Cell-specific Transcription of Leukotriene C$_4$ Synthase Involves a Kruppel-like Transcription Factor and Sp1*

(Received for publication, October 25, 1999, and in revised form, December 14, 1999)

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Leukotriene C$_4$ synthase (LTC$_4$S) is responsible for the biosynthesis of cysteinyl leukotrienes that participate in allergic and asthmatic inflammation. We analyzed 2.1 kilobases of the 5'-flanking region of the human LTC$_4$S gene, which contains three DNase I hypersensitivity sites, for its transcriptional activity when fused to a promoterless and enhancerless luciferase gene. Deletion analysis revealed a non-specific basal promoter region between nucleotides −122 and −56 upstream of the translation start site which contains a consensus Sp1 binding site and a putative initiator element (Inr) and cell-specific enhancer regions further upstream. A single mutation of either the Sp1 binding site between nucleotides −120 and −115 or the Inr (CAGC) between nucleotides −66 and −62 reduced the expression of the reporter gene by −60%, whereas double mutations decreased the expression by −80%. The incubation of nuclear extracts from THP-1 and K562 cells with a $[^{32}$P]-labeled oligonucleotide containing the Sp1 site or the Inr sequence gave gel-shifted complexes that were blocked by their respective cold oligonucleotides, and antisera specific for Sp1 and Sp3 provided supershifts for the former. Linker-scanning mutations of a cell-specific regulatory region revealed that mutations from nucleotides −165 to −125 reduced reporter activity. This region contains a tandem CACC repeat (at nucleotides −149 to −145 and −139 to −135). An oligonucleotide containing the distal CACC motif was gel shifted by THP-1 cell nuclear extract and was supershifted by antisera to Sp1 and Sp3. Cotransfection of an Sp1 expression plasmid into Drosophila SL2 cells with a −228 to −3 LTC$_4$S reporter construct transactivated the reporter gene, whereas mutations at the CACC repeat region reduced Sp1 transactivation by −86%. Similarly, the Kruppel-like factor Zf9/CPBP (core promoter-binding protein) transactivated the −228 construct in COS cells but not its CACC mutant construct. These findings indicate the involvement of Sp1 and an Inr in non-cell-specific regulation and a Kruppel-like transcription factor and Sp1 in the cell-specific regulation of the LTC$_4$S gene. These are the first such analyses of a member of a newly recognized superfamily of membrane-associated proteins involved in eicosanoid and glutathione metabolism, which contains key proteins involved in the generation of both prostanoids and cysteinyl leukotrienes.

LTC$_4$ synthase (LTC$_4$S) catalyzes the conjugation of leukotriene A$_4$ and glutathione (GSH) to form LTC$_4$. LTC$_4$ is implicated in the pathobiology of bronchial asthma by the efficacy of inhibitors of its biosynthesis and of receptor blockers for its constrictor metabolites in the treatment of this condition. LTC$_4$S is an 18-kDa perinuclear membrane protein that functions as a dimer (1, 2) and is expressed predominantly in mast cells, basophils, and eosinophils (3–5). LTC$_4$S was the first catalytic protein identified in a newly recognized superfamily of membrane-associated proteins involved in eicosanoid and GSH metabolism (MAPEG) (6). The MAPEG family members include the following: 5-lipoxygenase-activating protein, which is required for 5-lipoxygenase to metabolize released arachidonic acid to LTA$_4$; microsomal GSH S-transferase II and III, enzymes that are important in cellular protection mechanisms such as detoxification of xenobiotics and the peroxidation of lipid hydroperoxides (7) and also can conjugate LTA$_4$ to GSH; and GSH-dependent prostaglandin E synthase, a terminal enzyme of the cyclooxygenase pathway (8, 9) that provides prostaglandin E$_2$, a key prostanoïd inflammatory mediator. Thus, the MAPEG is the only superfamily with members that are critical to both the lipoxigenase pathway and the cyclooxygenase pathway of the arachidonic acid cascade.

LTC$_4$S is solely committed to the conjugation of LTA$_4$ to GSH and does not utilize xenobiotics as an alternative substrate. Site-directed mutational analysis of the catalytic mechanism for LTC$_4$S suggested that Arg-51 is involved in the opening of the epoxide ring of the LTA$_4$ and that Tyr-93 is responsible for GSH thiolate anion formation with resultant conjugation of the epoxide ring with the thiolate anion to yield LTC$_4$ (2). Arg-51 of LTC$_4$S is conserved in other members of the MAPEG family with catalytic function. Only Tyr-93 is conserved in 5-lipoxygenase-activating protein, which has no known enzymatic function.

