Analysis of the 5-lipoxygenase promoter and characterization of a vitamin D receptor binding site

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Abstract

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) and transforming growth factor beta (TGFβ) potently induce 5-lipoxygenase (5-LO) in myeloid cells. We analyzed vitamin D receptor (VDR) binding to putative vitamin D response elements within the 5-LO promoter and analyzed its function by reporter gene analysis. Binding of VDR and retinoid X receptor to the promoter region was shown in DNase I footprinting, electrophoretic mobility shift and chromatin immunoprecipitation assays. However, the identified VDR binding region did not mediate induction of reporter gene activity by 1,25(OH)₂D₃/TGFβ, neither in the 5-LO promoter context nor with the thymidine kinase (tk) promoter. Insertion of the rat atrial natriuretic factor VDRE in reporter plasmids containing the 5-LO promoter diminished induction by 1,25(OH)₂D₃/TGFβ as compared with the tk promoter. Similarly, low inductions were obtained when cells were transiently or stably transfected with constructs containing various 5-LO promoter regions. Concerning basal promoter activity, we identified a positive regulatory region (−779 to −229), which includes the VDR binding region, in 5-LO-positive MonoMac6 cells. In summary, the VDR/RXR complex binds to putative VDREs in the 5-LO promoter, but other sequences outside the 5-LO promoter seem to be responsible or additionally required for the prominent induction of 5-LO mRNA expression by 1,25(OH)₂D₃ and TGFβ.

Keywords: 5-lipoxygenase; Promoter; Vitamin D; Transforming growth factor beta

1. Introduction

Arachidonate 5-lipoxygenase (5-LO) is a key enzyme for leukotriene biosynthesis. Leukotrienes are involved in many pathological processes including asthma, rheumatic diseases and cancerogenesis. The enzyme catalyzes the first two steps in the conversion of arachidonic acid to leukotriene A₄ which is the precursor for the biologically active leukotrienes [1,2]. In the myeloid cell lines HL-60 and Mono Mac 6 (MM6), 1,25-

Abbreviations: ChIP, chromatin immunoprecipitation; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylase; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; rANF, rat atrial natriuretic factor; RLU, relative luminescence units; MM6, Mono Mac 6; TGFβ, transforming growth factor β; VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D responsive element

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VDRE consensus sequence or are in accordance with it, and thus represent further putative DR3- and DR4-type VDREs (Fig. 1A). In this study, we first addressed whether the VDR physically interacts with the putative VDREs within the 5-LO promoter. To define the mechanisms involved in the regulation of 5-LO expression by 1,25(OH)2D3 and TGFβ, we used deletion mutants of 5-LO promoter and performed reporter gene analysis with respect to (a) induction by 1,25(OH)2D3/TGFβ (b) induction by VDR/RXR expression (c) cell line characteristics (5-LO-positive and 5-LO-negative cell lines) and (d) genomic integration of reporter constructs (transient and stable transfection).

2. Materials and methods

2.1. 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 as described earlier [8]. Nuclease Extract columns for direct purification of pDNA were from Macherey-Nagel (Düren, Germany). Polyclonal poly(dIdC), poly(dIdC), poly(dAdT), poly(dAdT) and [γ-32P]dATP (3000 Ci/mmol) were from Amersham Pharmacia. Oligonucleotides were synthesized at Scandinavian Gene Synthesis AB (Köping, Sweden). Human recombinant VDR and RXRα protein were purchased from Biorenergy (Plymouth, Meeting, PA). The plasmid p(DR3)4tkluc (referred to as p(4x)ANF-tk) containing the four times concatenated rat ANF VDRE (AGGTCA TGAGAGAC) 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), were constructed as described previously [9].

2.2. Cells and cell culture

HL-60, U937 and RBL-1 cells were grown at 37 °C in a humidified atmosphere with 6% CO2 in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), streptomycin (100 μg/ml) and penicillin (100 U/ml). For cell culture of Mono Mac 6 (MM6) 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) [10]. 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% (v/v) FCS, 100 μg/ml streptomycin and 100 units/ml penicillin.

2.3. RT-PCR analysis

HL-60 cells were seeded at 4×10^5 cells/ml and incubated for 24 h with or without 1,25(OH)2D3 (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 [11] and RT-PCR analysis was performed as described previously [5]. The following PCR-primers were used at a concentration of 5 ng/μl: β-actin (24 cycles) 5′-GAGGAGGACA CCGTGCGCTGCT GA and 5′-CTAAGAGCAT T1GCTGGGGA CGATGGAGGG GCC, 5-LO (28 cycles) 5′-ACCACTTGAGC AGATCTGAGA CACGCG and 5′-GCAGTCTTGC TCTGTGTAAG ATGGG, CAT (35 cycles) 5′-CTATACAA CACCGTTCAG C and 5′-CGCCACGGGC ATACGACC.

