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Biochemistry

Characterization of the human 5-lipoxygenase gene promoter

(leukotrienes/transcriptional regulation/nuclear protein)

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ABSTRACT **Nucleotide sequences that direct transcription of the human 5-lipoxygenase gene have been examined by ligation to the chloramphenicol acetyltransferase gene and determination of chloramphenicol acetyltransferase activity in transfected HeLa and HL-60 cells. Various lengths of 5′-flanking sequences up to 5.9 kilobase pairs 5′ of the transcriptional initiation sites were tested. Two positive and two negative apparent regulatory regions were seen. Part of the promoter sequence (−179 to −56 from ATG), which includes five repeated GC boxes (the putative Sp1 binding sequence) was essential for transcription in both HeLa and HL-60 cells. Gel-shift assays (using the DNA fragment −212 to −88) revealed that the transcriptional factor Sp1 could bind to this region of the 5-lipoxygenase promoter. Furthermore, HL-60 nuclear extracts contained specific nuclear factor(s) binding to 5-lipoxygenase promoter DNA, which could not be detected in HeLa cell nuclear extracts.**

The enzyme 5-lipoxygenase (arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34) catalyzes transformation of arachidonic acid to (5*S*)-hydroperoxy-6-*trans*-6,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and further to leukotriene A_4 [(5*S*)-6-oxido-7,9,11-*trans*-14-*cis*-eicosatetraenoic acid]. The enzymes participating in leukotriene biosynthesis have been the subject for several recent studies, and cDNA clones corresponding to 5-lipoxygenase have been isolated (for review, see ref. 1). Also, the human 5-lipoxygenase gene has been isolated and characterized (2). Several features of the putative promoter region were characteristic for so-called housekeeping genes (3) (no TATAA or CCAAT, G+C-rich, multiple GGGCGG sequences). This paper describes further studies regarding the 5-lipoxygenase gene promoter.† In particular, a sequence containing five GGGCGG repeats was required for efficient transcription of chloramphenicol acetyltransferase (CAT) gene constructs, and gel-shift assays showed that transcription factor Sp1 could bind to DNA containing this G+C-rich region.

MATERIALS AND METHODS

Plasmid Constructions. Plasmid pUCOCAT was constructed from pCAT3M (4) and pUC19 (5). 5-Lipoxygenase gene promoter DNA was excised from recombinant phage 1x12A [5.9 kilobase (kb) *Kpn* I–*Bst*EII fragment] (2). Insertion into pUCOCAT provided 5LO5900CAT, from which several deletion derivatives were prepared (compare Fig. 2). A detailed description of the plasmid constructions can be obtained from the authors.

Cell Culture and DNA Transfection. HeLa and HeLaS3 cells (maintained in this institute) were cultivated in Dulbecco's modified Eagle's medium supplemented with 5% (vol/vol) fetal calf serum. HL-60 cells (American Type Culture Collection) and K-562 were maintained in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum. HepG2 and RBL1 cells were maintained in Eagle's medium and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% (vol/vol) fetal calf serum. Plasmids for DNA transfection were prepared by alkaline lysis and purified by CsCl centrifugation. For transfections the following methods were used: HeLa and HepG2, calcium phosphate precipitation followed by 15% (vol/vol) glycerol shock (2 min) (6); HL-60 and RBL1, DEAE-dextran (250 μg/ml) followed by chloroquine (0.1 or 0.2 mM, 60 min) (7); and K-562, electroporation [230 V, 960 μF, in phosphate-buffered saline (PBS)] (8). Cells were harvested within 48 hr after transfection, washed with PBS, and suspended in 100 μl of 0.25 M Tris·HCl, pH 7.8, for sonication. After centrifugation (15,000 × *g* for 10 min), the supernatants were assayed for protein (9), as well as for CAT activity.

CAT and Luciferase Assays. CAT activity was measured as described (10). To estimate transfection efficiencies of HeLa cells, the firefly luciferase containing plasmid pSV2AL 5′ was cotransfected, and the luciferase activity (11) was taken as an estimation of the transfection efficiency. CAT assay results from different transfection experiments could thus be normalized and compared.