The human LTC$_4$S gene is mapped to the 5q35 region of human chromosome 5 (10) in close proximity to the cluster of cytokine genes implicated in the polarization and function of the T cell of the Th2 phenotype in allergic and asthmatic disease. De novo induced LTC$_4$S transcript, protein, and function have been documented during differentiation and maturation of human eosinophils from cord blood progenitors with interleukin (IL)-3 and IL-5 in the presence of Matrigel over 2–4 weeks (11). Furthermore, mouse bone marrow-derived immature mast cells obtained with stem cell factor and IL-10 respond to stimulation with IL-3 in the presence of stem cell...
factor and IL-10 with marked up-regulation of LTC₄S transcript, protein, and function over a 2-week period (12). Although full regulation of LTC₄S expression requires consideration of gene transcription, post-transcriptional mRNA stability (13), and post-translational protein phosphorylation (14, 15), the developmental expression of LTC₄S in in vitro studies of the human eosinophil and the mouse mast cell prompts an initial focus on transcriptional regulation of this selectively expressed gene.

We now report the identification of multiple cis-acting elements in the LTC₄S promoter, including a proximal initiator-like element CAGAC (nucleotides −66 to −62) functioning in concert with an Sp1 site (nucleotides −120 to −115) to provide basal transcription and an upstream cell-specific cis-acting element CACCC (nucleotides −149 to −145) transactivated by Zf9/core promoter-binding protein (CPBP), a Kruppel-like factor, and Sp1. Within the context of the MAPEG superfamily of genes involved in the metabolism of released arachidonic acid, this is the first report of enhancing transacting factors functioning in concert with a TATA-less basilar initiator complex to regulate expression in transfected cells.

MATERIALS AND METHODS

Cells and Culture Conditions—Cell lines THP-1, K562, COS-7 (COS), and Drosophila Schneider cells (SL2) were purchased from American Type Culture Collection (Rockville, MD). The human LTC₄S-expressing promonocytic leukemia THP-1 cells and the non-LTC₄S-expressing chronic myelogenous leukemia K562 cells were grown and maintained in RPMI medium, and the monkey kidney COS cells were grown and maintained in Dulbecco’s modified Eagle’s medium; both media were supplemented with 10% fetal bovine serum. SL2 cells were grown at 22 °C in Schneider’s insect medium with 10% fetal bovine serum supplemented with 10% fetal bovine serum. SL2 cells were grown at 22 °C in Schneider’s insect medium with 10% fetal bovine serum supplemented with 10% fetal bovine serum.

Cell nuclei were isolated and digested with DNase I as described (16). The DNase I-treated genomic DNAs were digested with EcoRV, separated by electrophoresis on agarose gels, and hybridized to a 3²⁵P-labeled 275-bp KpnI-NcoI fragment consisting of the distal part of intron 1, and the proximal part of exon 1. Alternatively, the DNase I-treated genomic DNAs were digested with HpaI and hybridized with a 3²⁵P-labeled, 551-bp BspEI-HpaI fragment from the 3’-end region of the gene. DNase I hypersensitivity sites (HS) were determined by the appearance of new fragments that were not present in control DNA from cell nuclei not treated with DNase I.

Plasmid Constructs and Mutagenesis—A 1.4-kb SacI-KpnI fragment was obtained from PCR amplification of K562 genomic SacI clone 5.5 kb (10) and cloned into pFlashI, a promoterless and enhancerless luciferase reporter plasmid (SyntacSys, formerly Burlington, MA). The fragment encompassed nucleotides −1442 to −3 of the human LTC₄S gene promoter, numbered with respect to the transcription start site (+1), and was designated as −1442. To obtain a larger 5’-flanking fragment that covered the most distal DNase I HS, a 10-kb BamHI fragment of a P1 clone (10), which contains exon 1 and part of intron 1, was subcloned into pBlueScript. An additional 0.7 kb of human LTC₄S gene sequence 5’ of the −1442 SacI site was obtained by sequencing. A 2.1-kb fragment of the 5’-flanking region of the gene was prepared by PCR of the BamHI pBlueScript clone and subcloned into pFlashI as the −2189 construct. The deletion constructs of −1269, −557, and −228 were generated by digestion of the −1442 construct at the 5’-SacI site and a second internal restriction site, PstI, Apal, or NheI, respectively, and religation after the digested plasmid was blunted ended. Additional deletion constructs within −228 were generated by PCR with oligonucleotides and cloned into the reporter plasmid. Site-directed or linker-scanning mutagenesis was performed by PCR with two complementary constant oligonucleotides or with one constant oligonucleotide according to a modified overlap extension method as described (16). All constructs were verified by sequencing. The cDNA expression constructs used included pcDNA-Zf9 (17), pMcp-Sp1 (18), and pBOS-erythroid Kruppel-like factor (EKLF) from Dr. James J. Bieker (Mount Sinai School of Medicine, New York).