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.

Fig. 1. (A) Putative VDR binding sites/VDREs within the human 5-LO promoter. (B) Protection of regions in the 5-LO gene promoter by VDR and RXR. DNase1 footprinting was performed with the AluI (~364) to ScaI (~119) fragment of the promoter region of the 5-LO gene, radiolabelled at the AluI end. Lane 1: no VDR/RXR, BSA present as carrier protein (positive control). Lane 2: the probe was incubated with VDR protein (3 μg) and RXR protein (3 μg) and then digested 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 VDR/RXR, BSA present as carrier protein (positive control). Lane 7: Maxam-Gilbert A+G ladder. Electrophoresis was done with a 8% polyacrylamide sequencing gel. Star indicates the hypersensitive site (~268), and the heavily protected region from ~307 to ~299 is indicated. Parentheses indicate parts of the sequence conforming to DR3 and DR4 motifs.
2.4. Plasmid construction

The 5-LO promoter reporter gene deletion constructs were prepared using restriction enzymes and PCR methods. Starting with the plasmids K1 (a KpnI fragment of the genomic clone λx12A [12], which was cloned into plUC19, opened with KpnI). The plasmid contains the fragment between -6079 to +53 in relation to the 5-LO transcriptional start site and was provided by Dr. Shigeru Hoshiko and pGL3Basic (Promega). The 5-LO promoter-containing plasmid K1 was digested first by BstXI, the restriction site was blunted with T4 DNA polymerase, then digested with KpnI and ligated into the promoterless luciferase reporter vector pGL3Basic, which was opened with KpnI and Smal. The intermediate construct obtained was then partially digested with CferI, digested with Xhol 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 to +53 in relation to the transcription start (−6144 bp from the 5-LO ATG) using the restriction enzyme KpnI and then AffI (pN1), VanrI (pN2), NdeI (pN3), PvuII (pN5), EcoRI (pN6), EcoRV (pN8), Pmel (pN9), Pald (pN10) and BesI(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 5'-CTAGTGACAT GTCCTTCA TG ACCTCTGTG. Plasmid ptk was cloned using the Phospha LIGHT efficiency was monitored and normalized by cotransfection with pCMVSEAP kit (Boehringer Mannheim, Germany). Luciferase activity was determined by the Phospha-Light™ kit (Tropix) to determine the secreted placental alkaline phosphatase (SEAP) activity [14–18].

2.5. Transfection of myeloid cell lines by electroporation

Cells (MM6, HL-60, RBL-1 and U937) were plated into a 24-well tissue culture plate at a density of 6 × 10⁴ cells per well, so that 60–80% of the cells were confluent at the time of transfection (after about 24 h). 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. The medium was replaced by 200 μl of fresh serum free DMEM and incubated 37 °C in 5% CO₂ with the DNA-PPLUS-Lipofectin reagent complexes. After 5 h, 1 ml of DMEM containing 1% (v/v) 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.

2.6. Transfection of HeLa cells

Cells were plated into a 24-well tissue culture plate at a density of 6 × 10⁴ cells per well, so that 60–80% of the cells were confluent at the time of transfection (after about 24 h). 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. The medium was replaced by 200 μl of fresh serum free DMEM and incubated 37 °C in 5% CO₂ with the DNA-PPLUS-Lipofectin reagent complexes. After 5 h, 1 ml of DMEM containing 1% (v/v) 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.

2.7. Stable transfections of HL-60 cells

HL-60 cells were stably transfected with the 5-LO promoter-CAT construct p5-LO5900CAT [13] (referred to as pSL06079CAT), which was provided by Dr. Shigeru Hoshiko, containing the 5-LO promoter sequences from −6079 to +85 (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.

2.8. Luciferase assays

6 h (MM6, U937, HL-60, RBL-1) and 24 h (HeLa) after transfection, cells were washed once in PBS containing 0.5 mM MgCl₂, 0.5 mM CaCl₂ and lysed in 100 μl lysis buffer (Promega), containing a single-stranded DNA were combined in a solution containing 10 mM MgCl₂ and heated to 80 °C for 5 min, and slowly cooled down to room temperature. The annealed oligonucleotides were endlabelled with [32P]-γ-ATP complexes. After 5 h, 1 ml of DMEM containing 15% (v/v) FCS was added. 24 h after transfection, cells were transfected cells were selected for neomycin resistance at 600 μg/ml.

2.9. Preparation of nuclear extracts

MM6 cells were grown in presence of TGFβ (2 ng/ml) plus 1,25 (OH)₂D₃ (50 nM) as described [4]. After 4 days, cells (0.5–1 × 10⁶) were harvested and nuclear extracts were prepared according to the protocol of Shapiro et al. [19]. Nuclear extracts (protein content: 2–8 μg/ml) were immediately frozen in aliquots and stored at −70 °C for several months.

