-Smad3/-Smad4. Before transfection, cells were preincubated with TGFβ (1 ng/ml) for 24 h. 16 h after transfection, $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml) were added for another 8 h. Reporter gene activity was measured 24 h after transfection. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments and were normalized for transfection efficiency by cotransfection of pCMV-SEAP.

Identification of sequence elements with significance for Smad signalling in 5-LO gene regulation. In order to identify sequences in the coding region and introns that participate in Smad3/4 and TGFâ-mediated enhancement of 5-LO expression, deletion constructs from the vector pN10-cds were cloned and analyzed in reporter gene assays. The coding sequence was divided into four parts which were deleted. Part A ranges from exon 1 to 4, part B from exon 4 to 10, part C from exon 10 to 14 and part D corresponds to the end of exon 14. As shown in fig. 6A deletion of parts A, B or D reduced Smad3/4-mediated induction of 5-LO expression from 14.7-fold (pN10-cds) to 8.8-, 8.6- and 9.0-fold, respectively. A prominent decrease to 3.9-fold was observed, when part C was deleted. Comparably, the 5-LO promoter plasmid pN10 gives a 3.7-fold upregulation of 5-LO gene expression by Smad3/4 coexpression. Deletion of two or three parts of the coding sequence rather decreased the inhibitory effect (fig. 6B). In contrast to the Smad3/4 mediated effects, transcriptional activation by TGFâ was not affected by the deletion of parts of the coding region (figs. 7A+B).

Sequence analysis revealed the existence of several putative Smad binding elements (SBEs) in the 5-LO coding sequence and introns. Deletion of a 286 bp sequence in intron M from the vector pN10-cdsInJM comprising 4 putative SBEs (fig. 8A) led to a construct which is less responsive to coexpression of Smad proteins

(pdSBE2 10.4-fold, pN10-cdsInJM 18.4-fold) (fig. 8B), suggesting that these SBEs could be involved in the regulation of 5-LO mRNA expression by Smads. Again, the TGFâ-mediated upregulation of 5-LO expression was not altered by the deletion (fig. 8C).
Furthermore, the two most promising putative TGFâ responsive elements (TREs), located in exon 10 and intron J, were deleted from plasmid pN10-cdsInJM (fig. 8A). Deletion of TRE 1 significantly reduced the Smad-mediated enhancement of 5-LO expression from 16.2- to 6.7-fold (fig. 8B), whereas deletion of TRE 2 or of both led to no marked changes. The TGFâ-mediated transcriptional activation of 5-LO was not affected by either deletion (fig. 8C).

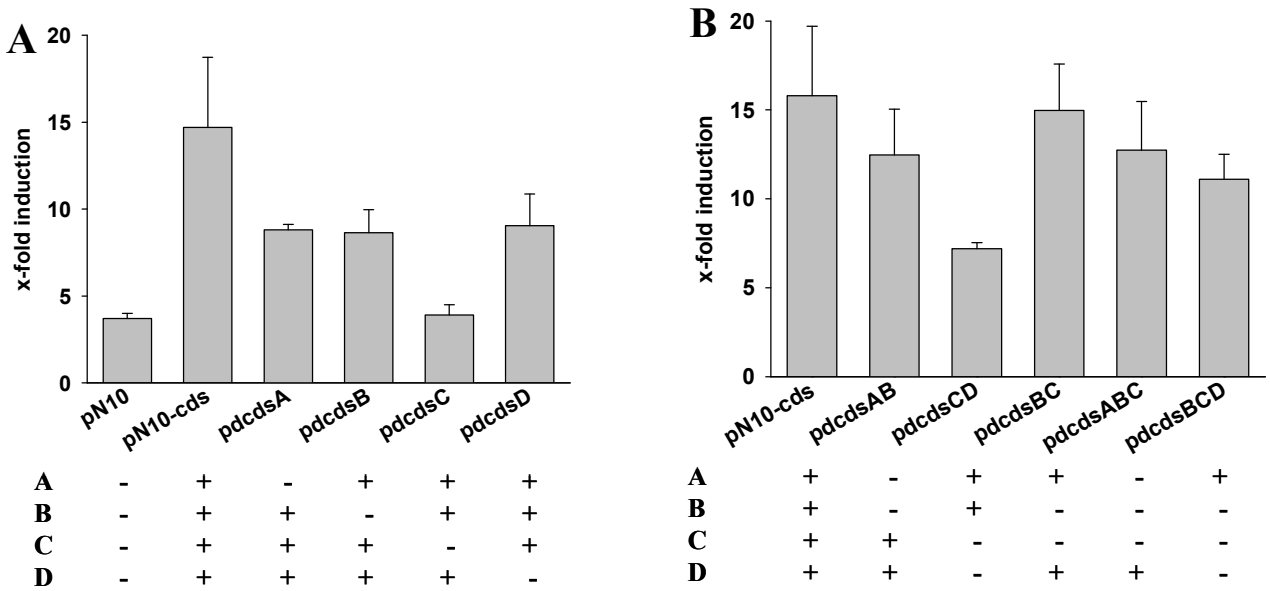

FIG. 6. **Effects of Smads3/4 on the transcriptional activity of coding sequence deletion plasmids in transiently transfected HeLa cells.** pN10-cds deletion constructs (A. Single deletions, B. double/triple deletions) were transiently transfected into HeLa cells with or without the expression vectors pCGN-Smad3 and pCGN-Smad4. Reporter gene activity was measured 24 h after transfection. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments and were normalized for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to pCMV-cotransfected cells.

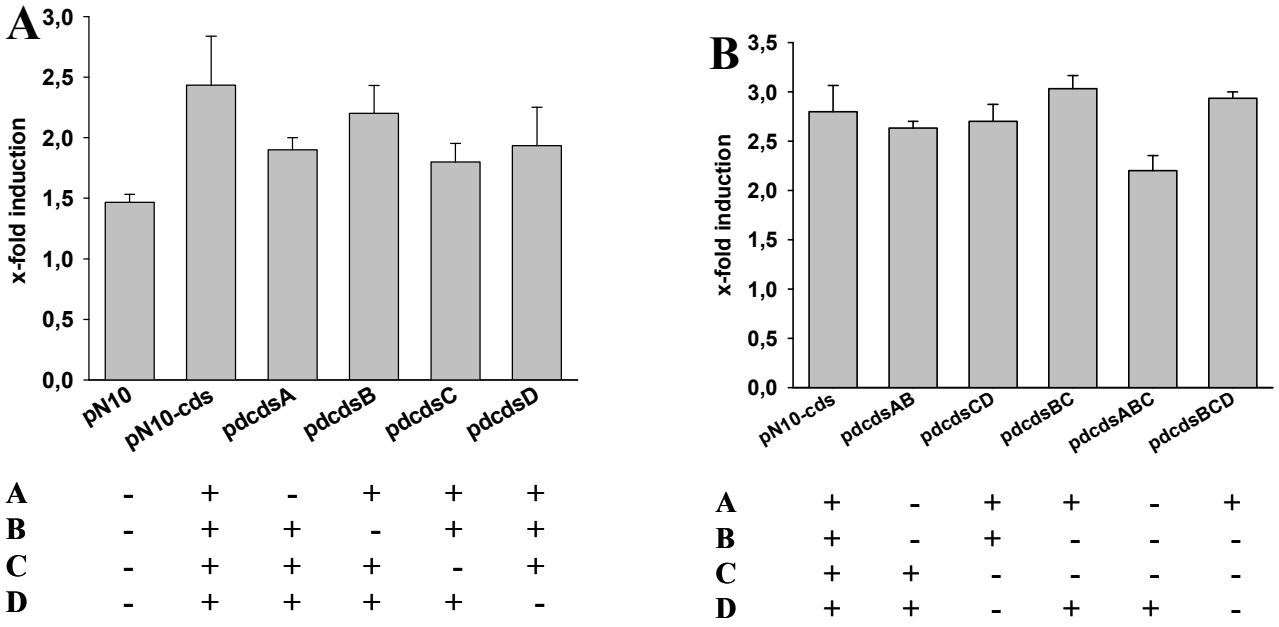

FIG. 7. **Effect of TGFb on the transcriptional activity of coding sequence deletion plasmids in transiently transfected HeLa cells.** pN10-cds deletion constructs (A. Single deletions, B. double/triple deletions) were transiently transfected into HeLa cells together with the expression vectors pCGN-Smad3 and pCGN-Smad4. Before transfection cells were preincubated with TGFβ (1 ng/ml) for 24 h. 16 h after transfection, TGFβ (1 ng/ml) was added for another 8 h before luciferase activity was measured. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments and were normalized for transfection efficiency by cotransfection of pCMV-SEAP. Inductions are expressed with respect to uninduced reporter activity.