Transfection—All DNAs used for transfection were purified by the cesium chloride method. THP-1 and K562 cells were transfected by the DEAE-dextran method (19) with modifications. Briefly, the cells were centrifuged and washed once with phosphate-buffered saline. The cell pellet was resuspended in STBS (25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, and 0.5 mM MgCl₂), and 10⁷ cells were placed in 1 ml of STBS containing 10 μg of reporter plasmid DNA and 100 μg/ml or 150 μg/ml DEAE-dextran for THP-1 or K562 cells, respectively. The cells were incubated for 20 min at room temperature, and the transfection was stopped by the addition of 10 ml of culture medium and centrifugation. The cell pellets were resuspended in 12 ml of fresh medium and cultured for 45 h. COS and SL2 cells were transfected with the calcium phosphate method using calcium chloride and DNA precipitation buffers (5 Primer → 3 Primer, Boulder, CO). About 2.5 × 10⁶ COS cells/6-cm dish were cotransfected with 3 μg of reporter constructs and 3 μg of cDNA plasmid expressing Zf9 protein or the corresponding plasmid pc1-neo. Calcium phosphate-DNA precipitates were removed after 16 h, and the cells were cultured for an additional 24 h. SL2 cells were plated at 30% confluence and transfected the same day with 1.5 μg of reporter constructs and 0.5 μg of cDNA plasmid expressing Sp1. Cells were harvested after 43 h of exposure to calcium phosphate-DNA precipitates.

Luciferase Assays—Luciferase activities were measured by a dual-luciferase reporter assay system (Promega, Madison, WI) and expressed as a fold increase over the activity of pFlashI. Transfection of the LTC₄S promoter by transcription factors was measured by the luciferase assay system (Promega) and expressed as a fold increase of LTC₄S reporter activity in the presence of a CDNA construct expressing a transcription factor over the activity in the absence of transcription factor. Transfection efficiency was normalized by renilla luciferase expression in experiments of cotransfection with COS and SL2 cells.

Electrophoretic Mobility Shift Assay—Small scale nuclear extracts were prepared from THP-1 and K562 cells as described (16). Electrophoretic mobility shift assays (EMSA) were performed in a 10-μl final volume, containing 5% glycerol, 1 mM MgCl₂, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5 μg of poly(dI-dC), and 1 μg of bovine serum albumin. Each binding reaction was carried out with 4 μg of nuclear extract and approximately 2 × 10⁶ cpm of labeled probe at room temperature for 20 min. For EMSA with oligonucleotides for EKLF binding, each reaction contained 0.5 μg of in vitro transcribed and translated EKLF (kindly provided by Drs. Brian A. Lewis and Stuart H. Orkin, Children’s Hospital Medical Center, Boston) in place of nuclear extract and was carried out in the absence of poly(dI-dC) (20). For supershift experiments, 1 μl of antiserum against Sp1 family members (Santa Cruz Biotechnology, Santa Cruz, CA) or EKLF (20) was added to a reaction mixture 20 min before the addition of 3²⁵P-labeled probe. The final reaction mixtures were separated on a 6% nondenaturing polyacrylamide minigel (Novex, San Diego, CA) in 0.5 × TBE at 100 V for 1 h.

The LTC₄S oligonucleotides used in EMSA are listed in Table I. Additional EMSA oligonucleotides used included an Sp1 gel shift oligonucleotide 5’-GAGAGCCCGGGCAGCAG-3’ (Santa Cruz Biotechnology), and its mutant 5’-ATTCCATCGTGGGGCGGAGAC-3’ and its mutant 5’-ATCGACTGGGTTGGGAGC-3’ and its mutant 5’-AGAGCCCTTACTTCGTGGAGA-CACCAC-3’ and its mutant 5’-AGAGCCCTTACTTCGTGGAGAAC-AC-3’ (21).

