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The 5-Lipoxygenase Promoter Is Regulated by DNA Methylation*

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5-lipoxygenase (5-LO), the key enzyme in leukotriene biosynthesis, is expressed in a tissue- and cell differentiation-specific manner. The 5-LO core promoter required for basal promoter activity has a unique (G+C)-rich sequence that contains five tandem Sp1 consensus sequences. The mechanisms involved in the regulation of cell type-specific 5-LO expression are unknown. Here we show that 5-LO expression is regulated by DNA methylation. Treatment of the 5-LO-negative cell lines U937 and HL-60TB with the demethylating agent 5-aza-2′-deoxycytidine (AdC) up-regulated expression of 5-LO primary transcripts and mature mRNA in a similar fashion, indicating that AdC stimulates *5-LO* gene transcription. Analysis of the methylation status of the 5-LO promoter revealed that the core promoter region was methylated in U937 and HL-60TB cells, whereas it was unmethylated in the 5-LO-positive parent HL-60 cell line. Reporter gene assays with 5-LO promoter constructs gave up to 68- and 655-fold repression of 5-LO promoter activity in HeLa and Mono Mac 6 cells by methylation. 1,25-dihydroxyvitamin D_3 and transforming growth factor-beta (TGFβ), potent inducers of the 5-LO pathway in myeloid cell lines, increased 5-LO RNA expression in HL-60TB and U937 cells, but co-treatment with AdC was required to achieve 5-LO expression levels in HL-60TB cells that were comparable with wild-type HL-60 cells. In reporter gene assays, 1,25-dihydroxyvitamin D_3 and TGFβ were unable to induce promoter activity when the 5-LO promoter constructs were methylated, which suggests that 5-LO promoter demethylation is a prerequisite for the high level induction of 5-LO gene expression by 1,25-dihydroxyvitamin D_3 and TGFβ and that the effects of both agents on 5-LO mRNA expression are not related to DNA methylation.

The enzyme 5-lipoxygenase (5-LO, arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34)[1] catalyzes the conversion of arachidonic acid to (5*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and further to leukotriene A_4 ((5*S*)-6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) (1, 2). 5-LO is expressed in a variety of immune competent cells including B-lymphocytes, granulocytes, monocytes, mast cells, and dendritic cells (3). 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 granulocyte-macrophage colony-stimulating factor (GM-CSF) (4), whereas interleukin-3 regulates the development of the 5-LO pathway in mouse mast cells (5). In the human myeloid leukemic cell lines HL-60 and Mono Mac 6, cell differentiation by 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and transforming growth factor-beta (TGFβ) leads to a strong induction of the 5-lipoxygenase pathway (6, 7). In Mono Mac 6 cells, the induction of 5-LO protein expression and activity by TGFβ and 1,25$(OH)_2D_3$ was accompanied by a 64-fold up-regulation of mature 5-LO mRNA and an up to 5-fold increase in 5-LO primary transcripts (7, 8), whereas no significant induction of 5-LO transcription was found in nuclear run-off assays (9). The human 5-LO gene promoter was first characterized by Hoshiko *et al.* (10). Several features of the putative 5-LO promoter region (such as the lack of TATAA or CCAAT boxes and repeated (G+C)-rich elements) are characteristic for so-called housekeeping genes. Previous data suggest that the transcription factors Egr-1 and/or Sp1 are required for basal 5-LO transcription and that they functionally interact with the 5-LO promoter and activate it via repeated response elements located between positions −212 and −88 bp, relative to the translational start site (10, 11). 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 (12). 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 (13).

As yet, no data are available on the mechanisms involved in the cell type-specific activation of the 5-LO promoter in response to cell differentiation signals and inflammatory stimuli. Expression of several genes with (G+C)-rich promoters has been shown to be regulated by DNA methylation (14). Whereas promoters of (G+C)-rich housekeeping genes are usually unmethylated, methylation of promoters of tissue-specific genes is usually linked with silencing of the respective genes.

Therefore, it was of interest to study the role of DNA methylation in the regulation of 5-lipoxygenase expression. The human myeloid cell lines Mono Mac 6 and HL-60 show prominent 5-LO gene expression and 5-LO activity after differentiation by TGFβ and 1,25$(OH)_2D_3$ (6, 7), whereas U937 cells and the HL-60TB cell line, a subline of the HL-60 cell line, show FLAP expression but lack expression of the *5-LO* gene (15, 16).

Here we show that the suppression of 5-LO expression in U937 and HL-60TB cells is a result of DNA methylation and that 5-LO promoter activity is regulated by methylation of CpG sites within the (G+C)-rich core promoter region.

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[1] The abbreviations used are: 5-LO, 5-lipoxygenase; AdC, 5-aza-2′-deoxycytidine; FCS, fetal calf serum; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 or calcitriol; TGFβ, transforming growth factor-beta; RT, reverse transcriptase; SEAP, secreted placental alkaline phosphatase.