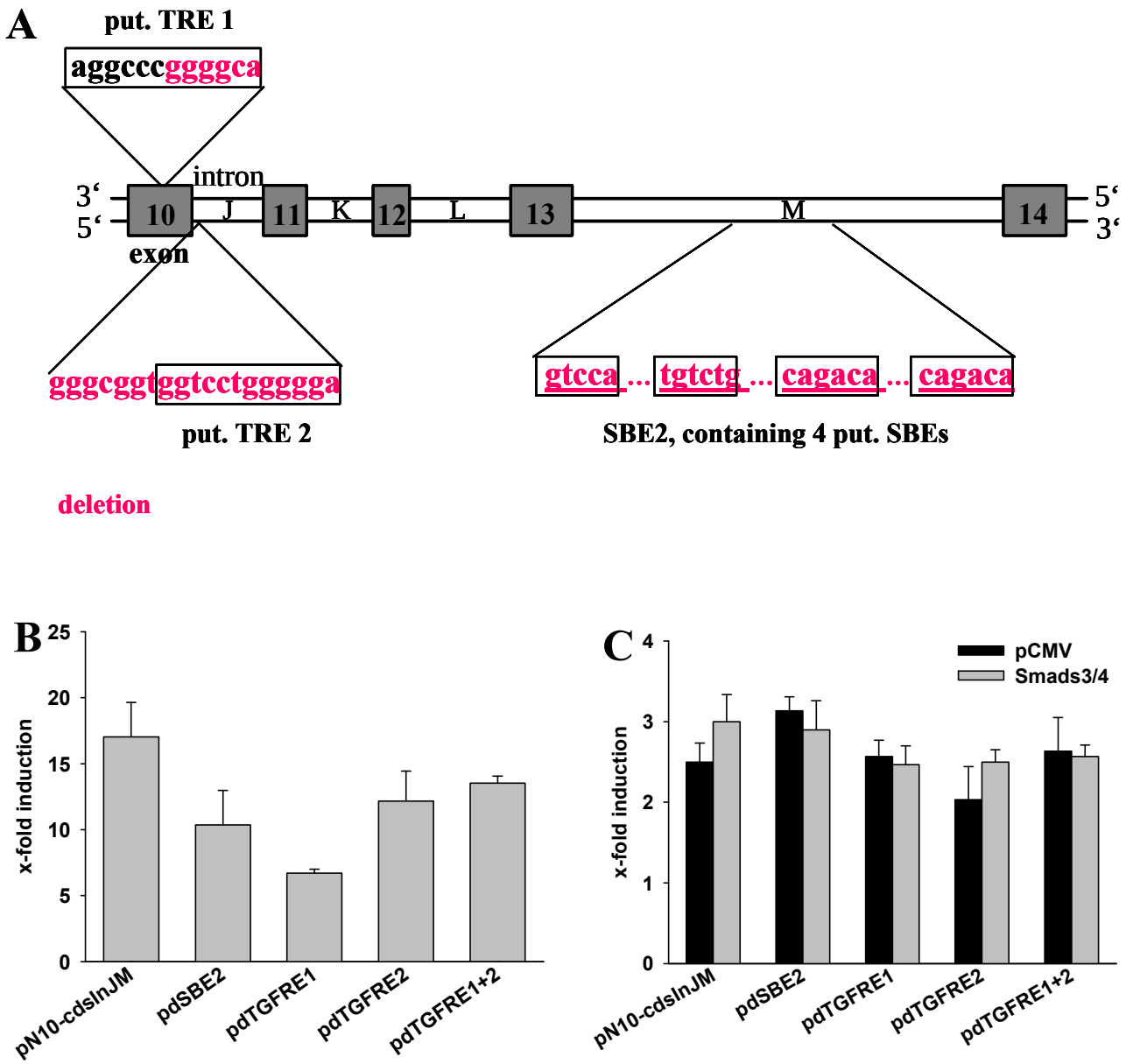

FIG. 8. **Influence of deletion of four putative SBEs and two putative TREs on TGFb- and Smad3/4- mediated 5-LO upregulation.** A) Schematic presentation of the mutated plasmid constructs. Deletion of a 286 bp fragment in intron M, containing four putative SBEs yielded pdSBE2. Two putative TREs, located in exon 10 and intron J were deleted from the plasmid pN10-cdsInJM in pTGFRE1 and pTGFRE2, respectively. B) Effects of Smad coexpression. HeLa cells were transiently transfected with the indicated plasmids together with or without the expression vectors pCGN-Smad3 and pCGN-Smad4. C) Effects of TGFβ. Cells were preincubated with or without TGFβ (1 ng/ml) for 24 h. 16 h after transfection, TGFβ (1 ng/ml) was added for another 8 h, before luciferase activity was measured. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments. Luciferase activity was normalized for transfection efficiency by cotransfection of pCMV-SEAP and determination of SEAP activity.

DISCUSSION

Previous reports have established a prominent role for $1,25(OH)_2D_3$ and TGFβ in the upregulation of 5-LO gene expression in myeloid cells during maturation [7, 8]. It was found that the about 40- to 60-fold induction of 5-LO mRNA in Mono Mac 6 cells cultured with TGFβ and $1,25(OH)_2D_3$ was due to both increased 5-LO gene transcription (about 5-fold) and to stimulated transcript elongation and maturation [16]. Both TGFβ and $1,25(OH)_2D_3$ were required for these effects.

In this study we further investigated the role of TGFβ and Smad signalling on 5-LO gene regulation. Having in mind that Smads have been identified as downstream effectors in TGFβ signalling (for review, see [17]), cotransfection studies with the receptor-regulated Smad3 and the cooperating Smad4 were performed. We found that coexpression of Smads strongly enhanced reporter gene activity in reporter gene constructs containing the 5-LO coding sequence. Interestingly, this effect did not depend on the 5-LO promoter and was also observed with the respective pGL3-basic and pGL3-promoter plasmids pGL3-ba-cdsInJM and pGL3-prom-cdsInJM.

Furthermore, the strong coding sequence-dependent induction of reporter gene activity was only observed with Smads but not with TGFβ as stimulus, indicating that this effect mainly depends on Smads but not on TGFβ signalling in general.

Deletion of exon 10 to exon 14 (part C) of the 5-LO coding sequence abolished the Smad effect suggesting that this part contains essential response elements required for Smad signalling. Deletion of other parts of the coding sequence also reduced inducibility by Smad3/4 which points to the existence of additional response elements in the other parts of the coding sequence.

In silico analysis of the 5-LO gene revealed putative SBEs within the promoter as well as in the 3'-UTR, the coding region and in particular in the region from exon 10 to 14. Interestingly, deletion of a Smad consensus sequence in exon 10 strongly reduced response to Smad3/4 coexpression. Additionally, reduction of the Smad effects was also observed when a short DNA stretch within intron M was deleted that contains 4 putative Smad binding elements suggesting that these elements could be involved in the regulation of 5-LO expression by the TGFβ/Smad pathway. Interestingly, coexpression of Smads did not significantly alter the response to TGFβ (figs. 4, 7A+B, 8C) and deletion of various parts of the coding sequence did not significantly change the effects of TGFβ on reporter gene activity. Furthermore, compared to Smads, the TGFβ-mediated effect is rather low and does not differ between the different plasmids and related deletion constructs in the reporter gene assys. The data suggest, that at least under the experimental conditions of the reporter gene assays, the cellular availability and maybe the nuclear localization of Smad proteins seems to be the limiting and critical parameter in the upregulation of reporter gene activity.

As found before for the regulation of cellular 5-LO mRNA expression in the human monocytic cell line Mono Mac 6, there is a synergistic effect between TGFβ/Smad and vitamin D signalling (fig. 5). Thus, the highest reporter gene activity was obtained when VDR/RXR and Smads were cotransfected and when TGFβ and $1,25(OH)_2D_3$ were added. Interaction between the TGFβ and $1,25(OH)_2D_3$ signalling pathways has been described. The VDR has been shown to physically and functionally interact with Smad3 and Smad7 in vitro and in vivo [18-20]. The affinity of Smads to SBEs is rather weak. Hence, probably additional DNA contacts via binding partners such as the VDR are required [17]. In another publication, multiple mechanisms responsible for TGFβ/$1,25(OH)_2D_3$ crosstalk have been reported: ligand-binding to the VDR induces TGFβ mRNA expression, the VDR enhances TGFβ receptor II expression in certain cell types, Smad3 activates the VDR and the crosstalk is dependent on the PI3-kinase pathway [18]. Our experiments suggest that 5-LO is another example for a gene where Smads and the VDR interact and activate transcription in concert.

In summary, our results provide evidence for the participation of the Smad signalling pathway in 5-LO gene regulation. Furthermore, we could demonstrate the involvement of the coding sequence in Smad signalling and identified several Smad binding elements located in exon 10 and intron M that seem to be relevant for the regulation of 5-LO expression by Smads. Further experiments will be required to prove binding of Smads to these sites.

Acknowledgements. We thank Astrid Brüggerhoff for expert technical assistance.