RESULTS

Detection of DNase I HS in the 5’-Region—The LTC₄S gene promoter contains multiple consensus sequences for transcription factors, including AP2 at nucleotide −968 and AP1 at nucleotide −900 in the 5’-upstream region and a GC-rich region at nucleotides −121 to −113 in the proximal promoter (10). Transcription of the LTC₄S promoter had been shown by primer extension analysis to initiate from multiple start sites at nucleotides −66, −69, and −96 with poly(A)’ RNA from in vitro derived eosinophils and KG-1 cells (10) or from a single site at nucleotide −78 using total RNA from THP-1 cells (22) (B. H. R.). To locate regions that might be involved in LTC₄S gene transcription, we conducted DNase I HS analysis in the 5’-flanking region. Nuclei prepared from LTC₄S-expressing THP-1 cells and treated with increasing amounts of DNase I were digested with EcoRV, separated by agarose gel electrophoresis, and probed with a 3²⁵P-labeled 275-bp KpnI-NcoI fragment of intron 1, and was subcloned into pBlueScript. An additional 0.7 kb of human LTC₄S gene sequence 5’ of the −1442 SacI site was obtained by sequencing. A 2.1-kb fragment of the 5’-flanking region of the gene was prepared by PCR of the BamHI pBlueScript clone and subcloned into pFlashI as the −2189 construct. The deletion constructs of −1269, −557, and −228 were generated by digestion of the −1442 construct at the 5’-SacI site and a second internal restriction site, PstI, Apal, or NheI, respectively, and religation after the digested plasmid was blunted ended. Additional deletion constructs within −228 were generated by PCR with oligonucleotides and cloned into the reporter plasmid. Site-directed or linker-scanning mutagenesis was performed by PCR with two complementary constant oligonucleotides or with one constant oligonucleotide according to a modified overlap extension method as described (16). All constructs were verified by sequencing. The cDNA expression constructs used included pcDNA-Zf9 (17), pMcp-Sp1 (18), and pBOS-erythroid Kruppel-like factor (EKLF) from Dr. James J. Bieker (Mount Sinai School of Medicine, New York).
probe (a in Fig. 1A) overlapping parts of exon 1 and intron 1 (Fig. 1A). DNA from control nuclei not treated with DNase I but digested with EcoRV gave rise to a single 9-kb fragment in Southern blot analysis. When the nuclear DNA was treated with increasing amounts of DNase I, the 9-kb fragment disappeared, and three smaller fragments appeared (Fig. 1B). These EcoRV fragments at sizes of 6.6, 1.4, and 2.1 kb correspond to 5′-regions at nucleotides −100, −900, and −1600 of the LTC4S gene, thereby revealing locations that might be actively involved in transcription (Fig. 1A).

To assess for possible DNase I HS in exon or intron regions, the DNase I-treated DNAs were digested with HpaI and hybridized with a 32P-labeled 551-bp BspEI-HpaI probe (b in Fig. 1A) overlapping parts of exon 5 and the 3′-flanking sequence (Fig. 1A). HpaI digestion of control DNA not treated with DNase I gave rise to an approximately 9-kb fragment. In comparison, three smaller fragments of 2.9, 3.7, and 4.4 kb appeared in DNase I-treated and HpaI-digested DNA (data not shown). The DNase I HS derived from HpaI digestion corresponded to the same regions derived from EcoRV digestion (Fig. 1A), uncovering no additional DNase I HS in exon and intron regions and supporting a focus on the first 2 kb of the 5′-region.

Cell-specific Expression of the LTC4S Promoter—2.1 kb of the LTC4S 5′-region was cloned into the reporter plasmid pFlashI (the −2189 construct) to include all three DNase I HS for promoter analysis. Plasmid DNAs of the −2189 construct and its deletion variants, the −1442, −1269, −557, and −228 constructs, were first tested for promoter activity in THP-1 cells, a LTC4S-positive cell line. After transfection, the cells were harvested and assayed for reporter gene expression. The −2189 construct was transcriptionally 12-fold more active than the cloning vector pFlashI, which contained no promoter or enhancer activity (Fig. 2A). Deletion of the 5′-sequence from nucleotides −2189 to −557 appeared to increase the reporter activity, which peaked at 23-fold with the −557 construct.

**FIG. 1. Genomic organization of the human LTC4S gene and analysis for DNase I HS.** Panel A, exons are marked by solid boxes. The nucleotide sequence of the LTC4S promoter is partially listed and numbered with respect to the translation start site (+1). A GC-rich sequence at nucleotides −121 to −113 and the transcription start sites at nucleotides −66, −69, and −96 are underlined, and the additional transcription start site at nucleotide −78 is in boldface. The relevant restriction enzyme sites are shown and abbreviated as follows: A, ApaI; B, BspEI; E, EcoRV; H, HpaI; K, KpnI; D, NcoI; N, NheI; P, PstI; and S, SacI. Locations of DNase I HS are indicated by numbered arrows (Roman numerals), and the two probes are labeled a and b. Panel B, DNase I HS are shown in Southern blot analysis with EcoRV-digested THP-1 cell nuclear DNA. Lanes 1–5 correspond to the nuclei from THP-1 cells treated with 0, 3, 9, 12, and 15 units, respectively, of DNase I followed by EcoRV restriction digestion of the precipitated DNA. The schematic presentation of DNase I HS fragments derived from restriction digestion with EcoRV or HpaI is depicted in panel A.