2.10. Electrophoretic mobility shift assays

The following oligonucleotides were used in the gel retardation assays: mouse osteopontin VDRE MO-23 mer: 5'-CAAAGGTTCAGGAGGTTCACG TGC, 5'-LO-23 mer: 5'-AGGCAGGACA AAGGGTGAG AAC GAC (−313 to −291); 5'-LO-56 mer: 5'-AGGCAGGACA AAGGGTGAG AACCAATCAG GAGAAGGAAG GAGAACAAAT CGAGTAGTGG ATGAG (−313 to −258).

To prepare double-stranded oligonucleotides, equal amounts of the complementary single-stranded DNA were combined in a solution containing 10 mM MgCl₂ and heated to 80 °C for 5 min, and slowly cooled down to room temperature. The annealed oligonucleotides were endlabelled with [32P]-γ-ATP complexes. After 5 h, 1 ml of DMEM containing 15% (v/v) 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.
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%, MgCl₂, 5 mM, EDTA 0.5 mM, DTT 0.5 mM, NaCl 50 mM, 0.05 mg/ml poly (dl–dC)·poly(dl–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 non-denaturing 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.

2.11. Western blot

Nuclear extracts (10 μg protein in 10 μl) were mixed with the same volume of 2 × sample loading buffer (SDS-b) and boiled for 5 min at 95 °C. Then, 4 μl of 0.1% bromophenol blue (1:1, v/v) were added and proteins were separated by SDS-PAGE using a mini Protein system (Bio-Rad) on a 10% gel. After electrophoresis 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, 1:1000 dilution (Santa Cruz, CA)) for overnight at 4 °C. The membranes were washed with TBS and incubated with 1:1000 dilution of alkaline phosphatase-conjugated rabbit IgG (Sigma) for 2 h at RT. After washing with TBS and PBS 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 MgCl₂).

2.12. DNase I footprinting

For DNase I footprinting, an oligonucleotide was obtained by restriction enzyme digestion of the plasmid 5LO931CAT[13]. First, the plasmid was digested with SacII. The smaller fragment was then purified by agarose gel electrophoresis and extracted with the QiAQuick gel extraction kit (Qiagen, Hilden, Germany). After digestion with SacII, the DNA was treated with calf intestinal alkaline phosphatase (MBI Fermentas, Lithuania) and radiolabelled at the 5′ ends using T4 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 smaller fragment was then purified by agarose gel electrophoresis through 2.0% agarose. 1.2 μg of the dye SybrGreen (1:2000 dilution) was added to each sample before loading. Gel images were scanned on a Fuji FLA3000 reader using ScienceLab99 software.

2.13. Chromatin immunoprecipitation assay

MM6 cells were treated with 50 nM 1,25 (OH)₂D₃. After the indicated time points, nuclear proteins were cross-linked to genomic DNA by adding formaldehyde for 15 min directly to the medium to a final concentration of 0.125 M and incubating for 5 min at room temperature on a rocking platform. After centrifugation, the medium was removed and the cells were washed twice with ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄·2 H₂O). The nuclei were isolated by resuspending the cell pellets in Pipes buffer (5 mM Pipes pH 8.0, 85 mM KCl, 0.5% NP-40, protease inhibitor cocktail (Roche Diagnostics)) and incubating for 10 min on ice. After centrifugation and removal of the supernatant, the pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris–HCl, pH 8.1) and incubated for 10 min on ice. The lysates were sonicated to result in DNA fragments of 300 to 1000 bp in length. Cellular debris was removed by centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors, 16.7 mM Tris–HCl, pH 8.1). Nonspecific background was removed by incubating the chromatin suspension with a salmon sperm DNA/protein A agarose slurry (Upstate Biotechnology, Lake Placid, NY, USA) for 30 min at 4 °C with agitation. The samples were centrifuged and the recovered chromatin solutions were incubated with 5 μl of indicated antibodies overnight at 4 °C with rotation. The antibodies against VDR (sc-1008), RRXs (sc-553), Pol II (sc-899), and IgG (sc-2027) were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). The immuno-complexes were collected with 60 μl of protein A agarose slurry (Upstate Biotechnology) for 2 h at 4 °C with rotation. The beads were pelleted by centrifugation for 1 min at 4 °C at 100×g and washed sequentially for 5 min by rotation with 1 ml of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–HCl, pH 8.1) and LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Finally, the beads were washed twice with 1 ml TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8.0). The immuno-complexes were then eluted by adding 250 μl elution buffer (1% SDS, 100 mM NaHCO₃) and incubation for 15 min at room temperature with rotation. After centrifugation, the supernatant was collected and the elution was repeated. The supernatants were combined and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and incubating overnight at 65 °C. The remaining proteins were digested by adding proteinase K (final concentration 40 μg/ml) and incubation for 1 h at 45 °C. Genomic DNA fragments were recovered by phenol chloroform extraction and ethanol precipitation using glycoGen as a carrier.