REFERENCES

[1] D. Steinhilber, Curr. Med. Chem. 6 (1999) 71-85.
[2] M. Mehrabian and H. Allayee, Curr. Opin. Lipidol. 14 (2003) 447-457.
[3] A. Helgadottir, A. Manolescu, G. Thorleifsson, S. Gretarsdottir, H. Jonsdottir, U. Thorsteinsdottir, N.J. Samani, G. Gudmundsson, S.F. Grant, G. Thorgeirsson, S. Sveinbjornsdottir, E.M. Valdimarsson, S.E. Matthiasson, H. Johannsson, O. Gudmundsdottir, M.E. Gurney, J. Sainz, M. Thorhallsdottir, M. Andresdottir, M.L. Frigge, E.J. Topol, A. Kong, V. Gudnason, H. Hakonarson, J.R. Gulcher and K. Stefansson, Nat. Genet. 36 (2004) 233-239.
[4] J. Uhl, N. Klan, M. Rose, K.D. Entian, O. Werz and D. Steinhilber, J. Biol. Chem. 277 (2002) 4374-4379.
[5] S.R. McColl, E. Krump, P.H. Naccache, P.E. Poubelle, P. Braquet, M. Braquet and P. Borgeat, J. Immunol. 146 (1991) 1204-1211.
[6] M. Murakami, K.F. Austen, C.O. Bingham, 3rd, D.S. Friend, J.F. Penrose and J.P. Arm, J. Biol. Chem. 270 (1995) 22653-22656.
[7] M. Brungs, O. Rådmark, B. Samuelsson and D. Steinhilber, Proc. Natl. Acad. Sci. U S A 92 (1995) 107-111.
[8] M. Brungs, O. Rådmark, B. Samuelsson and D. Steinhilber, Biochem. Biophys. Res. Commun. 205 (1994) 1572-1580.
[9] D. Härle, O. Rådmark, B. Samuelsson and D. Steinhilber, Adv. Exp. Med. Biol. 469 (1999) 105-111.
[10] O. Werz, M. Brungs and D. Steinhilber, Pharmazie 51 (1996) 893-896.
[11] H.W. Ziegler-Heitbrock, E. Thiel, A. Futterer, V. Herzog, A. Wirtz and G. Riethmuller, Int. J. Cancer 41 (1988) 456-461.
[12] N. Klan, S. Seuter, N. Schnur, M. Jung and D. Steinhilber, Biol. Chem. 384 (2003) 777-785.
[13] Y.Y. Zhang, O. Rådmark and B. Samuelsson, Proc. Natl. Acad. Sci. U S A 89 (1992) 485-489.
[14] C.S. Martin, P.A. Wight, A. Dobretsova and I. Bronstein, Biotechniques 21 (1996) 520-524.
[15] T.T. Yang, P. Sinai, P.A. Kitts and S.R. Kain, Biotechniques 23 (1997) 1110-1114.
[16] D. Härle, O. Rådmark, B. Samuelsson and D. Steinhilber, Eur. J. Biochem. 254 (1998) 275-281.
[17] J. Massagué, Nat. Rev. Mol. Cell Biol. 1 (2000) 169-178.
[18] L. Yang, J. Yang, S. Venkateswarlu, T. Ko and M.G. Brattain, J. Cell. Physiol. 188 (2001) 383-393.
[19] Y. Yanagi, M. Suzawa, M. Kawabata, K. Miyazono, J. Yanagisawa and S. Kato, J. Biol. Chem. 274 (1999) 12971-12974.
[20] J.K. Aschenbrenner, H.W. Sollinger, B.N. Becker and D.A. Hullett, J. Surg. Res. 100 (2001) 171-175.

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Trichostatin A and Structurally Related Histone Deacetylase Inhibitors Induce 5-Lipoxygenase Promoter Activity

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5-Lipoxygenase (5-LO) mRNA expression in Mono Mac 6 cells is induced by the histone deacetylase inhibitor trichostatin A (TsA). In order to study the effects of TsA and several structurally related compounds such as MD85, D237 and M232 on 5-LO promoter activity, we have analyzed the response of a 5-lipoxygenase (5-LO) promoter luciferase reporter gene construct to histone deacetylase inhibitors in transiently transfected Mono Mac 6 and HeLa cells. We show that the activity of 5-LO promoter constructs comprising the sequences – 778 to + 53 and of several successive deletions of the 5-LO promoter is strongly increased upon TsA treatment. The data suggest a significant involvement of histone deacetylases in the regulation of 5-LO gene transcription. The basal activity of the 5-LO promoter strongly depends on the presence of multiple Sp1-binding sites (GC-boxes), five of which are positioned in tandem. Deletion of the five tandemized GC-boxes in the 5-LO reporter gene construct revealed that the induction of 5-LO promoter activity by TsA seems to be independent of these GC-boxes. Methylation of 5-LO reporter gene constructs by M.*Hpa*II reduced 5-LO promoter activity but did not prevent induction of promoter activity by TsA, although the activated reporter gene activities were lower compared to the unmethylated plasmid, indicating the dominance of methylation over TsA-sensitive histone deacetylation in silencing of the 5-LO gene. The structure-activity data obtained for histone deacetylase inhibitors suggest that this assay system might serve as a cellular screening tool for the development of HDAC inhibitors.

Key words: DNA methylation / Histone deacetylase / 5-Lipoxygenase / Mono Mac 6 cells / Trichostatin A.

Introduction

5-Lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid to leukotriene A_4 [(5S)-6-oxido-7,9,11, *trans*-14-*cis*-eicosatetraenoic acid] which can be further converted to the biologically active leukotrienes that are mediators of inflammatory and allergic reactions (Samuelsson *et al.*, 1987; Ford-Hutchinson *et al.*, 1994). 5-LO expression occurs in a tissue- and cell-specific manner; a variety of immunecompetent cells including B lymphocytes, granulocytes, monocytes, mast cells and dendritic cells prominently express the 5-LO gene (Steinhilber, 1999).

Previous studies of the 5-LO promoter suggested that the transcription factors Egr-1 and/or Sp1 are required for basal transcription, and that they functionally interact with the 5-LO promoter and activate it *via* repeated response elements located between positions – 147 and – 23 bp relative to the transcriptional start site (Hoshiko *et al.*, 1990; Silverman *et al.*, 1998). Interestingly, naturally occurring mutations were found in the 5-LO promoter consisting of the deletion of one or two, or the addition of one Sp1-binding site (In *et al.*, 1997). These mutations only slightly alter 5-LO promoter activity in reporter gene assays but have a significant impact on the response of asthma patients to 5-LO inhibitors (Drazen *et al.*, 1999). Recently, we could show that the 5-LO promoter is regulated by DNA methylation, and methylation of CpG sites within the 5-LO promoter strongly reduces its activity (Uhl *et al.*, 2002). Since recent publications suggest a link between DNA methylation and histone acetylation, it was of interest to investigate whether changes in histone acetylation affect 5-LO promoter activity. The histone acetylation status is regulated by the equilibrium of histone acetyltransferase (HAT) and histone deacetylase activity (HDAC). Acetylation of the N-terminal region of the core histones by HATs alters nucleosome and higher order chromatin structure, facilitating transcriptional elongation and the binding of transcription factors to nucleosomes associated with regulatory DNA sequences (Davie, 1997). Recently, it was shown for 15-LO that its expression is increased by HDAC inhibitors (Kamitani *et al.*, 2001). In this study, the effects of HDAC inhibitors like trichostatin A (TsA) were investigated on cellular 5-LO mRNA expression in Mono Mac 6 cells and on 5-LO promoter activity after transient transfection of several 5-LO promoter luciferase reporter gene constructs into HeLa and Mono Mac 6 cells and subsequent determination of luciferase activity. 5-LO promoter-reporter gene constructs with or without the five tandem-

ized Sp1 binding sites were used for this study since recent studies indicated the involvement of multiple Sp1 binding sites in the transcriptional activation of other genes like Gα12 and WAF/Cip1 by HDAC inhibitors like sodium butyrate, trichostatin A or HC toxin (Nakano *et al.*, 1997; Yang *et al.*, 2001).

Previously, transforming growth factor β (TGFβ) and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] have been identified as strong inducers of 5-LO gene expression in the myeloid cell lines HL-60 and Mono Mac 6 (Brungs *et al.*, 1994, 1995; Härle *et al.*, 1998, 1999). Participation of histone acetylation in gene regulation by steroid hormones has become increasingly evident (Beato *et al.*, 1996) and TsA has been shown to synergistically enhance cell differentiation by 1,25$(OH)_2D_3$ and retinoic acid (Kosugi *et al.*, 1999). Therefore, it was of interest to study effects of the vitamin D receptor (VDR) and its interacting partner, the retinoid X receptor (RXR), on 5-LO promoter activity in the presence or absence of the ligand 1,25$(OH)_2D_3$ and TGFβ in combination with TsA.

Finally, we present a series of inhibitors of HDAC. These compounds were synthesized recently (Jung *et al.*, 1999) and are structurally related to the potent inhibitors trapoxin B and trichostatin A. We investigated structure-activity relationships (SARs) of several analogs for inhibition of HDAC in order to check whether the induction of 5-LO promoter activity by HDAC inhibitors may serve as a cellular screening system for potential inhibitors of histone deacetylase.