**TABLE I**

LTC4S oligonucleotides used in EMSA

| GC box sequence | −128 | CTGGAGATGGGGCGGGAGAGCAACC | −105 |
|-----------------|------|-----------------------------|------|
| GC box sequence mutant |      | ..C.CGA.                    |      |
| Inr-like motif   | −76  | GGAAGCTCTGAAGGAGGACT       | −62  |
| Inr-like mutant A|      | ..GG.                      |      |
| Inr-like mutant B|      | ..GG.                      |      |
| AP3 motif        | −172 | CTTGGCCTGTGTTGATAGGT       | −152 |
| Distal CACCC motif | −156 | ATGTCACCTCCCTCCTG        | −140 |
| Distal CACCC mutant |      | ..GAT.                    |      |
| Proximal CACCC/CCCTC motif | −144 | CGGTCGACCCCTCCCTGAGA     | −123 |
| Proximal CACCC/CCCTC mutant A |      | ..AA.                     |      |
| Proximal CACCC/CCCTC mutant B |      | ..G.                      |      |
| Proximal CACCC/CCCTC mutant C |      | ..C.                      |      |
Although further deletion from nucleotides −557 to −228 resulted in a reduction of promoter activity by 30%, the 17-fold increment over the cloning vector indicated that this −228 to −3 5′-region was important for the full promoter activity in THP-1 cells (Fig. 2A).

To characterize this proximal 225-bp promoter region further, several small deletion constructs were obtained by PCR and transfected into THP-1 cells. Reporter activity did not change when the sequence between nucleotides −228 and −175 was deleted (Fig. 2B). However, promoter activity decreased significantly from 23-fold to 12-fold when the sequence between nucleotides −175 and −122 was deleted (Fig. 2B), indicating that this region was important for the reporter activity in THP-1 cells. Because LTC4S expression is cell-specific, some of the LTC4S promoter constructs were transfected into K562, a LTC4S-negative cell line. Neither the −2189 nor the −557 constructs showed any reporter activity above the control plasmid with no insert in K562 cells (Fig. 2C) compared with 12- and 23-fold increases in activity, respectively, in THP-1 cells. Progressive deletion revealed a peak 3-fold increase in transcription activity for the reporter gene in K562 cells for the −122 construct compared with a 12-fold increase in THP-1 cells. Therefore, the −122 construct compared with a 12-fold increase in THP-1 cells. Results established that the LTC4S promoter region cloned contains positive cis-acting elements in two regions, between nucleotides −557 and −228 (Fig. 2A) and between nucleotides −175 and −122 (Fig. 2B), and that both regions regulate cell-specific (Fig. 2C) transcription of reporter gene in transient transfection assays.

**Functional Identification of Basal Promoter Elements**—Because the short −122 reporter construct showed basal promoter activity in both THP-1 and K562 cells, we examined the possible location of the non-cell-specific cis-acting elements by deletion analysis of the −122 construct. Deletion from nucleotides −122 to −76 removed the GC box sequence (Sp1 binding motif) between nucleotides −120 and −115 (Fig. 1A) and the two distal transcription start sites at nucleotides −96 and −78. This resulted in a modest decrease in reporter activity in THP-1 and K562 cells (Fig. 2, B and C). A marked decrease to near base-line reporter activity was observed in both THP-1 and K562 cells when an additional 12 bp, from nucleotides −76 to −64, was deleted (Fig. 2, B and C). This 12-bp region contains the two proximal transcription start sites. Sequence analysis of this region revealed the presence of CAGAC sequence located at nucleotides −66 to −62, which matches the conventional Inr consensus motif of CA_{1-2}TAPy (23). A 24-bp reporter construct (nucleotides −77 to −54) containing these start sites and the Inr-like motif was found to be transcriptionally as active as the −76 to −3 construct in both THP-1 and K562 cells. In comparison, a 32-bp construct (nucleotides −109 to −77) containing the two distal transcription start sites without the Inr-like sequence had little reporter activity (Fig. 2, B and C).

The functional importance of the Sp1 binding site at nucleotides −120 to −115 and the Inr-like motif in reporter expression was examined further by mutation of these sites in the context of the −228 construct (Fig. 3). A single mutation of either the Sp1 site or the Inr motif resulted in a reduction of the reporter activity in THP-1 cells from 18-fold for the wild type to 6- and 8-fold, respectively (Fig. 3C). A further decrease to 3-fold over base-line activity occurred when both sites were mutated (Fig. 3C, far right bar). These functional data suggest that both the Inr-like motif at nucleotides −66 to −62 and the Sp1 site at nucleotides −120 to −115 are positive cis-acting elements involved in nonspecific basal promoter activity as well as cell-specific expression, possibly through interaction with upstream elements.