2.14. PCR of chromatin templates

For the proximal promoter region, primer pairs were designed, optimized and controlled by running PCR reactions with 25 ng genomic DNA (input) as a template. The primer pair 5′-AAATGCTGCATGCAATCAGGAGAC (forward primer) and 5′-AGCTCTTCCCCACCTGTTCCCGTG (reverse primer) gave the best results. When running immunoprecipitated DNA (output) as a template, the following PCR profile was used: preincubation for 5 min at 94 °C, 50 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C and one final incubation for 10 min at 72 °C. The PCR products were separated by electrophoresis through 2.0% agarose. 1.2 μl of the dye SybrGreen (1:2000 dilution) was added to each sample before loading. Gel images were scanned on a Fuji FLA3000 reader using ScienceLab99 software.

3. Results

3.1. DNase I footprinting assay of regions with putative VDREs within the 5-LO promoter

Sequence analysis of the 5-LO promoter reveals the presence of several putative VDR binding sites from −309 to −262 in relation to the transcriptional start site (Fig. 1A). DNase I footprinting was performed using a 246 bp probe containing the sequence between the AluI site (−364) and the SacI site (−119) of the 5-LO gene promoter. When the probe was coincubated with RRX and VDR proteins, an apparent hypersensitive site appeared at −268. The 96 bp of the probe upstream (5′) of this position were protected from DNase I, whereas the downstream part of the probe (149 bp) was accessible for DNase I (Fig. 1B). 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 −295) proposed by us [7]. Protection against DNase I also comprised the putative DR4-
type VDRE and suddenly stopped at the hypersensitive site at −268. Apparently, binding of VDR plus RXR covers or bends the probe in such a way that access of Dnase I to the sequence upstream of −268 was prevented. The protection against DNase I was most prominent when RXR and VDR were combined, but also VDR alone (3 μg) gave a similar result (slightly weaker footprints). However, RXR alone (3 μg) did not bind to the probe (data not shown), and when BSA was added (as carrier protein, lanes 1 and 6 in Fig. 1B) to the binding reaction, there was no effect. Also, purified SP1 protein alone gave no footprint in this part of the 5-LO promoter, and in combination with VDR/RXR there was no additional protection against DNase I.  

3.2. EMSA studies: VDR/RXR-binding to putative VDREs in the 5-LO promoter

In order to confirm VDR/RXR binding to the putative VDREs, EMSAs were performed using synthetic oligonucleotides corresponding to the sequence −313 to −291 (5LO-23) and −313 to −258 (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. 2A, VDR or RXR alone 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 23-bp 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 MM6 cells, cultured in presence or absence of 50 nM 1,25(OH)2D3 plus 1 ng/ml TGFβ. A significantly stronger band was obtained with differentiated (1,25(OH)2D3 plus TGFβ)-treated MM6 cells, as compared to untreated cells (Fig. 2B). This correlated with the total amounts of VDR protein present in such extracts as determined by Western blot (Fig. 2C). When EMSAs were performed using purified VDR and RXR proteins as well as nuclear extracts (obtained from 1,25(OH)2D3/TGFβ-differentiated MM6 cells) side by side, the migration pattern was apparently the same for 5LO-23 and MO-23 (Fig. 2D). However, for 5LO-56 an additional more intense shift was observed after incubation with nuclear extracts from 1,25(OH)2D3/TGFβ-treated cells (but not with extracts from untreated cells), implying the...
occurrence of additional protein(s) induced by 1,25(OH)₂D₃/TGFβ that are capable to bind the VDR/RXR/5-LO-56 complex.

3.3. Chromatin Immunoprecipitation studies: binding of VDR/RXR to the proximal 5-LO promoter

To further investigate binding of VDR and RXR to the proximal 5-LO promoter, ChIP analyses were performed with primers that encompassed the promoter region from −305 to −8, including the putative VDREs, in 5-LO-positive MM6 cells. Association of the VDR and the RXR to the 5-LO promoter was observed independently of the treatment with 1,25(OH)₂D₃ for up to 2 h in comparable magnitudes (Fig. 3). In addition to VDR and RXR, RNA polymerase II was detected, which indicates that the promoter is active.