RNA Preparation and S1 Nuclease Protection Assays. For preparation of RNA ≈10^8 HeLa cells (10 to 14 15-cm dishes) were transfected with 100 μg of plasmid DNA per dish. After 36 hr, RNA was extracted (12). Poly(A)$^+$ RNA was purified on oligo(dT)-cellulose. The probe was prepared from 5LO292CAT by digestion with *Bgl* II, labeling of the 5′ end, and digestion with *Eco*RI. The labeled DNA probe was purified by PAGE. Poly(A)$^+$ RNA (40 μg) was hybridized with probe (3 × 10^5 cpm, 30 ng) in a solution containing 80% (vol/vol) formamide overnight at 53°C. Subsequently S1 nuclease digestion was performed (2).

DNA Sequence Analysis. This technique was performed as described (13).

Preparation of Nuclear Proteins and Detection of DNA-Binding Proteins. HeLaS3 and HL-60 nuclear extracts were prepared, and gel-retardation assay was performed as described (14, 15).

Oligonucleotides. 5′-GGGGCGGGGCTGCA-3′ and 5′-GCCCCGCCCCTGCA-3′ were hybridized, after phosphorylation ligation provided concatemers. The monomer and concatemer forms were used as Sp1 competitors in gel-retardation analysis.

RESULTS

Identification of the Promoter Region. To identify DNA sequences regulating 5-lipoxygenase gene expression, the region (−5900 to +20) was excised from the genomic clone 1x12A (2) and inserted into a plasmid already containing the CAT reporter gene (+1 is adenine in the ATG start codon).

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Abbreviations: CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38191).

The resulting plasmid (5LO5900CAT) was transiently introduced to HeLa and HepG2 cells, as well as to myeloid cell lines HL-60, K-562, and RBL1. Although several methods were tried, transfection efficiencies for the myeloid cells were low (see *Materials and Methods* for optimal procedure for each cell line). Nevertheless, the 5900-base pair (bp) DNA fragment clearly had promoter activity in all cells tested (Fig. 1). Before further analysis of the 5-lipoxygenase gene promoter, it was confirmed that the CAT expressions seen after transfection with 5LOCAT derivatives were truly controlled by the 5-lipoxygenase gene promoter. For this purpose poly(A)$^+$ RNA was prepared from HeLa cells transfected with three different 5LOCAT constructs, and the transcriptional initiation sites for the chimeric RNA species were determined by S1 nuclease protection assay (Fig. 2*A*). RNA–DNA hybrids originating between nucleotides −65 and −62 were found. This is practically identical to the initiation of 5-lipoxygenase gene transcription in human leukocytes, showing that observed CAT activities were controlled by the 5-lipoxygenase gene promoter.

Expression of endogenous 5-lipoxygenase in these cell lines was determined by Northern (RNA) blots. HeLa and HepG2 cells were negative, whereas K-562 (weak), RBL1, and HL-60 (particularly after treatment with dimethyl sulfoxide) cells were positive (data not shown). For further studies we choose the human epithelial cell line HeLa (for ease of transfection) and also the promyelocytic cell line HL-60, which expresses the 5-lipoxygenase gene endogenously.

Some aspects regarding transfection of HL-60 cells should be mentioned. Thus, when HL-60 cells were transfected with the negative control plasmid pUCOCAT (lacking a promoter before the CAT gene), unexpectedly high background CAT activity was obtained (≈80% of the CAT activity after transfection with positive control plasmid pSV2CAT containing simian virus 40 early promoter). For HeLa cells, the corresponding background was <1%. However, CAT activity in HL-60 could still be determined because addition of PMA to cells transfected with plasmid containing 5-lipoxygenase promoter stimulated CAT activity 1.5- to 3-fold (PMA at 30 ng/ml was present 24–48 hr after transfection). PMA also stimulated CAT activity after transfection with pSV2CAT but had almost no effect on cells transfected with pUCOCAT. PMA stimulation of the CAT activities were also seen with HeLa and K-562 cells (Fig. 1 and Table 1).