Results

TSA Induces 5-LO mRNA Expression in Mono Mac 6 Cells

In order to study the effects of TsA on 5-LO mRNA expression, Mono Mac 6 cells were cultured with TsA (330 nM) and/or TGFβ (1 ng/ml) and 1,25$(OH)_2D_3$ (50 nM) for 24 h. The cells were harvested, total RNA was extracted and reverse transcribed into cDNA. 5-LO and β-actin expression was analyzed by PCR employing 28 and 24 cycles, respectively (Brungs *et al.*, 1995). TsA alone lead to an about 11-fold increase in 5-LO mRNA expression, whereas induction by 1,25$(OH)_2D_3$/TGFβ was 43-fold (compare Brungs *et al.*, 1995) (Figure 1). Interestingly, combination of TsA with 1,25$(OH)_2D_3$/TGFβ did not increase the effect of 1,25$(OH)_2D_3$/TGFβ but rather inhibited (62%) the effect of both agents and lead to a 16-fold increase in 5-LO mRNA compared to the control. Thus, TsA alone strongly increases 5-LO mRNA expression in Mono Mac 6 cells but does not enhance the induction of 5-LO mRNA expression by 1,25$(OH)_2D_3$/TGFβ.

TsA Strongly Induces 5-LO Promoter Activity in Transiently Transfected Mono Mac 6 and HeLa cells

To determine the effects of histone acetylation on transactivation of the 5-LO gene promoter, TsA, a potent in-

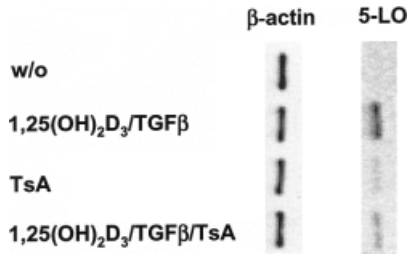

Fig. 1 Induction of 5-LO mRNA Expression in Mono Mac 6 Cells.
Cells were cultured for 24 h in the presence or absence of TsA (330 nM), TGFβ (1 ng/ml) and 1,25$(OH)_2D_3$ (50 nM). Cells were then harvested and total RNA was extracted (Chomczynski and Sacchi, 1987), transcribed into cDNA and analyzed by PCR as described (Brungs *et al.*, 1995). Twenty-four and 28 PCR cycles were used for β-actin and 5-LO amplification, respectively.

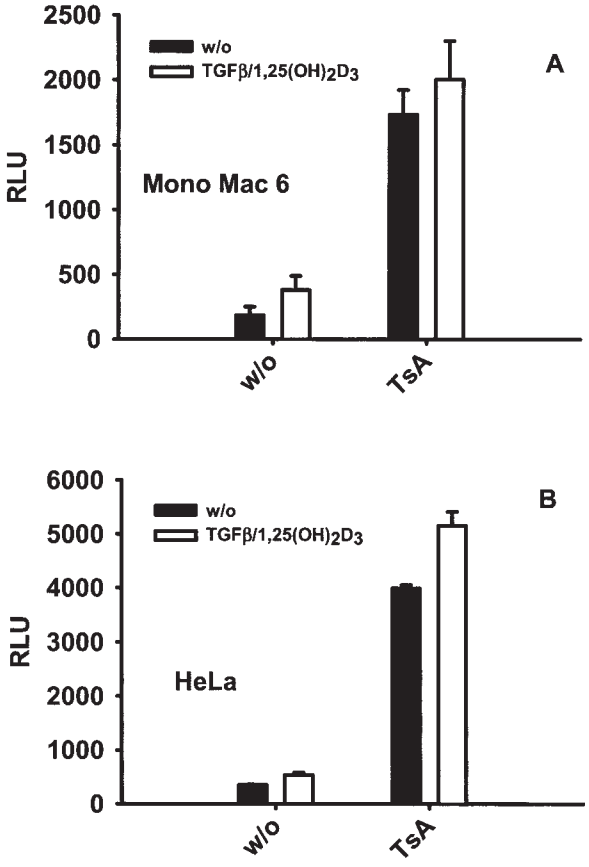

Fig. 2 Induction of 5-LO Promoter Reporter Gene Transcription by TsA.
Mono Mac 6 cells (A) and HeLa cells (B) were transfected with the 5-LO promoter luciferase reporter gene construct pN10 and the expression vectors pSG5hRXR and pSG5hVDR by electroporation and lipofection, respectively. 1,25$(OH)_2D_3$ (50 nM), TGFβ (1 ng/ml) and TsA (330 nM) were added immediately after transfection as indicated. After 6 h (Mono Mac 6) and 24 h (HeLa), cells were harvested and luciferase activity was determined. Results are presented as mean values ± SEM of triplicate transfections. Results were normalized for transfection efficiency by co-transfection of pCMVSEAP.

hibitor of histone deacetylases, was added to Mono Mac 6 and HeLa cells immediately after transfection with the 5-LO promoter/luciferase reporter construct pN10 (comprising the sequence −778 bp to +53 bp relative to the transcriptional start site) or with the pGL3Basic reporter plasmid, which lacks the promoter region as a negative control. When the expression vectors for the receptors VDR and RXR were cotransfected, TsA (330 nM) strongly potentiated 5-LO promoter-driven transcription 11-fold in HeLa cells, and 8-fold in Mono Mac 6 cells (Figure 2). In the absence of the receptor expression vectors, TsA (330 nM) induced the 5-LO promoter activity by 25- and 5-fold in HeLa and Mono Mac 6 cells, respectively (data not shown). Transcription from the promoterless pGL3Basic vector was unaffected by TsA in HeLa and Mono Mac 6 cell lines.

5-LO Promoter Activity Is Independent of $1,25(OH)_2D_3$/TGFβ in Reporter Gene Assays

No increase in 5-LO promoter activity could be seen for any construct in reporter gene assays in response to $1,25(OH)_2D_3$ and TGFβ in Mono Mac 6 and HeLa cells, both with or without TsA and in the presence (Figure 2) or absence (data not shown) of the expression vectors for VDR/RXR.

Interestingly, when the p(DR3)4tkluc plasmid containing a 4-times concatemerized VDRE in front of the thymidine kinase promoter was transfected together with VDR and RXR, transcription from this plasmid increased up to 34-fold (Mono Mac 6) and 8-fold (HeLa) after addition of TGFβ and $1,25(OH)_2D_3$. In the absence of the receptor expression vectors for VDR/RXR, induction of reporter gene activity by $1,25(OH)_2D_3$ and TGFβ was only 2.3-fold in Mono Mac 6 cells and 1.6-fold in HeLa cells (data not shown) which indicates the requirement of coexpression of both receptors in order to observe strong vitamin D effects under the experimental conditions of our reporter gene assays.

Interestingly, TsA (330 nM) potentiated the effects of $1,25(OH)_2D_3$/TGFβ on transcription from p(DR3)4tkluc (Figure 3). Compared to the untreated control, addition of TsA to HeLa cells led to a 5.5-fold induction of promoter activity and the combination of TsA with $1,25(OH)_2D_3$/TGFβ resulted in a 73-fold increase which was 12-fold higher than the promoter activity obtained with TsA alone.

TsA-Mediated Induction of 5-LO Promoter Activity Does Not Depend on the Five Tandemized GC-Boxes

In order to determine whether the induction of 5-LO promoter activity by TsA is dependent on the presence of the GC-rich region required for basal transcription (Uhl *et al.*, 2002), the constructs pN10 (comprising the region −778 bp to +53 bp containing five Sp1 sites in tandem, termed GC-boxes) and the deletion constructs pN11-16 (Figure 4B) were transfected into HeLa cells and luciferase activity was determined. Induction of reporter

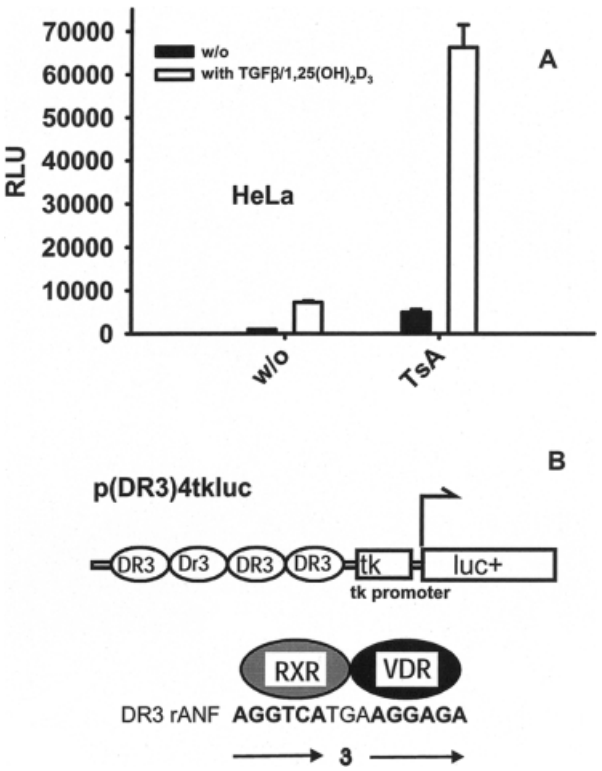

Fig. 3 Effects of TsA and $1,25(OH)_2D_3$/TGFβ on the Transcriptional Activity of p(DR3)4tkluc.
(A) HeLa cells were transfected with p(DR3)4tkluc and the expression vectors pSG5hRXR and pSG5hVDR by lipofection. After transfection, TsA (330 nM), $1,25(OH)_2D_3$ and TGFβ (50 nM and 1 ng/ml, respectively) were added as indicated and reporter gene activity was determined after 24 h. Results are presented as mean ± SEM of triplicate transfections, and were normalized for transfection efficiency by cotransfection of pCMVSEAP.
(B) Schematic representation of the luciferase reporter gene construct p(DR3)4tkluc with the direct repeat type (DR3) vitamin D response element (VDRE) of the rANF promoter in front of a thymidine kinase promoter.

gene activity by TsA was observed with plasmids pN10 to pN13, thus indicating an effect that seems to be independent of the GC-boxes since pN13 lacks the five tandemized GC-boxes. In HeLa cells, transcription from pGC0 that also lacks the GC box sequences from −111 bp to −78 bp was also induced 3.5-fold compared to the untreated control (data not shown). Further deletion of the 5-LO promoter (pN14, pN15, pN16) led to the loss of TsA inducibility. This could be due to the lack of a functional promoter which is in agreement with the very low transcriptional activity of these plasmids. In contrast, the absolute transcriptional activity of the construct pN13, containing sequences −78 bp to +53 bp and lacking the five Sp1 binding sites, was still 15-fold and 60-fold higher than the activity of the promoterless vector (pGL3Basic) in HeLa and Mono Mac 6 cells, respectively.