3.4. Analysis of promoter deletion mutants: effects of 1,25(OH)₂D₃/TGFβ and identification of regulatory regions

In order to (a) determine inducibility of the 5-LO promoter by 1,25(OH)₂D₃/TGFβ and (b) identify positive and negative regulatory regions within the promoter, MM6 and HeLa cells were transiently transfected (6 h for MM6, 24 h for HeLa) with plasmids encoding deletion constructs of the 5-LO promoter in front of the luciferase gene. Starting from construct pN0 (−6079 to +53) fourteen deletion mutants were cloned, ending with construct pN14 (−31 to +53). To ensure the availability of sufficient amounts of nuclear receptor proteins, cells were co-transfected with expression vectors for VDR and RXR.

As shown in Fig. 4A, the full-length 5-LO promoter within reporter gene construct pN0 (−6079 to +53) significantly induced luciferase activity (by 57-fold) versus the promoterless vector in reporter gene construct pN0 (−6079 to +53) that lacks the DNA sequence from −779 to +53, which gave 1290-fold and 1210-fold higher activities compared to the promoterless vector. Except for the pN11 construct (−229 to +53), there was no significant upregulation of the reporter gene activity in presence of 1,25(OH)₂D₃/TGFβ compared to untreated cells, indicating that 1,25(OH)₂D₃/TGFβ caused no transcriptional transactivation.

Also in HeLa cells, pN10 caused the most prominent upregulation of transcriptional activity among the reporter gene constructs (Fig. 4B). In contrast to MM6 cells, no positive regulatory region was found from −779 to −229 in this cell line. As found for MM6 cells, the 5-LO promoter reporter gene constructs were not significantly responsive to 1,25(OH)₂D₃/TGFβ stimulation in HeLa cells.

Taken together, the promoter region from −779 to −229 acted as a positive regulatory region with respect to basal promoter activity in the 5-LO-positive cell line MM6, but activity of the investigated promoter constructs of various length was not inducible by 1,25(OH)₂D₃/TGFβ.

3.5. Analysis of promoter deletion mutants: influence of VDR/RXR coexpression

In order to check the effects of VDR/RXR coexpression on 5-LO promoter activity, MM6 cells were transiently transfected with the 5-LO promoter reporter gene constructs pN9-pN14 or pGL3Basic in the 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. 4C). Coexpression of VDR/RXR did not significantly influence expression of the pN9 and pN10 reporter gene constructs.

However, coexpression of VDR/RXR in MM6 cells resulted in a 2- to 3-fold reduction of reporter gene activity for pN11, pN12, pN13 and pN14. Thus, coexpression of VDR and RXR does not significantly affect 5-LO promoter activity when the putative VDREs are present (plasmids pN9, pN10) but reduces 5-LO promoter activity in reporter gene constructs (pN11–pN14) that lack the DNA sequence from −779 to −229, which contains the putative VDREs. Obviously, these sequences are required for maximal promoter activity when expression of VDR/RXR is high.

3.6. Analysis of the proximal 5-LO promoter: influence of 1,25(OH)₂D₃/TGFβ and cell line specificity

In order to analyze cell specific effects concerning 5-LO promoter activity, the 5-LO-positive myeloid cell lines RBL-1, MM6, HL-60 and U937, as well as 5-LO-negative HeLa cells were used for reporter gene analysis. The cells were transiently transfected with the plasmid pN10. The luciferase reporter gene plasmid p(4x)R:ANF-tk, containing a 4-times concatemerized rat atrial natriuretic factor (rANF) VDRE in front of the thymidine kinase promoter, was used as a positive control.

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 MM6 cells, transcription

![Time (h) 0 1 2](Image 158x131 to 210x257)

![Input](Image 158x131 to 210x257)

![VDR](Image 158x131 to 210x257)

![RXR](Image 158x131 to 210x257)

![Pol II](Image 158x131 to 210x257)

![IgG](Image 158x131 to 210x257)

Fig. 3. ChIP analysis of VDR/RXR and RNA polymerase II binding to the 5-LO promoter. Mono Mac 6 cells (MM6) were differentiated with 50 nM 1,25(OH)₂D₃. Chromatin immunoprecipitation was performed using antibodies raised against VDR, RXR and RNA polymerase II. Anti-IgG-antibodies were used as unspecific control-antibodies. Cells were incubated for 0 h, 1 h and 2 h before cross-linking. The immunoprecipitated DNA fragments were subjected to PCR analysis using primer pairs encompassing the proximal 5-LO promoter including the putative VDREs (−305 to −8).
from the 5-LO promoter was about 1200-fold higher than from pGL3Basic. Of particular interest, in MM6, HL-60 and U937 cells, 5-LO mRNA expression (not shown, compare [3,4,20]) and nuclear protein binding to 5LO-23 and 5-LO-56 (Fig. 2) was increased in the presence of 1,25(OH)2D3 and TGFβ, whereas luciferase activity was not enhanced in either cell type (Fig. 5A).
transfection, 1,25(OH)_{2}D_{3} and TGFβ together with the expression vectors pSG5hRXR and pSG5hVDR. After pN10 and p(4x)rANF-tk were transiently transfected into the indicated cell lines constructs pN10 (A) and p(4x)rANF-tk (B) in various cell lines. The constructs 5B). However, in sharp contrast to pN10, treatment of myeloid p(4x)rANF-tk was cell type dependent in a similar fashion (Fig. 5). In analogy to pN10, also the basal transcription from the plasmid pCMVSEAP. Values were normalized for transfection efficiency by cotransfection of pcMVSEAP.