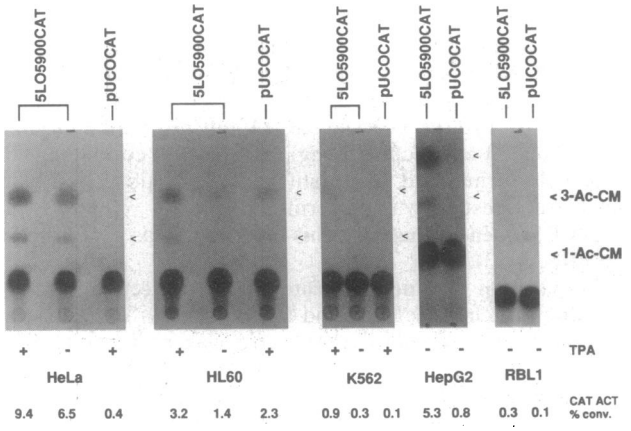

FIG. 1. Human 5-lipoxygenase promoter-driven expression of CAT. Autoradiograms show conversion of [^{14}C]chloramphenicol to monoacetylated derivatives by extracts from HeLa, HL-60, K-562, HepG2, and RBL1 cells, transfected with 20 μg of 5LO5900CAT or pUCOCAT. Transfected cells were treated with (+) or without (−) phorbol 12-myristate 13-acetate (PMA) (TPA) (30 ng/ml) 24–48 hr after transfection. Extracts (250–1000 μg of proteins—equivalent amounts for each set of experiments) were analyzed for CAT activity as described (10) (except that 5-fold more acetyl CoA was used for assays of K-562, HL-60, and RBL1 cells). Incubation times were 2 hr for HeLa and HepG2 cells, 8 hr for K-562 cells, and 16 hr for HL-60 and RBL1 cells. Acetylation of [^{14}C]chloramphenicol was determined by thin-layer chromatography. After autoradiography, acetylated products were excised, and radioactivity was counted in a liquid scintillation counter. Positions of chloramphenicol (CM), 1-acetylchloramphenicol (1-Ac-CM), and 3-acetylchloramphenicol (3-Ac-CM) are indicated by arrows. Conversions (%) of [^{14}C]chloramphenicol to acetylated forms are shown.

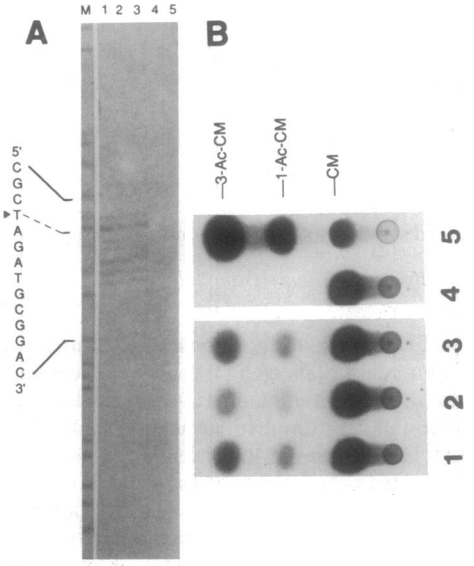

FIG. 2. Determination of transcriptional initiation sites of 5LO-CAT constructs in HeLa cells by S1 nuclease mapping. (*A*) Poly(A)$^+$ RNA isolated from transfected cells and used for S1 nuclease protection assays. Lanes: M, Maxam–Gilbert A+G reaction of the 5′ end-labeled probe; 1, RNA from cells transfected with 5LO931CAT; 2, RNA from cells transfected with 5LO727CAT; 3, RNA from cells transfected with 5LO292CAT; 4, RNA from cells transfected with pUCOCAT; 5, RNA from mock-transfected cells. (*B*) CAT assays of HeLa cells transfected with the 5LOCAT constructs were done in parallel with the S1 nuclease protection assays. One hundred micrograms of protein was incubated with [^{14}C]chloramphenicol and acetyl CoA for 2 hr. Lanes: 1–4, as in *A*; 5, pSV2CAT positive control.

It should also be noted that transfections of HL-60 cells (PMA treated) with 5-lipoxygenase gene chimeras gave relatively higher CAT activities than HeLa cells (PMA treated). Thus, in HL-60 cells 5LO5900CAT or 5LO179-14CAT gave CAT activities as high as pSV2CAT (the control plasmid with simian virus 40 promoter). In HeLa cells, the same chimeras gave CAT activities that were only 13 and 24% that obtained with pSV2CAT.