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MATERIALS AND METHODS

Reagents—Molecular biology reagents were from MBI Fermentas, Sigma, Life Technologies, Inc., Promega, or other sources indicated in the text. Insulin was a gift from Hoechst-Marion-Roussel (Frankfurt, Germany). Human TGFβ1 was purified from outdated platelets (according to Ref. 17). Nucleospin extract columns for direct purification of pDNA were from Macherey-Nagel (Düren, Germany). HPLC solvents were from Merck (Darmstadt, Germany).

Plasmid Constructs—Vectors pN10 and pN7 were obtained by insertion of 5-LO promoter fragments −843 to −12 (upstream of the 5-LO start codon) and −1547 to −12 into the promoterless luciferase reporter vector pGL3Basic (Promega), respectively. Plasmid constructs were analyzed by DNA sequencing. The pSG5VDR and pSG5RXR expression plasmids for the human vitamin D receptor (VDR) and the human retinoid X receptor alpha (RXRα) were obtained from Dr. Carsten Carlberg (Kuopio, Finland).

Cell Culture—HL-60 cells were obtained from ATCC; U937 and HL-60TB cells were from the Karolinska Institute (Stockholm) and grown at 37 °C in a humidified 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 units/ml). For a cell culture of Mono Mac 6 cells, which were kindly provided by Dr. H. W. L. Ziegler-Heitbrock (Munich), the growth medium was supplemented with 1× nonessential amino acids, sodium pyruvate (1 mM), oxalacetate (1 mM), and insulin (10 μg/ml) (18). HeLa cells were obtained from Dr. Müller (Pharmacological Institute, Frankfurt) and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin.

DNA Demethylation by 5-Azadeoxycytidine—U937 and HL-60TB cells (0.3×10^6 cells/ml) were treated with 100 nM (U937 cells) or 30 nM AdC (HL-60TB cells) for 72 h. The medium containing AdC was replaced every 12 h as described (19). After 72 h, the cells were seeded at a density of 0.35×10^6 cells/ml and grown without AdC for 4 days. AdC-treated cells are designated U937 AdC and HL-60TB AdC.

Cell Differentiation—Cells (0.3×10^6 cells/ml) were differentiated by $1,25(OH)_2D_3$ (50 nM) and TGFβ (U937, 10 ng/ml; Mono Mac 6, 2 ng/ml; HL-60, HL-60TB, and HeLa, 1 ng/ml). Differentiated cells were harvested for RNA isolation and determination of 5-LO activity after 2 and 4 days, respectively.

5-LO Activity Assay—5-LO activity assays with intact cells and cell homogenates were carried out as described, and the formed 5-LO products were analyzed by high pressure liquid chromatography (7). 5-LO activity is expressed as nanograms of 5-LO metabolites/10^6 cells and includes leukotriene B_4, the all-*trans* isomers of leukotriene B_4, and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE).

RT-PCR Analysis—Total RNA was isolated from cells as described (20). RT-PCR analysis was performed as described previously (8). The following PCR primers were used at a concentration of 5 ng/μl: β-actin (24 cycles), 5′-GAGGAGCACCCCGTGCTGCTGA-3′ and 5′-CTAGAAGCATTTGCTGTGGACGATGGAGGGGCC-3′; 5-LO (exon primers for mature mRNA, 30 cycles), 5′-ACCATTGAGCAGATCGTGGACACGC-3′ and 5′-GCAGTCCTGCTCTGTGTAGAATGGG-3′; 5-LO (intron primers for pre-mRNA, 33 cycles), 5′-AGATGAGTCATGCTCACAGAACT-3′ and 5′-AGATCAGCATTAGGAACTAGCTT-3′.

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

DNA Isolation—Genomic DNA was isolated by proteinase K digestion and a 2-fold phenol/chloroform/isoamylalcohol extraction. After precipitation by the addition of 2 volumes of ethanol and 0.5 volumes of ammonium acetate (7.5 M), the DNA was fished, washed twice with ethanol (80%, v/v), and dissolved in water. Plasmid DNA was isolated from *Escherichia coli* using NucleoBond extraction columns (Macherey-Nagel) according to the manufacturer's protocol.