EMSA of the Inr-like motif oligonucleotide (−76 to −62) (Table I) with nuclear extracts from K562 (Fig. 3A) and THP-1 cells (data not shown) showed a single DNA-protein complex. The complex formation was inhibited by a 100-fold molar excess of cold native oligonucleotide, Inr-like motif mutant A oligonucleotide with a CA/GG change at nucleotides −69 and −68, and the TdT Inr oligonucleotide. Complex formation was not inhibited by Inr-like motif mutant B oligonucleotide with a CA/GG change at nucleotides −66 and −65 or mutant TdT Inr oligonucleotide (Table I and Fig. 3A). Thus, the Inr mutation critical for protein binding is at the −66 proximal transcription start site, not at the −69 start site.

EMSA of the GC box sequence oligonucleotide (−128 to −105) (Table I) with THP-1 cell nuclear extract revealed the
formation of two DNA-protein complexes. The formation of these complexes was inhibited by a 100-fold molar excess of cold native oligonucleotide and by a consensus Sp1 gel shift oligonucleotide but not by a GC box mutant oligonucleotide (Table I) or a mutated consensus Sp1 gel shift oligonucleotide (Fig. 3B). A supershift assay with antisera against Sp1 family members showed that anti-Sp1 supershifted the upper complex and anti-Sp3 supershifted both the upper and the lower complexes. Neither anti-Sp2 nor anti-Sp4 supershifted these complexes (Fig. 3B).

Characterization of cis-acting Elements in the Cell-specific Promoter Region—Of the two regions found to affect the cell-specific promoter activity, the region from nucleotides −175 to −122 was selected for analysis because it was shorter and closer to the basal promoter than the region from nucleotides −557 to −228. To delineate the positive cis-acting elements in the 53-bp proximal cell-specific region, six linker-scanning mutants were generated in the context of the −228 construct. Each mutant contained mutations in a 7- to 11-bp stretch of the 53-bp region, whereas the total length of the wild-type construct was maintained. After transfection into THP-1 cells, the promoter activity of each mutant was assayed relative to the cloning vector and compared with that of the wild-type −228 construct. Mutations between nucleotides −175 and −165 (mutant 1) increased the reporter activity slightly over that of the wild type (Fig. 4B). Mutations between nucleotides −164 and −125 reduced reporter activity from 20-fold over that of the cloning vector to between 12- and 5-fold over base line compared with the wild-type construct. The most marked decrement was observed for the mutations between nucleotides −146 and −136 (mutant 5) (Fig. 4, A and B).

Sequence analysis of the 40-bp region between nucleotides −164 and −125 revealed the presence of multiple consensus cis-acting elements. They include one AP3 site (at nucleotides −160 to −154), tandem CACCC sites (at nucleotides −149 to −145 and −139 to −135), and one CCCTC site (at nucleotides −135 to −131) which overlaps the proximal CACCC site (Fig. 4A). These sequence motifs were investigated by EMSA with THP-1 cell nuclear extract and 32P-labeled oligonucleotides encompassing these sites (Table I).

EMSA with an oligonucleotide (nucleotides −141 to −123) containing the proximal CACCC (−139 to −135) with overlapping CCCTC (−135 to −131) sites (Table I) formed several gel shift complexes with a nuclear extract of THP-1 cell nuclear extract. Competitor oligonucleotides were added at a 100 × molar excess to lanes 2–5; including native cold oligonucleotide (lane 2), GC box mutant (lane 3), consensus Sp1 (lane 4), and consensus Sp1 mutant (lane 5). Antisera to Sp1, 2, 3, and 4 are shown in lanes 6–9, respectively. Panel C, effect of mutations at the Sp1 binding site and in the Inr-like motif region on the basal promoter activity in THP-1 cells. The luciferase activity is expressed as the fold increase over that of the cloning vector, pFlashI. The relative activity and standard deviation are calculated on the basis of three separate experiments.

**Fig. 3.** EMSA and mutational analysis of the basal promoter elements. Panel A, EMSA of the Inr-like motif oligonucleotide (−76 to −62) with a K562 cell nuclear extract. Competitor oligonucleotides were added at a 100 × molar excess to lanes 2–6, including the Inr-like motif, the Inr-like mutant A, the Inr-like mutant B, the TdT Inr, and the TdT Inr mutant, respectively. LTC₄S oligonucleotide sequences are listed in Table I. Panel B, EMSA of the Sp1 (GC box) containing oligonucleotide with THP-1 cell nuclear extract. Competitor oligonucleotides were added at a 100 × molar excess to lanes 2–5; including native cold oligonucleotide (lane 2), GC box mutant (lane 3), consensus Sp1 (lane 4), and consensus Sp1 mutant (lane 5). Antisera to Sp1, 2, 3, and 4 are shown in lanes 6–9, respectively. Panel C, effect of mutations at the Sp1 binding site and in the Inr-like motif region on the basal promoter activity in THP-1 cells. The luciferase activity is expressed as the fold increase over that of the cloning vector, pFlashI. The relative activity and standard deviation are calculated on the basis of three separate experiments.
a recombinant protein, formed a complex with the distal CACCC motif oligonucleotide containing a CACACCC sequence that encompasses the distal CACCC site; this complex was supershifted by anti-EKLF antibody (data not shown).