We also attempted to transfect HL-60 cells treated with dimethyl sulfoxide. However, dimethyl sulfoxide reduced

Table 1. Relative CAT activities of HeLa and HL-60 cells transfected with 5LOCAT constructs

	HeLa	HL-60
pSV2CAT	1.00 (0.31)	1.00
pUCOCAT	0.005 (0.004)	0.44
5LO5900CAT	0.13 (0.08)	0.98
5LO179-14CAT	0.24 (0.09)	1.02

HeLa (1 × 10^6 cells) and HL-60 (5 × 10^6 cells) were transfected with 20 μg of plasmids per 100-mm dish or 250-ml bottle. After 20–24 hr, PMA (30 ng/ml) was added and cultures were continued for 24 hr before assay. CAT activities were related to that obtained with pSV2CAT. The variation from mean value was <40% in HeLa cell and <35% in HL-60 cell experiments. Values in parentheses are the relative activities obtained without PMA stimulation.

CAT activities of transfected cells severely so that no reliable results were obtained.

Deletion Analysis of the Promoter Region. A set of plasmids with different deletions in the 5-lipoxygenase gene promoter was constructed, and HeLa cells were transfected. The resulting CAT activities, reflecting the activities of the corresponding promoter parts, are shown in Fig. 3.

First, it could be concluded that −179 to −56 stretch of the 5-lipoxygenase gene promoter was essential for full activity. Also, this DNA segment contained elements, apparently involved in induction by PMA of 5-lipoxygenase gene expression (Table 1). Successive deletion mutants revealed two positive regulatory elements (−5900 to −3700 and −931 to −854) and two negative regulatory elements (−3400 to −1557 and −727 to −292).

To rule out the possibility that these observations were from differences in translational efficiency, six plasmids lacking the translational initiation region were constructed (Fig. 3*B*). When HeLa cells were transfected with these plasmids, the relationships between the resulting CAT activities were practically the same as with the original plasmids (Fig. 3*A*).

Specific Interaction of 5-Lipoxygenase Gene Promoter DNA with Nuclear Proteins from HeLa and HL-60 Cells. A *Nar* I–*Bgl* II fragment containing the DNA (−212 to −88) essential for promoter activity was excised from plasmid 5LO292-88CAT. After labeling with [α-^{32}P]ddATP and terminal deoxynucleotidyltransferase, binding of proteins from nuclear extracts to the probe was examined by gel-retardation assay. First, nuclear extracts from HeLaS3 cells were examined; nonadherent HeLaS3 cells were used in this case for convenient preparation of nuclear proteins. When gel-retardation assay was done with HeLa proteins in the presence of nonspecific competitor poly(dI·dC), several shifted bands appeared in electrophoresis; the two major bands are marked with arrows in Fig. 4*A*. Next, the assay was done in the presence of monomers of the Sp1 competitor oligonucleotide [d(GGGGCGGGGCTCGA)]; the shifted bands were still formed. However, with concatemers of the Sp1 competitor, [d(GGGGCGGGGCTCGA)$_n$] formation of the shifted bands was inhibited. Similar findings were published regarding the Sp1-binding site(s) of the human methallothionein IIA promoter (15). These results indicate that transcription factor Sp1 was present in the HeLa cell nuclear extracts and could bind to this stretch of promoter DNA (−212 to −88).

Nuclear extracts were also prepared from HL-60 cells, either nondifferentiated or after treatment with PMA (24 hr) or dimethyl sulfoxide (120 hr). When gel-retardation assays were done with the three different HL-60 extracts, the same shifted bands appeared in all cases (see arrows in Fig. 4*B*; no difference between nondifferentiated and differentiated cells). However, these bands were not present in assays with nuclear proteins from HeLa cells. On the other hand, the major bands obtained with HeLa nuclear proteins were poorly formed with HL-60 nuclear proteins. This result indicates that different factors binding to the 5-lipoxygenase promoter region exist in HeLa and HL60 cells, respectively.