In order to determine if binding of multiple proteins (VDR/RXR heterodimers, coactivators) at the identified VDR binding region could prevent ligand dependent transcriptional activation by VDR/RXR-heterodimers, two series of analogous constructs with different promoter contexts, either the 5-LO promoter or the tk promoter, were cloned. The constructs contained either (a) the rANF VDRE instead of the proposed 5-LO VDRE[7] from the 5-LO promoter context generally supports 1,25(OH)_{2}D_{3}/TGFβ-mediated transcriptional activation, construct p(4x)rANF-N10, where the rANF VDRE was cloned into the proximal 5-LO promoter instead of the rANF VDRE in front of the tk promoter, was induced about 9-fold by 1,25(OH)_{2}D_{3}/TGFβ treatment. To check whether the 5-LO promoter context generally supports 1,25(OH)_{2}D_{3}/TGFβ-mediated transcriptional activation, construct p(4x)rANF-N10, where the rANF VDRE was cloned into the proximal 5-LO promoter instead of the VDR binding region (as detected by DNAse footprinting), and for evaluation of the influence of the promoter context, several reporter plasmids were employed (Fig. 6). First, the effect of the region between –309 and –262 on transcriptional activation was analyzed. Thus, construct p(4x)E/BR-tk containing the putative 5-LO VDREs (E/BR) cloned in front of the tk promoter as a four times concatemer, was used. As shown in Fig. 6, construct p(4x)E/BR-tk did not show 1,25(OH)_{2}D_{3}/TGFβ-induced activity as compared to the control plasmids pGL3Basic or ptk. In all three cases, inductions of about 2-fold were observed. In contrast, construct p(4x)rANF-tk, containing the four times concatemerized rANF VDRE in front of the tk promoter, was induced about 9-fold by 1,25(OH)_{2}D_{3}/TGFβ treatment. The context of the pN10 promoter led to a 50% lower induction by 1,25(OH)_{2}D_{3}/TGFβ than the tk promoter-based plasmid.

For detailed characterization of the region containing the putative VDREs between position –309 and –262 that matches the VDR binding region (as detected by DNAse footprinting), and for evaluation of the influence of the promoter context, several reporter plasmids were employed (Fig. 6). First, the effect of the region between –309 and –262 on transcriptional activation was analyzed. Thus, construct p(4x)E/BR-tk containing the putative 5-LO VDREs (E/BR) cloned in front of the tk promoter as a four times concatemer, was used. As shown in Fig. 6, construct p(4x)E/BR-tk did not show 1,25(OH)_{2}D_{3}/TGFβ-induced activity as compared to the control plasmids pGL3Basic or ptk. In all three cases, inductions of about 2-fold were observed. In contrast, construct p(4x)rANF-tk, containing the four times concatemerized rANF VDRE in front of the tk promoter, was induced about 9-fold by 1,25(OH)_{2}D_{3}/TGFβ treatment. To check whether the 5-LO promoter context generally supports 1,25(OH)_{2}D_{3}/TGFβ-mediated transcriptional activation, construct p(4x)rANF-N10, where the rANF VDRE was cloned into the proximal 5-LO promoter instead of the VDR binding region, was analyzed in comparison with p(4x)rANF-tk. The context of the pN10 promoter led to a 50% lower induction by 1,25(OH)_{2}D_{3}/TGFβ than the tk promoter-based plasmid.

