Identification of an IL-6 response element in the human LCAT promoter

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Abstract **LCAT is a key enzyme of reverse cholesterol transport that is essential to maintain HDL-mediated lipid transport and cholesterol homeostasis. Alterations in LCAT expression have a profound effect on plasma HDL cholesterol concentrations. Previously LCAT mRNA and activity were shown to be regulated by several inflammatory cytokines, including the pleiotrophic cytokine interleukin-6 (IL-6). A series of full-length and sequential deletion LCAT promoter constructs were used to determine whether inflammatory stimuli affect LCAT transcription and to further identify functional, cytokine-responsive promoter regions that mediate this response. Using transfected HepG2 cells, results indicate that treatment with IL-6 induced a 2.5-fold activation of full-length LCAT promoter activity. A minimal (−1514 bp to −1508 bp) IL-6 response element with high sequence homology to the signal transducer and activator of transcription (STAT) family member, STAT3, was mapped within the distal promoter and shown to be sufficient to mediate the IL-6 response. Further, overexpression of STAT3 significantly enhanced the effect of IL-6 on LCAT promoter activity. These data suggest that the IL-6 responsive transcription factor, STAT3, contributes to LCAT transcriptional regulation. The elucidation of distinct biochemical signaling pathways associated with inflammation may provide new insight into transcriptional regulation of genes involved in lipid metabolism.**—Feister, H. A., B. J. Auerbach, L. A. Cole, B. R. Krause, and S. K. Karathanasis. **Identification of an IL-6 response element in the human LCAT promoter.** ***J. Lipid Res.*** **2002.** 43: **960–970.**

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The acute phase response is mediated by several multifunctional cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and IL-6, and characteristically involves stimulation of specific plasma proteins, such as serum amyloid A (SAA) and C-reactive protein (CRP) (1). Chronic inflammation has also been associated with alterations in lipid metabolism, which include hypertriglyceridemia and significantly reduced HDL cholesterol (HDL-C) concentrations (2). The latter is considered an independent risk factor for increased susceptibility to coronary heart disease (3). This decrease in HDL-C has been associated with altered expression of several plasma proteins that directly regulate HDL metabolism, including CETP (4), hepatic lipase (5), apolipoprotein A-1 (apoA-1) (6), and LCAT (7, 8). The molecular mechanisms that underlie this change in HDL metabolism however, are not fully understood.

LCAT is a key enzyme of reverse cholesterol transport that is essential to maintaining HDL-mediated lipid transport and cholesterol homeostasis (9). LCAT is primarily synthesized and secreted from the liver, with minor expression detected in brain and testes (10). Located on the surface of plasma HDL, LCAT serves to hydrolyze and transfer the 2-acyl group of lecithin to esterify free cholesterol, promoting accumulation of cholesterol esters within HDL particles (11). In this manner, LCAT activity contributes to HDL-mediated efflux of free cholesterol from peripheral cell membranes into plasma for modification and transport back to the liver (3). Accordingly, as characterized in LCAT-deficient humans (12) and modeled by targeted disruption of the LCAT gene in transgenic mice (13, 14), the LCAT deficient phenotype is characterized by a dramatic decrease in plasma HDL and apoA-1 concentrations, an accumulation of discoidal HDL, impaired adrenal cholesterol delivery, and premature atherosclerosis. Overexpres-

Abbreviations: CRP, C-reactive protein; IFN-γ, interferon-γ; IL-6, interleukin-6; IL-1β, interleukin-1β; JAK, Janus kinase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; SAA, serum amyloid A; SAF, sequence binding factor; SR-BI, scavenger receptor class B, type I; STAT, signal transducer and activators of transcription; TGF-β, transforming growth factor; TNF-α, tumor necrosis factor-α.

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sion of human LCAT has been proven to enhance cholesterol efflux, increase HDL-C and apoA-1 plasma concentrations in transgenic mice, (15–17) and protect against diet-induced atherosclerosis in transgenic rabbits (18).

The expression of LCAT was previously shown to be regulated under a variety of conditions that mimic the inflammatory response. LCAT activity was significantly downregulated upon administration of endotoxin (lipopolysaccharide, LPS) and the proinflammatory cytokine TNF-α in vivo (6–8, 19). In cultured HepG2 cells, LCAT mRNA and activity was similarly shown to be regulated by TNF-α, IL-1β, transforming growth factor (TGF)-β, and the pleiotrophic cytokine IL-6 (20, 21). Conversely, treatment with the synthetic glucocorticoids dexamethasone and triamcinolone increased LCAT mRNA and plasma enzyme activity (22). The expression of LCAT is therefore sensitive to both pro- and anti-inflammatory conditions that are also known to influence plasma HDL-C concentrations.