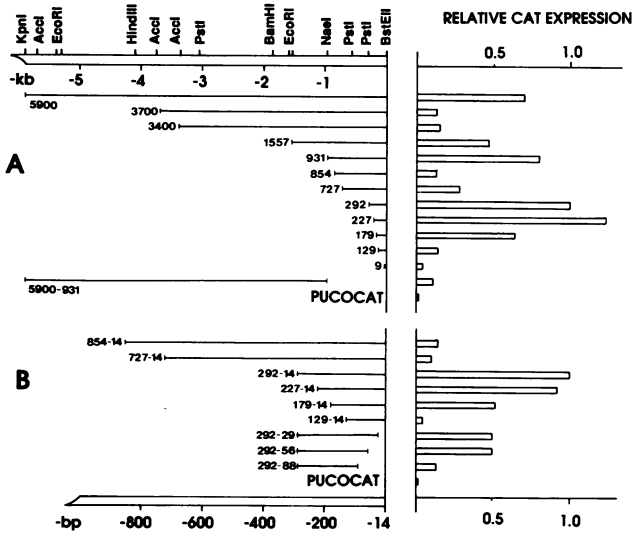

FIG. 3. Structure and activity of 5LOCAT chimeric genes. HeLa cells (1–2 × 10^6 cells) were transfected with 20 μg of recombinant plasmids and 1 or 2 μg of pSV2ALΔ5′. CAT assays were done by using 250–500 μg of protein and 2- to 6-hr incubations at 37°C. Results were normalized after luciferase assay of the same extract or an equivalent sample. (*A*) DNA fragment derived from the 5-lipoxygenase genomic clone 1x12A used for constructions is shown on top as a wide bar with restriction enzyme cleavage sites above. Nucleotide positions of the 5′-flanking sequence are numbered from the translation start codon. Relative CAT activities of transfected HeLa cells are expressed in relation to values obtained with 5LO292CAT. (*B*) CAT activities derived from deletion plasmids lacking the translational initiation region are related to values obtained with 5LO292-14CAT.