In order to determine if binding of multiple proteins (VDR/RXR heterodimers, coactivators) at the identified VDR binding region could prevent ligand dependent transcriptional activation by VDR/RXR-heterodimers, two series of analogous constructs with different promoter contexts, either the 5-LO promoter or the tk promoter, were cloned. The constructs contained either (a) the rANF VDRE instead of the proposed 5-LO VDRE [7] from position –309 to –295 in front of the adjacent VDR binding region from –294 to –262 (p(4x)rANF/BR-N10 and p(4x)rANF/BR-tk, compare Fig. 1) or (b) the rANF VDRE in front of a VDRE-free control sequence (p(4x)rANF/C-N10 and p(4x)rANF/C-tk). Indeed, both series of constructs revealed an inhibitory effect of the VDR binding site between position –294 and –262 of 33% and 42% respectively. Interestingly, spatial effects were observed with constructs containing the rANF VDRE in different distances. Construct p(4x)rANF/C-tk, containing a 60 bp spacer (C) between the rANF VDREs led to an about 40% higher induction (12.5-fold vs. 9-fold, respectively) compared to the direct concatemerization of the rANF VDREs in plasmid p(4x)rANF-tk. Finally, we checked whether the proposed VDRE sequence between position –309 and –295 alone was able to induce reporter gene activity. For this purpose, we analyzed the construct p(4x)rANF/C-tk. Obviously, the putative VDRE did not induce reporter gene activity, even not in combination with the tk promoter and with optimal spatial arrangement. In summary, neither the VDR binding site nor the proposed VDRE mediated induction of reporter gene activity by 1,25(OH)_{2}D_{3}/TGFβ. Furthermore, the natural 5-LO promoter context, represented by reporter plasmid N10 and its derivatives,
mediates less 1,25(OH)₂D₃/TGF-β-induced transcriptional activation than the heterologous, 1,25(OH)₂D₃/TGF-β-independent tk promoter.

3.8. Analysis of promoter deletion mutants: effect of stable transfection on induction by 1,25(OH)₂D₃/TGF-β

Finally, we investigated whether integration of the 5-LO promoter into the genome is required for the upregulatory effects of 1,25(OH)₂D₃ and TGF-β. HL-60 cells were stably transfected with 5-LO promoter-CAT constructs and cultured with or without 1,25(OH)₂D₃ and TGF-β. After 24 h, CAT and endogenous 5-LO mRNA expression was determined by RT-PCR, respectively. 1,25(OH)₂D₃ and TGF-β 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. 7A). In contrast, when the expression of endogenous 5-LO mRNA was determined, 1,25(OH)₂D₃ and TGF-β caused a 13.8-fold, 11.1-fold and 7.1-fold increased 5-LO expression in HL60-SV40, HL60-Basic, and HL60-6079-5LO, respectively (Fig. 7B). Thus, induction of 5-LO mRNA expression by 1,25(OH)₂D₃ and TGF-β seems to be mediated by regulatory elements located outside of the 5-LO promoter or by an interplay of elements within and outside of the promoter.

4. Discussion

Previous reports have established a prominent role for 1,25(OH)₂D₃ and TGF-β in the upregulation of 5-LO expression in myeloid cells during maturation [4]. It was found that the about 42- to 64-fold induction of 5-LO mRNA in Mono Mac 6 (MM6) cells cultured with 1,25(OH)₂D₃ and TGF-β was due to both increased 5-LO gene transcription (about 5-fold) and to stimulated transcript elongation and maturation but not due to alterations in mRNA half-life [6]. Both 1,25(OH)₂D₃ and TGF-β were required for these effects.
cells by 1,25(OH)₂D₃ and TGFβ caused markedly enhanced protein binding to VDREs from nuclear extracts, as compared to untreated cells (Fig. 2B–D). In DNase I footprinting studies, coinoculation with VDR and RXR led to protection of the promoter sequences matching the putative VDREs (Fig. 1B). Additionally, using ChIP analysis, we demonstrate in vivo binding of VDR and RXR to the respective 5-LO promoter site in MM6 cells (Fig. 3). Binding of VDR/RXR to the 5-LO promoter was not enhanced by 1,25(OH)₂D₃. This is in accordance with the results obtained in the reporter gene assay, which failed to demonstrate significant transcriptional activation of the 5-LO promoter upon stimulation with 1,25(OH)₂D₃ (plus TGFβ) in various myeloid cell lines (Fig. 5A), whereas expression of a reporter construct containing a 4-times concatenated rat ANF VDRE in front of the tk promoter (p(4x) rANF-tk) was strongly increased after treatment of these cell lines with 1,25(OH)₂D₃ and TGFβ (Fig. 5B). Moreover, in stably transfected HL-60 cells endogenous 5-LO mRNA was strongly upregulated by 1,25(OH)₂D₃ and TGFβ, whereas the CAT reporter gene, under the control of the entire 5-LO promoter fragment (−6079 to +85), was not responsive (Fig. 7). Together, we conclude that regulatory elements located outside of the 5-LO promoter are responsible or additionally needed for the strong induction of 5-LO mRNA expression by 1,25(OH)₂D₃ and TGFβ in myeloid cells.