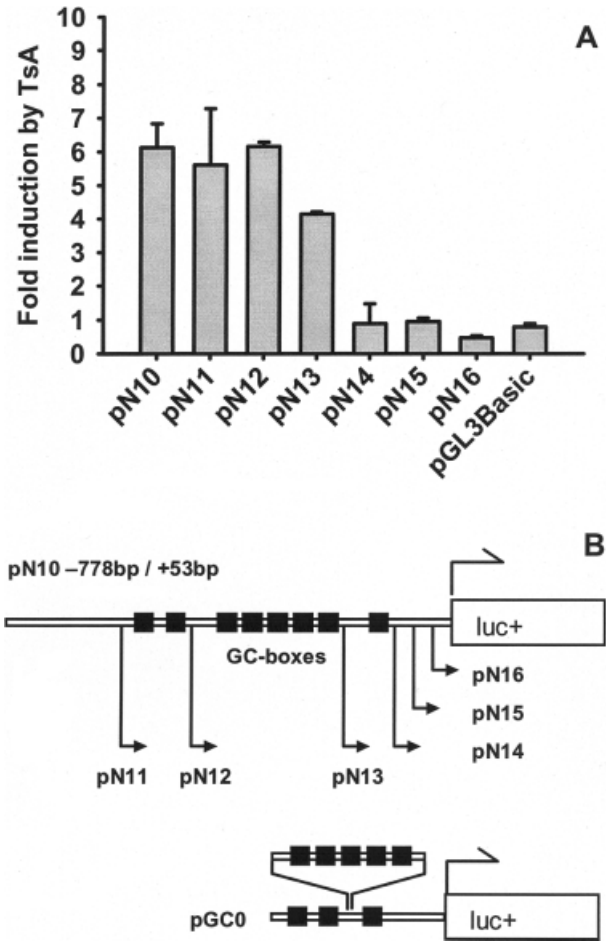

Fig. 4 Effect of Deletion of the Five Tandemized GC-Boxes on the Induction of 5-LO Promoter Activity by TsA.
(A) HeLa cells were transfected by the calcium phosphate precipitation method with the indicated promoter luciferase reporter gene constructs together with receptor expression vectors pSG5hRXR and pSG5hVDR. Immediately after transfection, TsA was added and the cells were incubated for 24 h prior to analysis of luciferase activity. Luciferase activity was normalized to transfection efficiency and is presented as the relative increase in promoter activity induced by TsA. The activity obtained in the absence of TsA was set to 1. Results are presented as mean ± SEM of triplicate transfections.
(B) Schematic representation of the 5-LO promoter luciferase reporter gene constructs.

DNA Methylation Dominates over Histone Acetylation in the Regulation of 5-LO Promoter Activity

Next, we performed transient transfection experiments using *in vitro* methylated plasmid DNA (pDNA). pDNA of the construct pN10 was methylated *in vitro* at CCGG residues by *Hpa*II methyltransferase (MTase), purified and transfected into HeLa cells by lipofection. *Hpa*II MTase methylates the sites –453 bp, –75 bp, –56 bp, –45 bp, –39 bp and +35 bp in relation to the 5-LO transcriptional start site, but does not methylate the GC-boxes in the 5-LO promoter. Methylation decreased the transcriptional activity of the 5-LO promoter reporter gene construct pN10 3.8-fold (Figure 5A), whereas a CMV promoter-driven luciferase reporter gene construct or the promoterless vector pGL3Basic were not affected (Figure 5B). Inhibition of 5-LO promoter activity by methylation was lower (1.7-fold) in the presence of TsA (330 nM). Thus, with the unmethylated plasmid, TsA gave a 12-fold induction of promoter activity, whereas a 27-fold increase by TsA was observed when the plasmid is methylated (Figure 5A). Furthermore, no effect of $1,25(OH)_2D_3$/TGFβ was observed under the indicated assay conditions. These data suggest that TsA treatment partially reversed methylation-dependent suppression of 5-LO promoter activity.

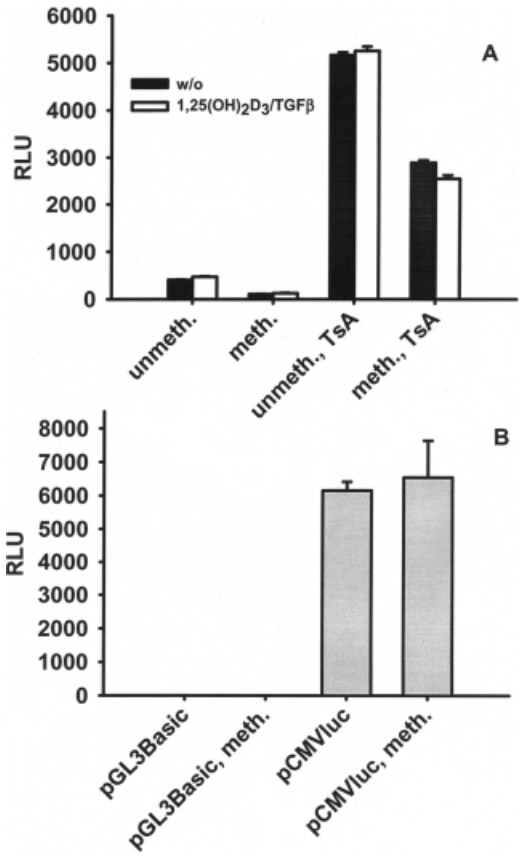

Fig. 5 Effect of CpG Methylation on 5-LO Promoter Activity.
(A) HeLa cells were transiently transfected with the 5-LO promoter luciferase reporter gene construct pN10, which was *in vitro* methylated by M.*Hpa*II or mock methylated. After transfection, cells were grown for 24 h with or without TGFβ (1 ng/ml) and $1,25(OH)_2D_3$ (50 nM).
(B) Effects of CpG methylation on pGL3Basic and pCMVluc reporter gene activity. Results are given as luciferase activity normalized to SEAP activity (mean ± SEM of at least three experiments).

Evaluation of TsA Analogs

Finally, our transient transfection system was used for the evaluation of TsA analogs and structurally related compounds without HDAC inhibitory activity (Hoffmann *et al.*, 2000). A pharmacophore for inhibitors of HDAC has been postulated, which is related to structural elements of the potent inhibitor TsA (Figure 6). Hydroxamic acids, such as TsA and the analogs MD85, D237 and M232, are potent HDAC inhibitors (Jung, 1997, 2001; Jung *et al.*, 1999; Remiszewski *et al.*, 2002). Since we found that TsA strongly induces 5-LO promoter reporter gene transcription, we wanted to use this assay to investigate the effects of these analogs. HeLa cells were transiently transfected with the 5-LO promoter luciferase reporter gene construct pN10 by lipofection and the cells were grown for 24 h with the indicated concentrations of the test compounds (Figure 7).

TsA, D237, MD85 and M232 showed prominent induction of transcriptional activity of pN10 in HeLa cells (Figure 7), whereas the compounds MD83A, M293 and M309 were inactive at 2.2 μM, 2 μM and 2.2 μM, respectively. TsA-mediated induction of pN10 luciferase reporter gene expression in HeLa cells was independent of the pDNA amount in the range of 0.1 – 10 μg (data not shown). In order to obtain EC_{50} values, HeLa cells transiently transfected with pN10 were incubated with the indicated concentrations of HDAC inhibitors (Figure 7). We found EC_{50} values of 37 nM for TsA and of 2.9, 5.0 and 0.3 μM for compounds M232, MD85 and D237, respectively. The obtained EC_{50} values (Figure 7) are in agreement with previous results in other assay systems (Table 1; Jung *et al.*, 1999; Hoffmann *et al.*, 2000; Remiszewski *et al.*, 2002).