Bisulfite Sequencing—Genomic DNA (20–30 μg) was cleaved with *Kpn*I (5 units/μg DNA) for 2 h at 37 °C. The DNA was precipitated by ethanol and washed once, and the pellet was dissolved in 23 μl of water at 4 °C overnight. The DNA then was bisulfite-treated as described in (21) with the following modifications. The bisulfite incubation was carried out in a thermocycler (Techne Cyclogene) for 6 h with an initial alkaline denaturation step at 95 °C for 7 min followed by shock cooling on ice. The samples were incubated at 50 °C with bisulfite (3 M) under mineral oil for 6 h in the dark. The incubation was interrupted by heating to 95 °C for 30 s every 60 min. The bisulfite-modified DNA was purified with NucleoTrap CR (Macherey-Nagel) according to the manufacturer's protocols and desulfonated by addition of 0.1 volumes of 3 N NaOH at 37 °C for 15 min. After 2 min on ice, 0.2 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol were added to precipitate the DNA. The precipitated DNA was washed once with 80% (v/v) ethanol, dried, and dissolved in 20–50 μl of water overnight at 4 °C. The modified DNA was amplified by nested PCR with 35 cycles for each amplification using primers specific for the noncoding strand of the *5-LO* gene with the sequences (R = reverse, F = forward primer): 5′-ATTCTCTCATAAATCRCACTTCCTC-3′ (R−552/−528); 5′-CTCTCATAAATCRCTCTTCCTCTACA-3′ (R−549/−525); 5′-TTGTAGAAGGGTTTGTTTAGTAGGT-3′ (F+107/+131); 5′-AATTATTGGTTGTTAGTGGTTAYGGTGAT-3′ (F+14/+44).

Annealing temperatures were 62 and 57 °C for the first and second amplification, respectively. The amplified fragments were directly sequenced (377 sequencer, PerkinElmer Applied Biosystems). Additional primers used only for sequencing were: 3′-TACCCCAATCCCCTA-5′ (R−306/−290, sequences from 5′ → 3′); 3′-ACAACACTAAATACAAACACC-5′ (R−71/−51, sequences from 3′ → 5′); 3′-TAGCTGCGGCGCATT-5′ (F−215/−200, sequences from 5′ → 3′); 3′-GTATGGTGTGGGTTGTGG-5′ (F−13/+5, sequences from 5′ → 3′). The Big-Dye Terminator Cycle Sequencing kit (PerkinElmer Applied Biosystems) has been used under the recommended conditions for DNA sequence determination.

In Vitro DNA Methylation—Plasmid DNA was incubated for 24 h at 37 °C with *Sss*I methylase (New England BioLabs) at 1 unit/μg pDNA in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9) supplemented with 160 μM *S*-adenosylmethionine. For transfections, methylated pDNAs were purified by Nucleospin extract columns (Macherey-Nagel). Complete methylation at CG sites was confirmed by *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. The cells were then resuspended at 46×10^6 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). 40 micrograms of supercoiled plasmid DNA in 30 μl of water were added to the cell suspension. After 5 min at room temperature, electroporation was performed at 975 μF and 200 V using a Bio-Rad GenePulser. The cuvettes were immediately cooled on ice for 20 min. Then 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 the cell transfer as indicated. 6 h after transfection cells were harvested for luciferase assay.

Transfection of HeLa Cells—24 h prior to transfection, 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. Plasmid DNA (0.4 μg) was diluted into serum-free Dulbecco's modified Eagle medium and precomplexed with 5 μl of PLUS reagent (Life Technologies, Inc.) 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. The medium then was replaced by 200 μl of fresh serum-free medium, and the DNA-PLUS-Lipofectin reagent complexes were added to the cells and incubated for 5 h at 37 °C in 5% CO_2. 1 ml of medium containing 15% (v/v) FCS was added. 24 h after transfection, cells were washed once in phosphate-buffered saline (pH 7.4), and luciferase activity was determined as described below.

Luciferase Assays—After transfection of Mono Mac 6 (6 h) and HeLa (24 h), cells were washed once in phosphate-buffered saline containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$ and lysed in 100 μl of lysis buffer (Luciferase Reporter Gene Assay constant light signal kit, Roche Molecular Biochemicals). Luciferase activity was determined by monitoring light emission with a Microlumat Plus LB96V EG&G Berthold luminometer. Light emission signal was integrated for 5 s. Transfection efficiency was monitored and normalized by cotransfection with 1 μg (MonoMac6) and 1 μg (HeLa) pCMVSEAP using the Phospha-Light™ kit (Tropix) to determine the secreted placental alkaline phosphatase (SEAP) activity. Expression vectors pSG5VDR and pSG5RXR were cotransfected in all reporter gene experiments. pCMVluc was used as positive control.

RESULTS

Induction of the 5-LO Pathway in HL-60TB and U937 Cells by 5-Azadeoxycytidine—As shown previously, undifferentiated HL-60 and Mono Mac 6 cells do not exhibit significant 5-LO

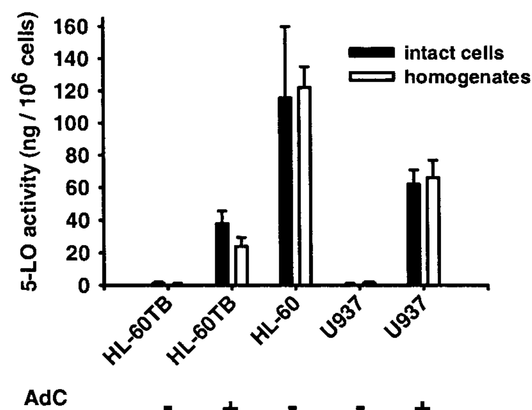


FIG. 1. **Effect of AdC on 5-LO activity in HL-60TB and U937 cells.** HL-60TB and U937 cells were grown in the absence or presence of AdC for 3 days. The cells were then cultured in the absence of additives for 4 days before differentiation was initiated by addition of TGFβ and $1,25(OH)_2D_3$. After 4 days cells were harvested, and 5-LO activity was determined. Values are given as mean + S.E. of three independent experiments.