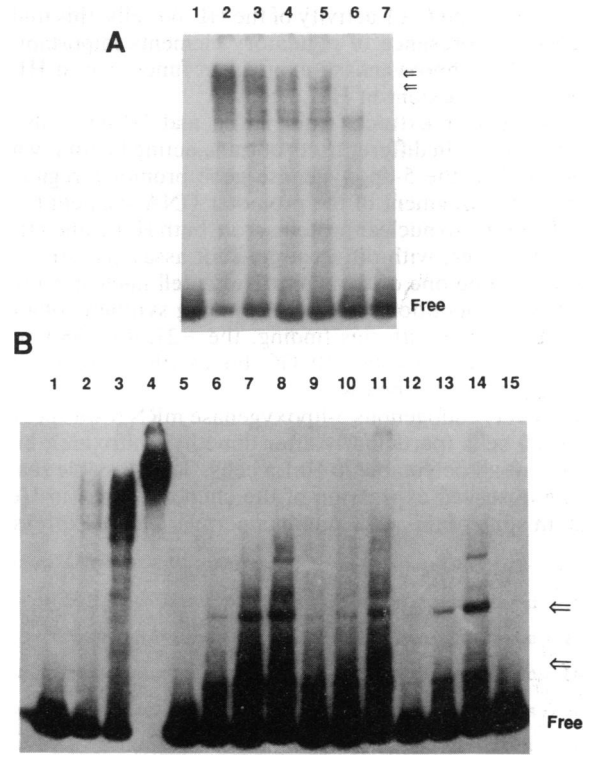

FIG. 4. Gel-retardation analysis of nuclear protein extracts from HeLa and HL-60 cells. (*A*) Binding reactions were conducted using the radiolabeled *Nar* I–*Bgl* II fragment (2 × 10^5 cpm, 90 fmol, 3.5 ng), poly(dI·dC) (200 μg/ml), and nuclear extracts from HeLa cells (2 μg of protein). Lanes: 1 and 7, probe; 2, probe with extract; 3, probe plus 50 molar excess of monomer Sp1 competitor with extract; 4, probe plus 250 molar excess of monomer competitor with extract; 5, probe plus 50 molar excess of concatemer form of Sp1 competitor with extract; 6, probe plus 250 molar excess of concatemer competitor with extract. Bands blocked by the concatemer form of the Sp1 competitor are indicated by arrows. Free DNA is also indicated. (*B*) Comparison of gel-retardation patterns obtained with HeLa and HL-60 nuclear extracts. Lanes: 1, 5, and 15, probe; 2–4, probe plus extract of HeLa cells (1.0, 3.0, and 9.0 μg of protein, respectively); 6–8, probe plus extract of nontreated HL-60 cells (1.0, 3.0, and 9.0 μg of protein, respectively); 9–11, probe plus extract of PMA-treated HL-60 cells (1.0, 3.0, and 9.0 μg of protein, respectively); 12–14, probe plus extract of dimethyl sulfoxide-treated HL-60 cells (1.0, 3.0, and 9.0 μg of protein, respectively). Distinct bands seen with HL-60 nuclear extracts are indicated by arrows.

DISCUSSION

The human 5-lipoxygenase gene was recently cloned and characterized (2). The promoter was found to be G+C-rich, and it lacked TATA and CCAAT sequences. In this study the 5-lipoxygenase gene promoter has been further analyzed. Chimeric genes between various parts of the promoter and the CAT reporter gene were constructed. Evidence for the essential role for a −179 to −56 DNA stretch (containing the transcriptional initiation sites and most of the GC boxes) was obtained.

Expression of the human 5-lipoxygenase gene appears to be regulated in a cell-specific manner. Substantial production of 5-lipoxygenase metabolites is well documented for cells of myeloid lineage such as granulocytes, basophils, and macrophages. Differentiation of immature cells is associated with enhanced 5-lipoxygenase activity, as shown in studies using HL-60 cells (16–18), chicken myelomonocytic cells (19), and mouse mastocytoma cells (20). Some of our findings may reflect the cell-specific expression of 5-lipoxygenase.

Thus, the relative CAT activity obtained after transfection of HL-60 cells was higher than in HeLa cells (see *Results* and Table 1). Assuming that this result was not only due to the high background CAT activity of the HL-60 cells, this finding suggests the presence of regulatory elements important for cell-specific 5-lipoxygenase expression functional in HL-60 but to a lesser extent in HeLa cells.

Also, nuclear extracts from HeLa and HL-60 cells appeared to contain different sets of trans-acting factors, which could bind to the 5-lipoxygenase gene promoter region. A radiolabeled fragment of the promoter DNA segment (−212 to −88) bound to nuclear proteins from both HeLa and HL-60 cells—however, with different gel-shift assay patterns. Sp1 appeared to be one of the major HeLa cell nuclear proteins based on competition with a Sp1-binding synthetic oligonucleotide. In line with this finding, the −212 to −88 stretch contains 6 (of the total 10) GC boxes (the putative Sp1-binding motifs; see below).

However, endogenous 5-lipoxygenase mRNA was present in HL-60 cells (particularly after dimethyl sulfoxide), but it was not at all detectable in HeLa cells. The probable reason for the observed expression of the chimeric genes in HeLa cells, in spite of this cell being nonpermissive for expression of endogenous 5-lipoxygenase, is that additional regulatory region(s) are located outside the analyzed part of the promoter (upstream of the −5900 *Kpn* I site or in intron sequences). For HeLa cells, in particular, similar findings were reported regarding ϵ-globin (21) and retinol-binding protein (22).