Thus, inhibition of HDAC activity and induction of 5-LO promoter activity depends on the presence of the hydroxamic acid group and the length of the spacer between the hydroxamic acid group and the second amide

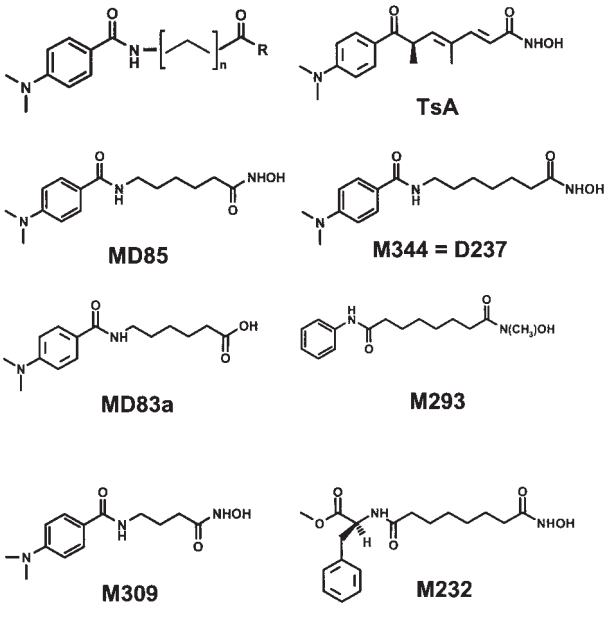

Fig. 6 Structures of HDAC Inhibitors.

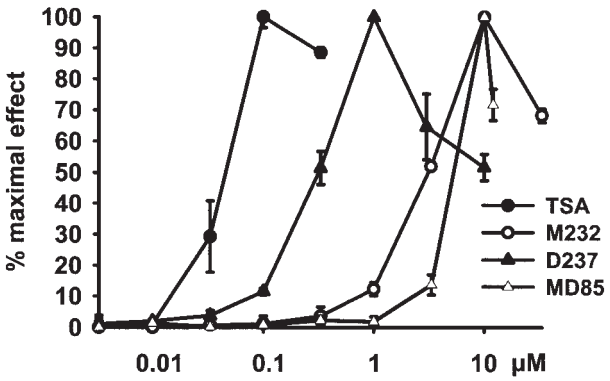

Fig. 7 Induction of 5-LO Promoter Activity by TsA Analogs. Concentration-dependent induction of pN10 luciferase reporter gene transcription was determined in transiently transfected HeLa cells after treatment of the cells with the indicated test compounds for 24 h. Results are given as mean ± SEM of at least three experiments.

bond with maximal activity at five and six methylene groups (compound MD85 and compounds M232 and D237, respectively; see Figure 6). The activity decreases drastically when the spacer becomes shorter than four methylene groups (M309), which is in agreement with previous results (Jung *et al.*, 1999). MD83a, the corresponding carboxylic acid derivative to MD85, was completely inactive.

Discussion

Analysis of the sequences immediately upstream from the transcription initiation site of 5-LO showed no TATAA or CCAAT boxes but repeated G+C-rich elements, such as repeated Sp1 and Egr-1 transcription factor binding sites located between positions – 147 bp and – 23 bp relative to the transcriptional start site. Previous data suggested that this part of the promoter, which binds the transcription factors Egr-1 and/or Sp1, is required for basal 5-LO transcription (Hoshiko *et al.*, 1990; Zhang *et al.*, 1994; In *et al.*, 1997; Silverman *et al.,* 1998; Silverman and Drazen, 2000). In this study, we wanted to investigate possible interactions between histone acetylation, DNA methylation and $1,25(OH)_2D_3$/TGFβ signalling in the regulation of 5-LO gene transcription.

In agreement to previous observations in nuclear run on assays (Härle *et al.*, 1999), no $1,25(OH)_2D_3$/TGFβ-dependent induction of 5-LO promoter activity was observed in reporter gene assays in HeLa and Mono Mac 6 cells, with or without cotransfection of expression vectors for the human receptors VDR and RXR. In contrast, in control experiments we detected a strong induction of the rANF type vitamin D response element (VDRE)-containing reporter gene construct p(DR3)4tkluc by $1,25(OH)_2D_3$/TGFβ that depended on the coexpression of VDR and RXR (Figure 3), indicating that our assay systems are suitable to detect transcriptional activation by these agents. These results suggest that induction of

Table 1 EC_{50} and IC_{50} Values of TsA and TsA Analogs in Different Assays.

Compound	Rat liver HDAC and fluorescent substrate (Hoffmann *et al.*, 2000)	Rat liver HDAC and labeled histones (Hoffmann *et al.*, 2000)	P21 promoter induction assay (Remiszewski *et al.*, 2002)	5-LO promoter transcription assay
TsA	0.0133 ± 0.001 μM	0.022 ± 0.002 μM	0.4 ± 0.3 μM	0.038 ± 0.0008 μM
M232	1.0000 ± 0.700 μM	13.400 ± 1.000 μM	–	2.900 ± 0.4000 μM
D237	–	–	2.1 μM	0.300 ± 0.0400 μM
MD85	0.4800 ± 0.020 μM	1.380 ± 0.040 μM	>10 μM	5.000 ± 0.2000 μM

5-LO gene expression by $1,25(OH)_2D_3$/TGFβ does not occur at the transcriptional level. This conclusion is in agreement with previous data from nuclear run-on assays and RT-PCR analyses (Härle *et al.*, 1998, 1999).

Addition of TsA to Mono Mac 6 cells induced 5-LO expression, and similar results were obtained in reporter gene assays with the 5-LO promoter. In the absence of $1,25(OH)_2D_3$/TGFβ, TsA increased transcription of the 5-LO reporter gene in HeLa and Mono Mac 6 cells with or without the coexpression of the receptors VDR and RXR (Figure 2). Thus, the TsA-dependent increase in 5-LO promoter activity was independent of the presence of $1,25(OH)_2D_3$/TGFβ and VDR/RXR coexpression.

In the case of the p(DR3)4tkluc construct, the $1,25(OH)_2D_3$/TGFβ-dependent induction of reporter gene transcription was potentiated by TsA, indicating a synergistic mechanism of vitamin D signal transduction and histone deacetylation (Figure 3), which is in agreement with previous observations made with other targets (Yanagi *et al.*, 1999; Massagué and Wotton, 2000; Rachez and Freedman, 2000). However, TsA induction of 5-LO promoter activity seems to be independent of VDR signaling.

Recent data obtained with human myeloid cell lines show that the basal 5-LO promoter activity is silenced by DNA methylation within the CpG-rich core promoter region (Uhl *et al.*, 2002). Furthermore, in the 5-LO negative myeloid cell lines U937 and HL-60 TB, 5-LO promoter demethylation is required in order to achieve high cellular induction of 5-LO expression by $1,25(OH)_2D_3$/TGFβ. Since methylated CpG sites are recognized by a variety of methyl-CpG-binding proteins that are associated either directly or indirectly with histone deacetylases (Ballestar and Wolffe, 2001), chromatin condensation could be one regulatory mechanism of 5-LO gene transcription (Ponton *et al.*, 1996; Ng *et al.*, 1999; Rice and Allis, 2001; Singal *et al.*, 2001). It has been shown that transcription from Sp1-driven promoters can be repressed by a tight association of the transcription factor Sp1 with histone deacetylase 1 in a multiprotein complex (Doetzlhofer *et al.*, 1999). However, with respect to 5-LO it is obvious that inhibition of HDACs by TsA and the subsequent induction of 5-LO promoter transcription does not depend on the presence of the five tandemized Sp1 and Egr-1 sites of the 5-LO proximal core promoter region, which were found to be essential for basal transcription in previous studies (Uhl *et al.*, 2002). These results suggest that there are binding sites for transcription factors outside of the five tandemized GC-boxes which can recruit histone deacetylases to the 5-LO promoter.

Interestingly, *in vitro* methylation of the 5-LO promoter reporter gene construct by M.*Hpa*II led to a prominent decrease in transcriptional activity, although the multiple CpG sites within the five tandemized GC-boxes are not methylated. In our transfection experiments, TsA treatment reduced methylation-dependent repression, indicating that recruitment of histone deacetylases could be involved. In addition, histone deacetylase-independent mechanisms of repression seem also to play a role, since the presence of TsA could not completely prevent the methylation-dependent repression. Thus, our data indicate the dominance of methylation *versus* TsA-sensitive histone deacetylation in silencing the 5-LO promoter, resulting in a trichostatin A-resistant repression (Boeke *et al.*, 2000; Yu *et al.*, 2000). This is similar to other reports which showed that transcriptionally silenced genes in cancer cell lines can be partially reactivated in the presence of a DNA methyltransferase inhibitor, but complete reactivation was not achieved (Singal and Ginder, 1999; Rice and Allis, 2001).

Taken together, our results support an active role of HDAC in transcriptional regulation of the 5-LO gene. Moreover, for the $1,25(OH)_2D_3$-inducible p(DR3)tkluc construct it was found that TsA and $1,25(OH)_2D_3$ are able to synergistically increase reporter gene activity in transient reporter gene assays.