activity. However, after differentiation with TGFβ and $1,25(OH)_2D_3$ there is a strong up-regulation of cellular activity (Ref. 7 and Fig. 1). In contrast, HL-60TB and U937 cells have no significant 5-LO activity after differentiation with TGFβ and $1,25(OH)_2D_3$ (Fig. 1). However, pretreatment of U937 and HL-60TB cells with AdC leads to high 5-LO activity after differentiation.

Similar effects were observed when 5-LO pre-mRNA and mature mRNA were analyzed by RT-PCR (Fig. 2). In HL-60 cells, TGFβ and $1,25(OH)_2D_3$ up-regulated 5-LO pre-mRNA expression, but only modest levels of 5-LO pre-mRNA were observed in HL-60TB cells after differentiation with TGFβ and $1,25(OH)_2D_3$. However, after pretreatment of HL-60TB cells with AdC, similar 5-LO pre-mRNA levels were found as in wild-type HL-60 cells. In U937 cells, AdC enhanced expression of 5-LO pre-mRNA and mature mRNA about 2- and 4-fold, respectively (Fig. 2). Stronger effects of AdC were observed in HL-60TB cells. AdC up-regulated 5-LO pre-mRNA and mature mRNA about 6.6- and 23-fold in undifferentiated cells and 4.8 and 8.5-fold in cells differentiated with $1,25(OH)_2D_3$ and TGFβ, respectively. Thus, similar to Mono Mac 6 cells, the changes in 5-LO RNA levels were more pronounced when mature RNA was analyzed compared with pre-mRNA in HL-60 and U937 cells. Furthermore, the data indicate that all three cell lines respond to TGFβ and $1,25(OH)_2D_3$ and show 5-LO RNA induction, although the achieved mRNA levels were lower in HL-60TB and U937 cells. Interestingly, AdC treatment also enhanced 5-LO RNA expression in undifferentiated cells, which suggests that its effects are at least in part independent of the cellular actions of TGFβ and $1,25(OH)_2D_3$.

Analysis of 5-LO Promoter Methylation Patterns—Activation of gene expression by AdC is assumed to be caused by inhibition of DNA methylation, which subsequently leads to partial demethylation of CpG sites and up-regulation of the activity of methylation-sensitive gene promoters. Because basal 5-LO promoter activity is induced by multiple Sp1/Egr-1 binding sites located in a C+G-rich region with many putative CpG methylation sites, it was of interest to study the methylation pattern of the 5-LO core promoter in HL-60, HL60-TB, and U937 cells and to correlate it with the 5-LO expression data shown above.

Genomic DNA isolated from all three cell lines was digested with *Kpn*I and subjected to DNA methylation analysis by genomic bisulfite sequencing as described under "Materials and Methods." Fig. 3 shows the methylation pattern of the