Recombinant EKLF did not form a complex with the AP3 motif containing oligonucleotide or the oligonucleotide with the proximal CACCC site that overlaps the CCCTC site (data not shown).

Transactivation of the LTC₄S Promoter by Sp1 and Zf9/CPBP through the CACCC Motif—To establish a function for the putative transcription factors recognizing the distal CACCC site in gel shift analysis, we cotransfected the reporter construct containing the 228 to 2 promoter fragment together with cDNA clones expressing various transcription factors. Because Sp1 is ubiquitously expressed in mammalian cells, we used Drosophila SL2 cells for Sp1 cotransfection experiments. Cotransfection of the −228 reporter construct with pPac-Sp1 transcription factor increased reporter activity 12-fold compared with a 4-fold transactivation increment of mutant 5, containing mutations in the CACCC repeats (Figs. 4A and 6C). Similarly, pPac-Sp3 transactivated the −228 reporter construct in SL2 cells (data not shown). Transfection of EKLF expression plasmid into the K562 cells did not increase the expression of the reporter gene (data not shown); however, Zf9/CPBP, a Kruppel-like subgroup member involved in inducible gene expression (17), increased the reporter activity when cotransfected with the −228 construct in COS cells. The reporter activity of the −228 construct increased 4-fold in the presence of a Zf9 expression plasmid pCi-neo-Zf9 compared with cotransfections with the control plasmid pCi-neo (Fig. 6D). The transactivation by Zf9/CPBP was entirely lost with the mutant 5 construct and partially diminished with the mutant 6 construct, containing mutations in both or one CACCC motif, respectively. Thus, we established that the distal CACCC is one of the cis-acting elements that plays a crucial role in cell-specific transcription of LTC₄S.

DISCUSSION

The regulatory cis-acting elements and candidate transcription factors for the proximal core promoter involved in the transcription of the human LTC₄S gene in THP-1 cells are described in the present study and depicted schematically in Fig. 7. The core promoter of the LTC₄S gene is located within 228 bp upstream of the translation start site and is composed of a non-cell-specific basal promoter region and a cell-specific upstream enhancer region. The basal promoter region contains an Inr-binding motif of CAGAC and an Sp1 binding GC box, whereas the enhancer region contains a tandem CACCC site that binds Sp1 and a Kruppel-like transcription factor.

Deletional analysis of 2.1 kb of the 5′-flanking sequence of the human LTC₄S gene revealed that two regions (nucleotides −557 to −228 and −175 to −122) were responsible for cell-specific promoter activity in THP-1 cells (Fig. 2, A and C), and one region (nucleotides −122 to −62) was responsible for non-cell-specific basal promoter activity in both THP-1 and K562
though the formation of the DNA-protein complex was inhibited by an oligonucleotide containing the classic TdT Inr (Fig. 3A). The fact that the mutation of either the GC box or the Inr of the LTC₄S promoter reduced reporter activity by more than 50% in THP-1 cells (Fig. 3C) suggests that both of these elements are functionally important and are required for transcription initiation and for full promoter activity.

Linker-scanning mutagenesis of the region immediately upstream of the basal promoter revealed a 40-bp region between nucleotides −164 and −125 (Fig. 4B) important for cell-specific transcription of the reporter gene. This region contains an AP3 binding site and a tandem repeat of a CACCC site (nucleotides −149 to −145 and −139 to −135). The proximal CACCC site overlaps a CCCTC site, and the distal CACCC is encompassed by a CACACC site (Fig. 4A). EMSA with an oligonucleotide containing the AP3 site (Table I) yielded a DNA-protein complex with a THP-1 cell nuclear extract (data not shown), but the nature of the binding protein was not pursued because the putative AP3 factor has not been cloned. The nuclear extract of THP-1 cells formed two complexes (α' and β') (Fig. 5A) with the proximal CACCC/CCCTC motif oligonucleotide (Table I). However, only the mutation of the CCCTC motif (mutant A) abolished the ability to compete with the native oligonucleotide. Therefore, it is likely that the CCCTC motif is involved in binding to the transcription factors in the formation of complexes α' and β'.