The results obtained with the TsA analogs in our transient transfection experiments with the 5-LO promoter reporter gene in HeLa cells are similar to previously reported potencies of these histone deacetylase inhibitors obtained with other assay systems. We could confirm a clear dependence of the inhibitory activity on the spacer length with maximal inhibition at 5 – 6 methylene groups, which points to a competition with an acetyl-lysine. The strong difference between hydroxamates and the carboxylates can be explained by differences in affinity for the zinc ion at the active site of histone deacetylases (Finnin *et al.*, 1999). The inhibitors of HDAC presented here were previously shown to induce histone hyperacetylation in leukemic cells, which correlated with their ability to induce terminal cell differentiaton (Jung *et al.*, 1999). Since the obtained EC_{50} values in this model are in agreement with the extent of HDAC inhibition found in other *in vitro* systems, our assay may be a useful tool for the prediction of the potency of HDAC inhibitors.

Materials and Methods

Materials

Molecular biology reagents were purchased from MBI Fermentas (St. Leon-Rot, Germany), Sigma (Deisenhofen, Germany), GIBCO (Karlsruhe, Germany), Promega (Mannheim, Germany) and NEB (Frankfurt, Germany) or other sources as indicated in the text. Insulin was a gift from Aventis (Frankfurt, Germany). Human TGFβ1 was purified from outdated platelets according to Werz *et al.* (1996). Nucleospin extract columns for direct purification of pDNA were obtained from Macherey-Nagel (Düren, Germany). The VDRE plasmid p(DR3)4tkluc and the pSG5VDR and pSG5RXR expression plasmids for the human vitamin D receptor (VDR) and the human retinoid X receptor α (RXRα) were obtained from Dr. Carsten Carlberg (Kuopio, Finland).

Construction of Reporter Plasmids

The 5-LO promoter containing plasmid K1 was digested first by *BstE*II. The restriction site was blunted with T4 DNA polymerase and then digested with *Kpn*I and ligated into the promoterless luciferase reporter vector pGL3Basic, which was opened with *Kpn*I and *Sma*I. The intermediate construct obtained was then partially digested with *Cfr42*I, digested with *Xho*I and blunted with T4 DNA polymerase before final religation (pN0). pN10 and pN11 were constructed by digestion of pN0, which contains the promoter region from +53 bp in relation to the transcription start (–12 bp from the 5LO translation start) up to position –6079 bp (–6144 bp from the 5-LO ATG) using the restriction enzymes *Kpn*I and *Pau*I or *Bst X*I, respectively. Overhangs were blunted by T4 DNA polymerase treatment and religated using the T4 DNA ligase. Plasmids pN12-pN16 and pGC0 were obtained by PCR deletion, using pN10 as the template, the reverse primer TAT CGA TAG AGA AAT GTT CTG GCA (pN12, pN13, pN14, pN15 and pN16), the forward primers CGC GTG AAG AGT GGG AGA GAA GTA CTG CGG (pN12), CAG CCG GGA GCC TGG AGC CAG ACC (pN13), AGG GAC CAG TGG TGG GAG GAG GCT (pN14), GCT AGA TGC GGA CAC CTG GAC CGC (pN15) and GGC TCC CGG CGC TCG CTG CTC (pN16) or the primers Rapid GC1: CAG CCG GGA GCC TGG AGC CAG ACC and Rapid GC 2: CCG CAG TAC TTC TCT CCC ACT CTT CAC GCG (pGC0) and *Pfu* I DNA polymerase. Following temperature cycling, the products were treated with *Dpn*I, plasmid ends were phosphorylated with T4 polynucleotide kinase and ligated with T4 DNA ligase. Plasmid constructs were verified by DNA sequencing.

Cell Culture

Mono Mac 6 cells which were kindly provided by Dr. H.W.L. Ziegler-Heitbrock (Munich, Germany) were grown at 37 °C in a humified atmosphere with 6% CO_2 in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), streptomycin (100 µg/ml) and penicillin (100 U/ml), 1× nonessential amino acids, sodium pyruvate (1 mM), oxalacetate (1 mM) and insulin (10 µg/ml) (Ziegler-Heitbrock *et al.*, 1988). HeLa cells were obtained from Dr. Müller (Pharmacological Institute, Frankfurt, Germany) and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin and 100 units/ml penicillin.

In vitro DNA Methylation

Ten µg plasmid DNA were incubated for 24 h at 37 °C with *Hpa*II methyltransferase at 1 unit/µg pDNA in 50 mM Tris-HCl, 10 mM EDTA, 5 mM 2-mercaptoethanol (pH 7.5), supplemented with 80 µM S-adenosylmethionine in a final volume of 50 µl. For transfections, methylated pDNAs were purified on Nucleospin extract columns. Complete methylation at CCGG sites was confirmed by endonuclease *Hpa*II digestion of the plasmids.

Transfection of Mono Mac 6 Cells

Mono Mac 6 cells (2×10^5 cells/ml) were grown for 48 h, harvested by centrifugation at 1000 g for 5 min at room temperature and washed twice at room temperature with RPMI-1640 without FCS and L-glutamine. Then, cells were resuspended at 4.6×10^7 cells/ml in RPMI-1640 without supplements, and 0.3 ml of the cell suspension were placed into a 0.4 cm electroporation cuvette (Bio-Rad, Munich, Germany). Forty micrograms of supercoiled luciferase reporter gene plasmid DNA and 1 µg internal standard pCMVSEAP in 30 µl water were added to the cell suspension. In cotransfection experiments, 5 µg of the expression vector pSG5VDR and 5 µg pSG5RXR were added. After 5 min at room temperature, electroporation was performed at 975 µF and 200 V using a Bio-Rad GenePulser (Klan and Steinhilber, 2003). The cuvettes were immediately cooled on ice for 20 min. The cells were transferred into 10 ml of RPMI-1640 containing 10% FCS, insulin, glutamine and nonessential amino acids. $1,25(OH)_2D_3$ (50 nM) and TGFβ (1 ng/ml) were added immediately after cell transfer as indicated. Six hours after transfection, cells were harvested for determination of luciferase activity.

Transfection of HeLa Cells

When HeLa cells were transfected by the calcium phosphate precipitation method, cells were plated into a 24-well tissue culture plate at a density of 4×10^4 cells per well 24 h before transfection, so that 60–80% of the cells were confluent at the time of transfection. For the transfection, 0.8 µg plasmid DNA of the luciferase reporter gene construct, 0.1 µg pSG5RXR, 0.1 µg pSG5VDR and 0.01 µg internal standard pCMVSEAP were used per well. The calcium phosphate-DNA coprecipitation was performed by overnight incubation. The medium was changed 16 h after transfection and the cells were incubated for 24 h with TsA at 330 nM. Subsequently, cells were harvested, the medium was exchanged by 100 µl fresh medium and luciferase activity was determined.

When HeLa cells were transfected by lipofection, cells were plated into a 24-well tissue culture plate at a density of 6×10^4 cells per well for 24 h, so that 60–80% of the cells were confluent at the time of transfection. Plasmid DNA of the luciferase reporter gene construct (0.4 µg) and internal standard pCMVSEAP (0.01 µg) were diluted into serum free Dulbecco's modified Eagle medium and precomplexed with 5 µl of PLUS reagent (GIBCO) by incubation at room temperature for 15 min. Precomplexed plasmid DNA was mixed with 25 µl of 1:50 diluted Lipofectin reagent and incubated for 30 min at room temperature. Then, the medium was replaced by 200 µl of fresh serum free medium and the DNA-PLUS-Lipofectin reagent complex was added to the cells and incubated for 5 h at 37 °C in 5% CO_2. Then, 1 ml of medium containing 15% (v/v) FCS was added. Twenty-four h after transfection, cells were washed once in phosphate-buffered saline pH 7.4 (PBS) and luciferase activity was determined as described below. In cotransfection experiments, 0.05 µg of the expression vector pSG5VDR and 0.05 µg pSG5RXR were included. pCMVluc (0.4 µg) was used as positive control.

Luciferase Assays

Six hours (Mono Mac 6) and 24 h (HeLa) after transfection, cells were washed once in PBS containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and lysed in 100 µl lysis buffer (Luciferase Reporter Gene

Assay constant light signal kit, Boehringer Mannheim, Germany). Luciferase activity was determined by monitoring light emission with a Microlumat Plus LB96V EG&G Berthold luminometer. Light emission signal was integrated for 5 seconds. Transfection efficiency was monitored and normalized by co-transfection with pCMVSEAP using the Phospha-Light™ kit (Tropix) to determine the secreted placental alkaline phosphatase (SEAP) activity.

Acknowledgments

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References

Ballestar, E. and Wolffe, A. P. (2001). Methyl-CpG-binding proteins. Targeting specific gene repression. Eur. J. Biochem. *268*, 1–6.

Beato, M., Candau, R., Chavez, S., Mows, C. and Truss, M. (1996). Interaction of steroid hormone receptors with transcription factors involves chromatin remodelling. J. Steroid Biochem. Mol. Biol. *56*, 47–59.

Boeke, J., Ammerpohl, O., Kegel, S., Moehren, U. and Renkawitz, R. (2000). The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A. J. Biol. Chem. *275*, 34963–34967.

Brungs, M., Rådmark, O., Samuelsson, B. and Steinhilber, D. (1994). On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGFβ. Biochem. Biophys. Res. Commun. *205*, 1572–1580.