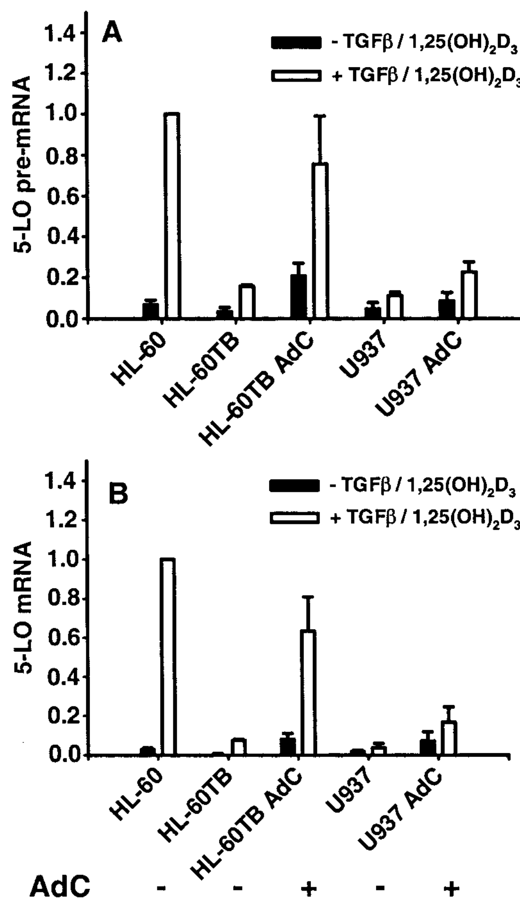
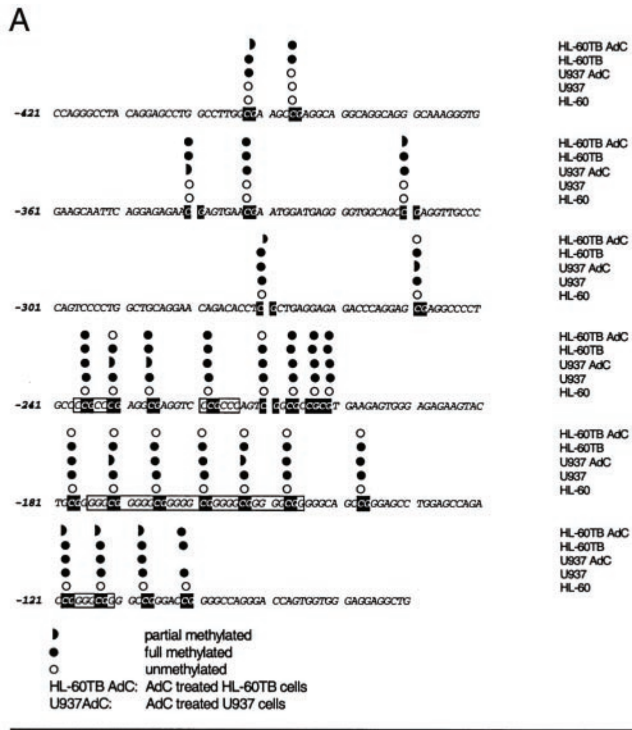


FIG. 2. **RT-PCR analysis of 5-LO RNA expression in HL-60, HL-60TB, and U937 cells.** Cells were grown in the absence or presence of AdC for 3 days. AdC was then removed, and after 4 days in culture cell differentiation was initiated by $1,25(OH)_2D_3$ and TGFβ where indicated in the legend. 5-LO pre-mRNA (*A*) and mature mRNA (*B*) were analyzed by RT-PCR and quantified as described under "Materials and Methods." Values are given as mean + S.E. of three independent experiments.

5-LO core promoter region of the indicated cell lines. In HL-60 cells showing prominent 5-LO expression the 5-LO core promoter was completely unmethylated, whereas it was heavily methylated in HL-60TB and U937 cells that show only low 5-LO expression. In U937 cells all CpG sites of the core promoter required for basal activity were methylated, whereas CpG sites located more upstream were unmethylated. Thus, a good correlation exists between the methylation status of the 5-LO promoter and 5-LO gene expression in HL-60, HL-60TB, and U937 cells (Figs. 2 and 3). Treatment of HL-60TB and U937 cells with AdC led to partial demethylation of the 5-LO promoter, although the demethylating effect of AdC was stronger in HL-60TB than in U937 cells (Fig. 3).

5-LO Promoter Activity Is Methylation-sensitive—To check whether 5-LO promoter activity is regulated by $1,25(OH)_2D_3$/TGFβ and/or DNA methylation, reporter gene assays were performed. The plasmids N10 and N7 and two deletion constructs (pN10ΔGC and pN7ΔGC) lacking the core region containing the five direct and two inverted GGGCGG repeats were used (Fig. 4). HeLa and Mono Mac 6 cells were transiently transfected with these plasmids, and promoter activities were determined as described under "Materials and Methods."

Deletion of the region −258 to −96 containing the Sp1 and Egr-1 binding sites close to the transcription initiation site (plasmids N10ΔGC and N7ΔGC) significantly reduced 5-LO promoter activity in transfected HeLa cells (Fig. 5*A*) and al-



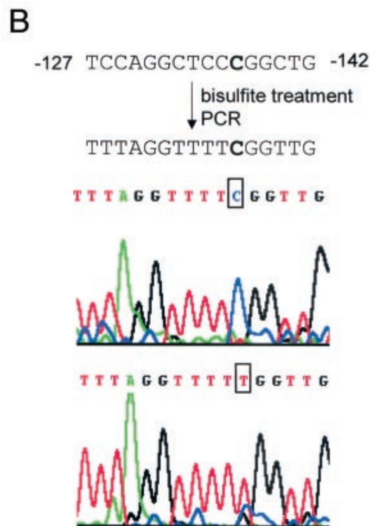


FIG. 3. **Methylation pattern of the 5-LO core promoter.** Genomic DNA was isolated from the indicated cell lines. Cells were treated for 3 days with AdC before DNA preparation as indicated. DNA methylation was analyzed by bisulfite sequencing. *A*, methylation pattern of the 5-LO promoter in various cell lines. Sp1 binding sites are boxed. *B*, example of bisulfite sequencing of the 5-LO promoter (position −127 to −142). In HL-60TB cells, no C/T conversion occurs at the CG-site (−137), indicating methylation (*upper panel*). AdC treatment of the HL-60TB cells leads to demethylation of this site, which is indicated by C/T conversion (*lower panel*).