Table 2. Sequence elements in human 5-lipoxygenase gene

Inverted repeat	−141 GCCGGGAGCCT -131
	−26 CGGCCCTCGGA -36
Repeated sequence	
I	−921 TGGGAGAGGC
II	−475 GGGGAGAGCA
III	−354 CAGGAGAGAA
IV	−270 GAGGAGAGAC
V	−259 CAGGAGCGAG
VI	−195 TGGGAGAGAA
Core sequence	RGGAGAGA
Homology box I (21, 22)	GGAGAGGNT
Homology box II (21, 22)	GAGGAGANT
Sp1-binding sequence	
I	CGTGGGGCGGCGGC
II	GAGTGGGCGGGAAC
III	CCTCGGGCGGGGCA
IV	AGCTGGGCGGGACC
V–IX	GGGGCGGGGGCGGGGGCG
X	GACCGGGCGGGGCC
Consensus sequence	GGGCGG
c-Ha-*ras* (24)	GGGGCGGGGGCGGGGGCG
hMT-IIA (15, 25)	CCGGGCGGGGCC
Myb product-binding sequence	ATAACGGTTTATT
Consensus sequence	YAACGGNT
mim-1 (27)	ATAACGGTTTTTT
AP-2-binding sequence	
I	AGTCCCCTGGCT
II	TGGCTCCAGGCT
Consensus sequence	CCCCAGGC
Simian virus 40 (28)	AGTCCCCAGGCT
NF-κB-binding sequence	TAGGGAGTCCCCG
Consensus sequence (29)	GGGANTYYCC

hMT, human metallothionein.

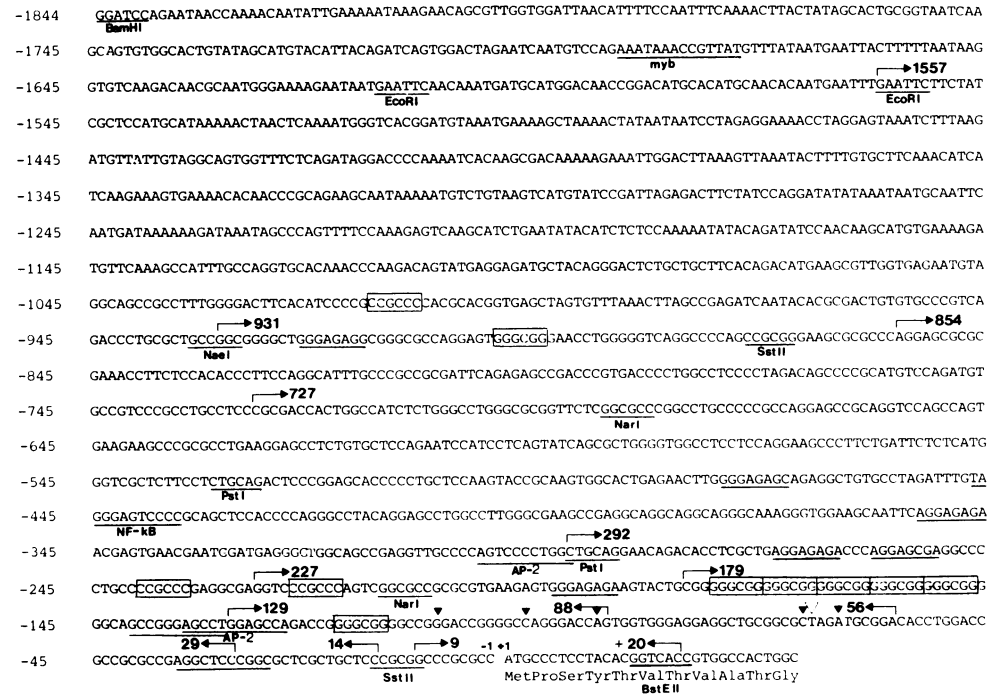

FIG. 5. Nucleotide sequence of the 5′-flanking region of the human 5-lipoxygenase gene. Nucleotides are numbered from the translational start codon; part of this sequence (−532 to +33) has been released (2). ▽, Major transcriptional initiation sites in leukocytes; ▼, possible minor initiation site. Sp1-binding sequences are boxed. Other putative transcriptional factor-binding motifs, inverted repeat structure, and repeated sequences are underlined. End points of deletion constructs used for the transfection experiments described in Fig. 2 and Fig. 3 are shown.