Brungs, M., Rådmark, O., Samuelsson, B. and Steinhilber, D. (1995). Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. Proc. Natl. Acad. Sci. USA *92*, 107–111.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Davie, J. R. (1997). Nuclear matrix, dynamic histone acetylation and transcriptionally active chromatin. Mol. Biol. Rep. *24*, 197–207.

Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E. and Seiser, C. (1999). Histone deacetylase 1 can repress transcription by binding to Sp1. Mol. Cell Biol. *19*, 5504–5511.

Drazen, J. M., Yandava, C. N., Dubé, L., Szczerback, N., Hippensteel, R., Pillari, A., Israel, E., Schork, N., Silverman, E. S., Katz, D. A. *et al.* (1999). Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. Nature Genet. *22*, 168–170.

Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. and Pavletich, N. P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature *401*, 188–193.

Ford-Hutchinson, A. W., Gresser, M. and Young, R. N. (1994). 5-Lipoxygenase. Annu. Rev. Biochem. *63*, 383–417.

Härle, D., Rådmark, O., Samuelsson, B. and Steinhilber, D. (1998). Calcitriol and transforming growth factor-β upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. Eur. J. Biochem. *254*, 275–281.

Härle, D., Rådmark, O., Samuelsson, B. and Steinhilber, D. (1999). Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-β and 1,25-dihydroxyvitamin D3. Adv. Exp. Med. Biol. *469*, 105–111.

Hoffmann, K., Brosch, G., Loidl, P. and Jung, M. (2000). First non-radioactive assay for *in vitro* screening of histone deacetylase inhibitors. Pharmazie *55*, 601–606.

Hoshiko, S., Rådmark, O. and Samuelsson, B. (1990). Characterization of the human 5-lipoxygenase gene promoter. Proc. Natl. Acad. Sci. USA *87*, 9073–9077.

In, K. H., Asano, K., Beier, D., Grobholz, J., Finn, P. W., Silverman, E. K., Silverman, E. S., Collins, T., Fischer, A. R., Keith, T. P. *et al.* (1997). Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription. J. Clin. Invest. *99*, 1130–1137.

Jung, M. (2001). Inhibitors of histone deacetylase as new anticancer agents. Curr. Med. Chem. *8*, 1505–1511.

Jung M., H. K., Brosch G., and Loidl P. (1997). Analogues of trichostatin A and trapoxin B as histone deacetylase inhibitors. Bioorg. Med. Chem. Lett. *7*, 1655–1658.

Jung, M., Brosch, G., Kolle, D., Scherf, H., Gerhauser, C. and Loidl, P. (1999). Amide analogues of trichostatin A as inhibitors of histone deacetylase and inducers of terminal cell differentiation. J. Med. Chem. *42*, 4669–4679.

Kamitani, H., Taniura, S., Ikawa, H., Watanabe, T., Kelavkar, U. P. and Eling, T. E. (2001). Expression of 15-lipoxygenase-1 is regulated by histone acetylation in human colorectal carcinoma. Carcinogenesis *22*, 187–191.

Klan, N. and Steinhilber, D. (2003). Transient transfection of the human myeloid cell line Mono Mac 6 using electroporation. Biotechniques *34*, 142–147.

Kosugi, H., Towatari, M., Hatano, S., Kitamura, K., Kiyoi, H., Kinoshita, T., Tanimoto, M., Murate, T., Kawashima, K., Saito, H. *et al.* (1999). Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. Leukemia *13*, 1316–1324.

Massagué, J. and Wotton, D. (2000). Transcriptional control by the TGF-β/Smad signaling system. EMBO J. *19*, 1745–1754.

Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., et al. (1997). Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J. Biol. Chem. *272*, 22199–22206.

Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D. and Bird, A. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nature Genet. *23*, 58–61.

Ponton, A., Thirion, J. P. and Sirois, P. (1996). Changes in chromatin conformation regulate the 5-lipoxygenase gene expression during differentiation of HL60 cells. Prostaglandins Leukot. Essent. Fatty Acids *55*, 139–143.

Rachez, C. and Freedman, L. P. (2000). Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. Gene *246*, 9–21.

Remiszewski, S. W., Sambucetti, L. C., Atadja, P., Bair, K. W., Cornell, W. D., Green, M. A., Howell, K. L., Jung, M., Kwon, P., Trogani, N. *et al.* (2002). Inhibitors of human histone deacetylase: synthesis and enzyme and cellular activity of straight chain hydroxamates. J. Med. Chem. *45*, 753–757.

Rice, J. C. and Allis, C. D. (2001). Histone methylation *versus* histone acetylation: new insights into epigenetic regulation. Curr. Opin. Cell Biol. *13*, 263–273.

Samuelsson, B., Dahlén, S.-E., Lindgren, J.-Å., Rouzer, C. A. and Serhan, C. N. (1987). Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science *237*, 1171 – 1176.

Silverman, E. S., Du, J., De Sanctis, G. T., Rådmark, O., Samuelsson, B., Drazen, J. M. and Collins, T. (1998). Egr-1 and Sp1 interact functionally with the 5-lipoxygenase promoter and its naturally occurring mutants. Am. J. Respir. Cell. Mol. Biol. *19*, 316 – 323.

Silverman, E. S. and Drazen, J. M. (2000). Genetic variations in the 5-lipoxygenase core promoter. Description and functional implications. Am. J. Respir. Crit. Care Med. *161*, S77-S80.

Singal, R. and Ginder, G. D. (1999). DNA methylation. Blood *93*, 4059 – 4070.

Singal, R., van Wert, J. and Bashambu, M. (2001). Cytosine methylation represses glutathione S-transferase P1 (GSTP1) gene expression in human prostate cancer cells. Cancer Res. *61*, 4820 – 4826.

Steinhilber, D. (1999). 5-lipoxygenase: a target for antiinflammatory drugs revisited. Curr. Med. Chem. *6*, 69 – 83.

Uhl, J., Klan, N., Rose, M., Entian, K. D., Werz, O. and Steinhilber, D. (2002). The 5-lipoxygenase promoter is regulated by DNA methylation. J. Biol. Chem. *277*, 4374 – 4379.

Werz, O., Brungs, M. and Steinhilber, D. (1996). Purification of transforming growth factor β 1 from human platelets. Pharmazie *51*, 893 – 896.

Yanagi, Y., Suzawa, M., Kawabata, M., Miyazono, K., Yanagisawa, J. and Kato, S. (1999). Positive and negative modulation of vitamin D receptor function by transforming growth factor-β signaling through smad proteins. J. Biol. Chem. *274*, 12971 – 12974.

Yang, J., Kawai, Y., Hanson, R. W. and Arinze, I. J. (2001). Sodium butyrate induces transcription from the G α(i2) gene promoter through multiple Sp1 sites in the promoter and by activating the MEK-ERK signal transduction pathway. J. Biol. Chem. *276*, 25742 – 25752.

Yu, F., Thiesen, J. and Stratling, W. H. (2000). Histone deacetylase-independent transcriptional repression by methyl-CpG-binding protein 2. Nucleic Acids Res. *28*, 2201 – 2206.

Zhang, D. E., Hetherington, C. J., Tan, S., Dziennis, S. E., Gonzalez, D. A., Chen, H. M. and Tenen, D. G. (1994). Sp1 is a critical factor for the monocytic specific expression of human CD14. J. Biol. Chem. *269*, 11425 – 11434.

Ziegler-Heitbrock, H. W., Thiel, E., Futterer, A., Herzog, V., Wirtz, A. and Riethmuller, G. (1988). Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. Int. J. Cancer *41*, 456 – 461.

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11 Publikationsliste

Klan, N., **Seuter, S.**, Schnur, N., Jung, M., Steinhilber, D. (2003). Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity. *Biol. Chem*., 384, 777-785.

Klan, N., **Seuter, S.**, Sorg, B., Dishart , D., Rådmark, O., Habenicht, A., Carlberg, C., Werz, O. und Steinhilber, D. (2004) Vitamin D-responsive elements within the human 5-lipoxygenase gene promoter bind vitamin D receptor, but do not confer vitamin D-dependent transcriptional activation. *manuscript in preparation*

Seuter, S. und Steinhilber, D. (2004). The Coding Sequence Mediates Induction of 5-Lipoxygenase Expression by 1,25$(OH)_2D_3$ and TGFβ. *submitted*

Seuter, S. und Steinhilber, D. (2004). Significance of the TGFβ/Smad signalling system in coding sequence-dependent 5-lipoxygenase gene regulation. *manuscript in preparation*

Kongressbeiträge und –teilnahmen

Teilnahme an der *"11th International Conference on ADVANCES IN PROSTAGLANDIN AND LEUKOTRIENE RESEARCH: Basic Science And New Clinical Applications*". Florenz, Italien, Juni 2000

Seuter, S., Klan, N., Steinhilber, D. (2002)

Induction of 5-lipoxygenase gene expression by transforming growth factor β and 1,25-dihydroxyvitamin D_3 depends on co-expression of vitamin D and retinoic acid receptors and regulatory elements outside of the promoter region.

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Seuter, S., Klan, N., Steinhilber, D. (2002)

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Seuter, S., Klan, N., Rådmark, O., Steinhilber, D. (2003)

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