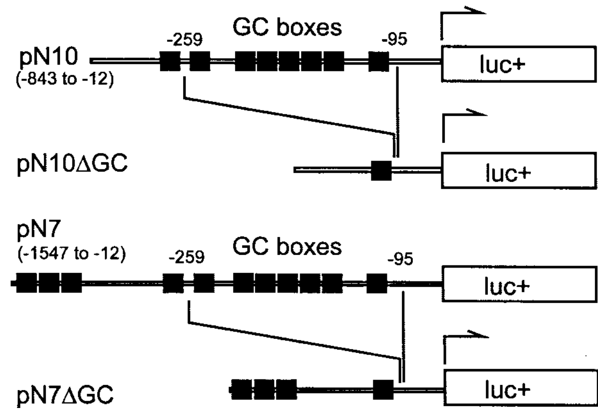


FIG. 4. **Schematic representation of the 5-LO promoter luciferase reporter gene constructs.**

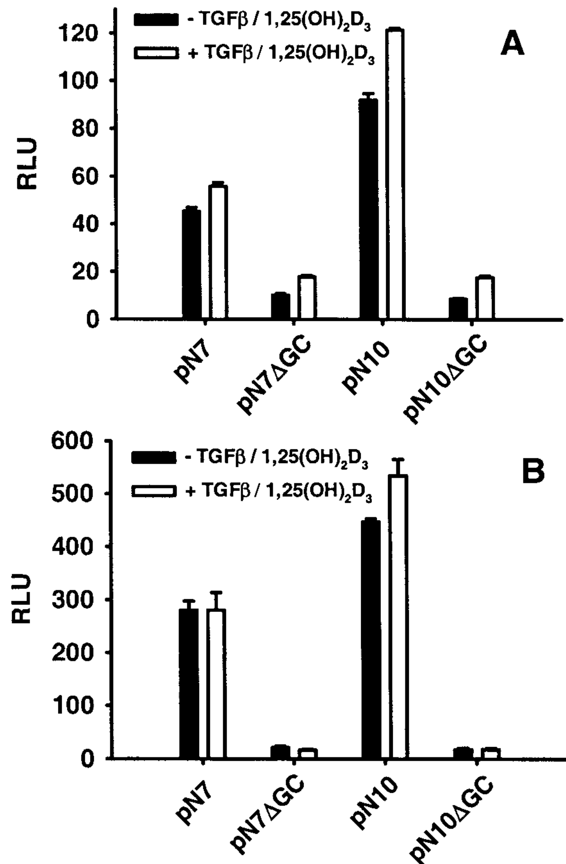


FIG. 5. **Deletion mutant analysis of the 5-LO promoter.** HeLa cells (*A*) and Mono Mac 6 cells (*B*) were transiently transfected with the indicated plasmids. After transfection cells were grown with or without TGFβ (1 ng/ml) and $1,25(OH)_2D_3$ (50 nM), and 5-LO promoter activity was determined. Results are given as luciferase activity normalized to cotransfected SEAP reporter activity (mean + S.E. of at least three experiments).

most abolished reporter gene expression in the 5-LO-positive monocytic cell line Mono Mac 6 (Fig. 5*B*). Surprisingly, $1,25(OH)_2D_3$ and TGFβ did not increase 5-LO promoter activity in reporter gene assays in Mono Mac 6 cells (Fig. 5), although both agents strongly induced 5-LO gene expression in this cell line (7) and although at least one vitamin D response element was present in the 5-LO promoter (22). In control experiments, $1,25(OH)_2D_3$ and TGFβ strongly activated (25-fold) a promoter construct containing the 4×-concatemerized DR3-type pig osteopontin vitamin D response element (core sequence ATGGGTCATATGGTTCA) in front of the thymidine kinase promoter. From these results it was concluded that there is no induction of 5-LO promoter activity by $1,25(OH)_2D_3$ and TGFβ, at least under the experimental conditions of transient reporter gene assays.

To study the effect of DNA methylation on 5-LO promoter activity and to investigate possible relationships between DNA methylation and $1,25(OH)_2D_3$/TGFβ signaling in the regulation of 5-LO promoter activity, the N10 and the N10ΔGC plasmids were methylated by *Sss*I methylase *in vitro*. Subsequently, Mono Mac 6 and HeLa cells were transfected with the