EMSA with the distal CACCC-containing oligonucleotide and THP-1 cell nuclear extract resulted in the formation of three DNA-protein complexes, complex a and two upper complexes identified as Sp3 and Sp1-Sp3 by supershift analyses (Fig. 6B). Therefore, the transcription factors of the Sp1 family bind to both the CACCC motif (an inverted GT box) (25) and the GC box. The transcription factor involved in complex a is not known; however, EKLF, a Kruppel-like protein, gel shifted the distal CACCC site, suggesting that a member of that family is involved in complex a formation.

The involvement of a Kruppel-like factor and Sp1, through binding to the distal CACCC site, in cell-specific transcription of LTC₄S was supported further by cotransfection experiments. Sp1 transactivated the −228 construct in transfected SL2 cells (Fig. 6C). EKLF itself did not transactivate the −228 construct in K562 cells (data not shown); however, another Kruppel-like protein, Zf9/CPBP (17), was able to transactivate the −228 construct in transfected COS cells (Fig. 6D). Mutations of the proximal site or overlapping both the proximal and the distal CACCC sites substantially reduced the ability of both Sp1 and Zf9/CPBP to transactivate the reporter constructs (Fig. 6, E and F). Together with the data from transfection with linker-scanning mutants in THP-1 cells (Fig. 4B), these results indicate that the distal CACCC site is important for the cell-specific transcription of the reporter gene. Although mutation of the proximal CACCC site decreased the reporter activity, the involvement of the proximal CACCC site in the transcription regulation of the LTC₄S gene is not conclusive because gel shift data showed the binding of transcription factors to the CCCTC motif (complexes α' and β') and not the proximal CACCC motif (Fig. 5A).

Zf9 binds to a GC box and transactivates collagen α1(I) (17) and transforming growth factor β1 promoters (26). Zf9 binds avidly to a tandem repeat of the GC box in the transforming growth factor β1 promoter but minimally to a single copy of its cognate recognition site (26). CPBP, the human homolog of Zf9, binds to a CACCC motif and transactivates the TATA-less promoter of pregnancy-specific glycoprotein in COS cells (27). The transactivation of the LTC₄S promoter/reporter construct (Fig. 6D) was comparable to the collagen α1(I) and the preg-
nancy-specific glycoprotein promoters by Zf9/CPBP in rat hepatic stellate and COS cells (17, 27). These results suggest that a Kruppel-like factor, such as Zf9/CPBP, is involved in the regulation of the cell-specific transcription of LTC₄S. The Kruppel-like factor may work in concert with coactivator partner(s) in THP-1 cells, such as the proteins that bound to the proximal CACC/CCCTC sequence, as in complex a’ and b’ (Fig. 5B). Furthermore, interaction between Zf9/CPBP and Sp1 proteins has recently been reported (26), suggesting that Zf9/CPBP may interact with Sp1 bound to the proximal GC box to transactivate the LTC₄S promoter.

Like LTC₄S, LTA₄ hydrolase and 5-lipoxygenase are also TATA-less genes (28, 29). Deletional and mutational analyses of the 5-lipoxygenase promoter in a reporter construct revealed a GC-rich region that is important for transcription (28). Gel shift assay demonstrated that a 5 tandem repeat sequence in the GC-rich region bound to transcription factors Sp1 and Egr-1 (30), both of which transactivate the 5-lipoxygenase promoter in SL2 cells (31). Naturally occurring mutations in this repeat region have also been identified in the human 5-lipoxygenase gene, raising the possibility of clinical implications in asthma (30). In our cotransfection experiment in SL2 cells, Egr-1 did not transactivate the LTC₄S reporter gene (data not shown), whereas Sp1 did (Fig. 6C).

Although there is another enhancing region upstream of the 228-bp proximal core promoter, the more proximal enhancer elements do functionally interact with the basal promoter to provide transcription in THP-1 cells and transactivation with cotransfection. Thus the 228-bp proximal core promoter contains both the basal and the cell-specific cis-acting elements for a TATA-less human gene whose encoded protein is narrowly distributed and is solely responsible for LTC₄ generation in cells central to allergic and asthmatic pathobiology.

Acknowledgments—We thank Drs. Robert Tjian (pPac-Spi), Scott L. Friedman (pCI-neo-Zf9), James J. Bieker (pBOS-EKLF), and Brian A. Lewis and Stuart H. Orkin (rEKLF and anti-EKLF) for generously sharing their valuable reagents with us; Dr. Laurie H. Gimcher for critical review of the manuscript; and Karen K. Beutel and Allison B. McKenzie for technical assistance.

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