Several general features of the 5-LO promoter region have been elucidated in previous studies. Noteworthy are the lack of TATAAA or CCAAT boxes and the presence of repeated G+C-rich elements [12,13], which are characteristic for so-called housekeeping genes. A major transcriptional start site was found 65 bp upstream of the translation start codon (ATG). Sequence analysis indicated the presence of putative cis-acting control elements such as multiple Sp1 sites and putative binding sites for transcription factors like AP-2 and NFκB. Five tandem Egr-1/Sp1 consensus binding sites from −111 to −82 have been shown to be functional in reporter gene studies [21]. Recent results underlined the importance of the 5-LO core promoter region from −193 to −31 and revealed a strong involvement of DNA methylation in the regulation of cell-type specific 5-LO gene expression [20]. Moreover, treatment with the histone deacetylase (HDAC) inhibitor Trichostatin A of MM6 and HeLa cells, which were transiently transfected with the 5-LO promoter reporter gene construct pN10, indicated a strong involvement of HDAC in regulating HDAC in the regulation of 5-LO promoter activity [22]. However, the data indicated, that DNA methylation or histone acetylation were not related to the prominent effects of 1,25(OH)₂D₃/TGFβ on the increase in 5-LO mRNA observed in MM6 and HL-60 cells [22].

In order to characterize the 5-LO promoter comprehensively, also with respect to basal promoter activity, we analyzed 14 promoter deletion mutants by means of reporter gene assays using HeLa and MM6 cells (Fig. 4A–B). We could confirm previous findings that the promoter region containing the five tandemized Sp1-sites (construct pN12) is essential for basal promoter activity. For both cell lines, a positive regulatory region located between −5814 and −5396 was identified. Hoshiko et al. [13] identified a positive regulatory region (−6079 to −3635) which comprises our sequence. On the other hand, in HeLa cells
we did not observe the presence of a negative regulatory region −662 to −227 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 (−5396 to −4895) which contains two putative binding sites for the transcription factor p53 that match the consensus sequence (GAACATGTCC) to more than 80%.

In contrast to HeLa cells, we found a positive regulatory region (−779 to −229) within the 5-LO promoter in reporter gene assays with the human monocytic cell line MM6. Interestingly, putative VDREs are located in that region, but as stated above, in reporter gene experiments 1,25(OH)2D3/TGFβ did not alter 5-LO promoter activity in any cell type investigated. These findings are in agreement with results from nuclear run-off assays [5], where increased transcription of the 5-LO gene after treatment with both agents could not be demonstrated.

Of interest, the coexpression of VDR and RXR was found to be essential for the strong induction of reporter gene activity of the positive control plasmid p(4x)rANF-tk by 1,25(OH)2D3/TGFβ, whereas only marginal effects of 1,25(OH)2D3/TGFβ were observed in most of the investigated cell lines without receptor coexpression, which could be due to the low endogenous expression 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 in MM6 cells. 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 −779 to −229, which contains the putative VDREs (Fig. 4C). One possible explanation could be that transcription factors (e.g. VDR/RXR heterodimers) bind to this positive regulatory region and recruit coactivators that are required for high 5-LO promoter activity. However, with 5-LO promoter constructs that lack this region the VDR does not bind anymore and instead sequesters coactivators from the 5-LO promoter.

To exclude an inhibitory effect of the promoter context on the function of the putative VDREs, we used reporter gene constructs containing the VDR binding site (as identified by means of DNase footprinting) within its native 5-LO promoter context or in conjunction with a heterologous promoter (Fig. 6). In both cases, the putative VDREs were not functional. Furthermore, we could demonstrate that embedding of the well-characterized rANF VDRE within the context of the proximal VDR binding site led to a marked decrease of its function as compared to control constructs containing proximal VDRE-free DNA sequences of the same length. This suggests that the proximal VDRE-like sequences rather suppress than induce transcriptional activation by 1,25(OH)2D3.

In our experiments with the stably transfected cell line HL-60 we observed a strong induction of endogenous 5-LO mRNA by 1,25(OH)2D3/TGFβ but no increase in CAT and β-actin mRNA (Fig. 7A). Therefore, we could exclude that lack of bridging factors or coactivators hampered 1,25(OH)2D3/TGFβ-induced transcription under the experimental conditions of transient transfection assays.

Collectively, our data reveal the presence of a positive regulatory region within the proximal 5-LO promoter (−779 to −229) that also comprises a VDR binding site. Although VDR/RXR are capable to bind to the putative VDREs in the 5-LO promoter, no induction of reporter gene activity by 1,25(OH)2D3/TGFβ, neither in transient transfection experiments nor after stable transfection, could be observed. Thus, the regulatory sequences involved in the prominent induction of 5-LO mRNA expression by 1,25(OH)2D3 and TGFβ in the human myeloid cell lines HL-60 and MM6 seem to be located outside this part of the 5-LO promoter, or sequences of other parts of the 5-LO gene are additionally needed for 1,25(OH)2D3/TGFβ mediated induction of 5-LO gene expression.

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