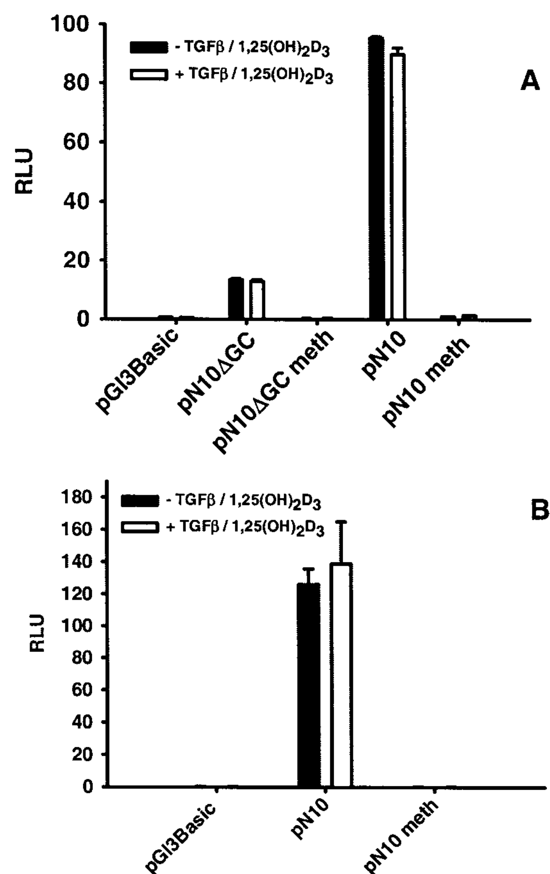


FIG. 6. **Effect of CpG methylation on 5-LO promoter activity.** HeLa cells (*A*) and Mono Mac 6 cells (*B*) were transiently transfected with the 5-LO promoter luciferase reporter gene constructs pN10 and the corresponding deletion variant pN10ΔGC. The plasmids were methylated by *Sss*I methylase or mock methylated. After transfection, cells were grown with or without TGFβ (1 ng/ml) and 1,25(OH)$_2$D$_3$ (50 nM). pGL3Basic was used as negative control. Results are given as luciferase activity normalized to cotransfected SEAP reporter activity (mean + S.E. of at least three experiments).

methylated or unmethylated plasmids, and promoter activity was determined. Methylation completely suppressed 5-LO promoter activity in both cell lines, and transcriptional activities of the methylated N10 and N10ΔGC plasmids were comparable with the negative control (pGL3Basic promoterless plasmid) (Fig. 6). Interestingly, 1,25(OH)$_2$D$_3$ and TGFβ could not activate transcription from the methylated constructs in HeLa and Mono Mac 6 cells, indicating that both hormones can not induce demethylation of the 5-LO promoter under these experimental conditions (Fig. 6). The relative changes in the 5-LO promoter activities by DNA methylation are summarized in Table I. Methylation of the 5-LO promoter plasmid pN10 led to a 68- and 655-fold repression of promoter activity in HeLa and Mono Mac 6 cells, respectively, whereas transcriptional repression by DNA methylation was much lower with the cytomegalovirus-driven pCMVluc vector (18- and 7-fold, respectively), which was used as positive control. Similar results were obtained when the 5-LO promoter insert of the pN7 plasmid was selectively methylated. Thus, the 5-LO promoter (−863 to −12) was excised from the pN7 plasmid, *in vitro* methylated by *Sss*I methylase, reinserted into the plasmid, and transfected into HeLa cells. Under these conditions, methylation led to a 38-fold repression of 5-LO promoter activity.

TABLE I
Effects of DNA methylation by SssI methylase on luciferase reporter gene activity

Cells	Plasmid	Effects of DNA methylation (-fold repression ± S.E., n = 3)	
		− TGFβ/1,25(OH)$_2$D$_3$	+ TGFβ/1,25(OH)$_2$D$_3$
Mono Mac 6 cells	pN10	655 ± 120	631 ± 161
	pCMVluc	7 ± 1	9 ± 1
HeLa cells	pN10	68 ± 8	95 ± 5
	pCMVluc	18 ± 4	19 ± 2

DISCUSSION

The *5-LO* gene is expressed in a tissue- and cell differentiation-specific manner (3). In view of this, the features of the 5-LO promoter (such as the (G+C)-rich region, multiple GC-boxes, and lack of TATA or CCAAT sequences) were rather surprising, because the 5-LO promoter resembles promoters for so-called housekeeping genes that are ubiquitously expressed (23). Previous reports demonstrated that the promoter region −212 to −88 containing multiple Sp1 and Egr-1 binding sites is important for basal transcriptional activity (10, 11). This promoter region has been found to be transcriptionally active in cells lines (*e.g.* HeLa cels) that do not show endogenous 5-LO expression (10).

Naturally occurring mutations were found in the 5-LO promoter that obviously 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 (13). Beside binding sites for Egr and Sp transcription factors, consensus sequences for other transcription factors like NF-κB, GATA, myb, and AP family members are present in the promoter region (24). However, to date the mechanisms involved in the cell-specific and/or stimulus-dependent regulation of the 5-LO promoter are unknown.