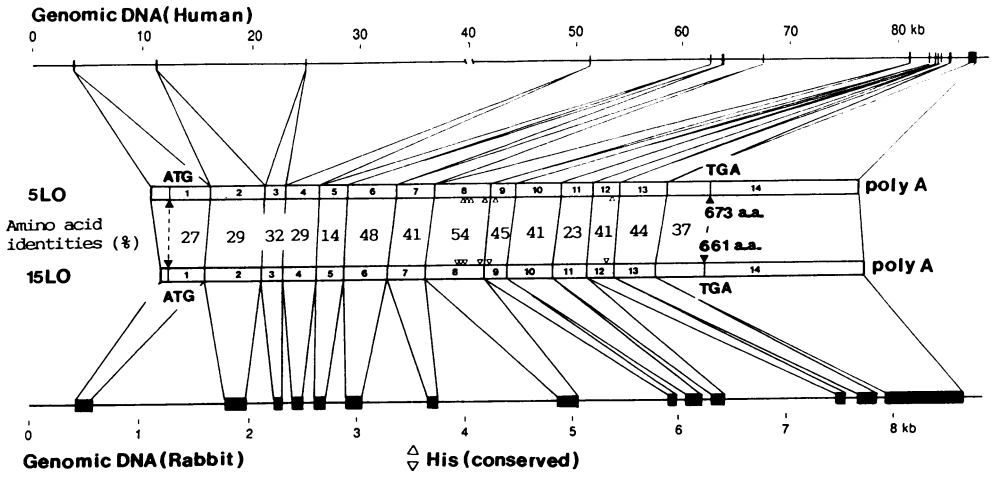

FIG. 6. Comparison of human 5-lipoxygenase (5LO) and rabbit 15-lipoxygenase (15LO) genes. Exon portions are indicated by thick bars in the genomic sequences. Amino acid identities of deduced amino acid sequences of the two genes are shown as percentage identity in each exon. Conserved histidine residues are indicated by open triangles.

To identify regulatory DNA elements of the 5′ portion, a 1844-nucleotide stretch of the 5-lipoxygenase gene promoter was sequenced (see Fig. 5). Several motifs were recognized as possible cis-acting sequences by binding to characterized transcriptional factors (23). The 10 Sp1-binding core motifs have already been mentioned; 5 of these were located in a cluster. Similarity to Sp1-binding sequences of the c-Ha-*ras* promoter (24) and human metallothionein IIA gene promoter (25) was high.

Repeated sequences weakly homologous to the silencer region I and II of the ε-globin gene (21) as well as to the silencer boxes I and II of the chicken lysozyme gene (26) were found (Table 2). One of these is located in the negative regulatory region (−727 to −292) that appeared in HeLa cell transfection experiments. Another DNA sequence homologous to the binding motif for transcription factor NF-κB (29) was also located in this region (−727 to −292). This transcriptional factor is involved in macrophage differentiation, and PMA induces formation of NF-κB in HL-60 cells (7).

Transcription factor AP-2 mediates many effects of PMA on gene expression (29). Sequences similar to the AP-2-binding motif were identified in the 5-lipoxygenase gene promoter at −135 and −302. The apparent PMA stimulation of CAT activities in cells transfected with 5-lipoxygenase gene promoter chimeras (Table 1 and Fig. 1) may reflect regulation of 5-lipoxygenase gene expression, possibly related to PMA-primed cell differentiation.

Finally, a DNA sequence was found that matched the binding motif for the *myb* protooncogene product described (28) for the *mim-1* gene promoter. A study using chicken myelomonocytic cells and *myb*-containing oncovirus indicated a down-regulatory effect of the myb protein on 5-lipoxygenase gene expression with concomitant inhibition of macrophage lineage differentiation (19). Another observation in line with this reasoning is that c-*myb* protooncogene expression declines in HL-60 cells during maturation (27).

The rabbit erythroid cell-specific 15-lipoxygenase gene was recently cloned and sequenced (30). Most striking was the identical exon/intron organization of the 5-lipoxygenase and 15-lipoxygenase genes (Fig. 6). Also the 15-lipoxygenase gene promoter was G+C-rich, and DNA including a GC box was found to be important for expression of 15-lipoxygenase gene chimeras in both fibroblasts (nonpermissive host) and erythrocytes (permissive host). The similar gene organizations of human 5-lipoxygenase and rabbit 15-lipoxygenase genes suggest conserved expression mechanisms and a common ancestral gene. However, the different cell specificities for expression of the various lipoxygenases indicate that different regulatory DNA elements have been added to the genes in this family after gene divergence.

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