When we studied 5-LO expression in a variety of cell lines and sublines, two myeloid cell lines (U937 and HL-60TB cells) that lack 5-LO expression in response to well known differentiation agents were identified. We found that the DNA demethylating agent AdC activates 5-LO expression in these cell lines. When methylation of CpG sites in the 5-LO promoter was analyzed, we found that the 5-LO promoter was heavily methylated in U937 and HL-60TB cells, whereas it was completely unmethylated in the 5-LO-positive cell line HL-60. Furthermore, reporter gene assays revealed that methylation of the 5-LO core promoter by *Sss*I methylase leads to an almost complete loss of transcriptional activity. Thus, taken together, our data clearly suggest that 5-LO promoter activity is regulated by DNA methylation and that suppression of 5-LO expression in the respective cell lines is obviously caused by DNA methylation.

In vertebrates, the genomes of somatic cells are globally methylated (with the exception of CpG-rich regions (CpG islands). CpG methylation correlates with alterations in chromatin structure and gene silencing (25). Aberrant methylation of promoter regions has been shown to be involved in cancer development via the suppression of tumor suppressor genes. Approximately half of all genes in mouse and humans contain CpG islands. These are mainly housekeeping genes that have a broad expression pattern, but 40% of the genes with a tissue- or cell type-specific expression pattern are also represented. Usually DNA demethylation is linked to activation of gene transcription. Several enzymes with DNA methylase activity were identified and functionally characterized (for review see Ref. 26), but less is known about enzymes and signaling pathways involved in demethylation of CpG islands. At present, the mechanisms that lead to cell type-specific and cell differentiation-related methylation and demethylation of CpG islands are unknown. Thus, 5-LO might represent an interesting candidate to elucidate such mechanisms.

Much more knowledge has been gained about the connection between the methylation status of gene promoters and transcriptional activity of the respective genes. Methylated CpG sites are recognized by a variety of methyl-CpG-binding proteins (*e.g.* MBDs1–3, MeCP2), which are associated either directly or indirectly with histone deacetylases (27). Thus, chromatin condensation mediated by recruitment of histone deacetylases seems to be one mechanism of gene silencing by CpG methylation. Concerning 5-LO, inhibition of histone deacetylases by trichostatin A (at 330 nM) did not lead to activation of 5-LO gene expression in U937 and HL-60TB cells, although general cellular histone acetylation by trichostatin A was detectable by AUT-gel electrophoresis (data not shown), suggesting that repression of 5-LO gene expression by DNA methylation does not depend primarily on recruitment of trichostatin A-sensitive histone deacetylases but seems to be mediated by other mechanisms. For 15-lipoxygenase, it has been shown that trichostatin A leads to the up-regulation of 15-LO mRNA expression (28), whereas AdC has no effect (data not shown). Another gene of the leukotriene pathway that is regulated by DNA methylation is the leukotriene B4 receptor BLT1. It has been shown with reporter gene assays that methylation of the BLT1 promoter reduces its activity to about 15% of the control (29).

Recently, we have shown that up-regulation of 5-LO mRNA expression in Mono Mac 6 cells by $1,25(OH)_2D_3$ and TGFβ is in part due to the induction of transcript elongation and mRNA maturation (8, 9), whereas no significant effects of both agents were found on 5-LO promoter activity in Mono Mac 6 cells with reporter gene assays (this study) and nuclear run-on assays (9). Here we have shown with reporter gene assays that in contrast to $1,25(OH)_2D_3$ and TGFβ, DNA methylation regulates 5-LO promoter activity. First, there was a strong inhibition of 5-LO promoter activity by DNA methylation. Second, AdC treatment of U937 and HL-60TB cells induced 5-LO expression and led to 5-LO promoter demethylation. AdC-induced 5-LO promoter demethylation was much stronger in HL-60TB cells than in U937 cells, which correlated with a stronger induction of 5-LO pre-mRNA (1.8- *versus* 6.6-fold) and mature mRNA (3.9- *versus* 23-fold), respectively. It has to be considered that modest changes in the methylation status of CpG-sites (*e.g.* from 90 to 70%) that could already affect 5-LO promoter activity are hardly detectable by the methylation-specific DNA sequencing method.

Taken together, our experiments suggest that demethylation of the 5-LO promoter is required for high 5-LO gene transcription. Both AdC treatment and $1,25(OH)_2D_3$/TGFβ stimulated 5-LO mRNA expression and the combination of both treatments had additive affects. In contrast to DNA methylation, $1,25(OH)_2D_3$/TGFβ did not affect 5-LO promoter activity under our experimental conditions but rather seemed to be related to post-transcriptional effects such as transcript elongation and maturation. Furthermore, in reporter gene assays we found no evidence that $1,25(OH)_2D_3$ and TGFβ can induce promoter activity when 5-LO promoter constructs are methylated, suggesting that both effects are independent from each other. Our data obtained with HL-60TB and U937 cells demonstrate that 5-LO promoter demethylation is required for the high level induction of 5-LO gene expression by inducers such as $1,25(OH)_2D_3$ and TGFβ.

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