Several well-characterized mediators of cytokine activation regulate hepatic gene expression during the acute phase response. TNF-α and IL-1β are classic activators of the transcription factor nuclear factor-κB (NF-κB) (23). CCAAT/enhancer-binding protein (CEBP/β; NF-IL-6), SAA-activating sequence binding factor (SAF), and signal transducers and activators of transcription (STATs) are known downstream mediators of IL-6-activated pathways (24–26). Alterations in gene expression, however, are typically not dependent upon activation of a single mediator, but instead likely result from interaction of multiple overlapping signal transduction pathways (27, 28). Transcriptional regulation of SAA and CRP gene expression, for example, was shown to involve recruitment and coactivation of several combinations of these intermediary proteins, shown to direct either synergistic (29, 30) or competitive (31, 32) promoter transactivation. The induction of either pro- or anti-inflammatory acute phase gene expression is therefore reliant, at least in part, upon the nature of these protein-protein interactions.

Recently *cis*-acting, cytokine-specific response elements have been identified within the promoters of several genes involved in lipid metabolism, including the LDL receptor (33), the human macrophage scavenger receptor A (34), and scavenger receptor class B, type I (SR-BI) (35). Characterization of LCAT transcriptional activity however, is limited to identification of the minimal 5′ upstream sequence necessary for basal activity (36) and Sp1/Sp3 mediated transactivation (37). The identification of defined, cytokine-inducible regulatory elements that contribute to the control of LCAT transcription has not been shown.

The purpose of this study was to determine whether inflammatory stimuli affect LCAT transcription and to further identify functional, cytokine-responsive promoter regions that mediate this response. Our data suggest that IL-6 induced activation of the Janus kinase (JAK)-STAT signal transduction pathway contributes to regulation of LCAT transcription in hepatic cells.

MATERIALS AND METHODS

Reagents

Recombinant human IL-6, IL-1β, and TNF-α were obtained from R & D Systems (Minneapolis, MN). LPS (*Escherichia coli* serotype 0111:B4) was purchased from Sigma (St. Louis, MO). All synthetic oligonucleotide primers were purchased from GibcoBRL Life Technologies. *Pfu*-DNA polymerase (Stratagene, La Jolla, CA) was used for PCR amplification of LCAT promoter sequence. All vector DNA was isolated from transformed DH5α competent cells (GibcoBRL) using MiniPrep Express™ (Bio101, Inc., Vista, CA) or Qiagen plasmid purification MidiPrep kits (Qiagen Inc., Valencia, CA).

Construction of LCAT promoter reporter constructs

The human LCAT promoter sequence (GenBank Accession No: X51966) was PCR amplified from a human BAC clone library (RPCI-11) provided by the Parke Davis Laboratory of Molecular Genetics (Alameda, CA). The full-length clone (−2651/+6 bp) and truncated derivatives (−1464/+6 bp, −847/+6 bp, −103/+6 bp) were obtained using the following 5′ primers, respectively: 5′ CCCAAGCTTCCCATCTTGAAAACTTGGC 3′; 5′ CCCAAGCTTTCCTATGGGACCAAGGCTAT 3′; 5′CCCAAGCTTGCCCGTTGATTCTGTTGTTG 3′; 5′ CCCAAGCTTTCCGGCAATCTCTGGCCACAA 3′ (underlined nucleotides indicate a *Hind*III site). A common 3′ primer was used in each PCR reaction: 5′ CGGAATTCAGAGAAGCGGCACTGGGCTGT 3′ (underlined nucleotides indicate an *Eco*R1 site). All promoter constructs were cloned into the intermediate vector pZERO-2 (Invitrogen) for sequence confirmation, and subsequently subcloned into the pGL3-Basic luciferase reporter (Promega) using adjacent *Nhe*1/*Sma*1 restriction sites. The pGL3-532LCAT (−532/+6 bp) construct was generated by internal restriction enzyme digest within the full-length promoter fragment (*Bam*H1/*Eco*R1) and similarly subcloned into pGL3-Basic. To further delineate the distal LCAT promoter region necessary to mediate cytokine responsiveness, various promoter fragments (−2651 bp to −1437 bp) were similarly ligated into the minimal SV-40 containing pGL3 promoter luciferase reporter (Promega). The largest distal promoter construct (−2651/−1437 bp) and subsequent truncated fragments (−2312/−1437 bp, −1890/−1437 bp, −1744/−1437 bp, −1639/−1437 bp, and −1528/−1437 bp) were PCR amplified from the full-length template (pGL3 Basic − 2.6 LCAT) using the following 5′ primers, respectively: 5′ CGGGGTACCCATCTTGAAAACTTGGCCT 3′; 5′ CGGGGTACCTTCATTGGTGTGTGAA 3′; 5′ CGGGGTACCAGATGAAGAAGGACCTGGCT 3′; 5′ CGGGGTACCATAACAGTGACATCTCT 3′; 5′ CGGGGTACCGAGAAGTCAGTGCCCTTTGT 3′; 5′ CGGGGTACCAAGCTAGTGCTCATTT 3′ (underlined nucleotides indicate a *Kpn*1 site). A common 3′ primer was used for all: 5′ CTAGCTAGCAGGAGATAGCCTTGGTCCCATA 3′ (underlined nucleotides indicate a *Nhe*1 site). All distal promoter fragments were subcloned into the pGL3 promoter vector using adjacent *Kpn*1/*Nhe*1 restriction sites.

For the functional mutation analysis, site-specific cluster mutations were introduced into the wild-type pGL3 Promoter + 203 bp LCAT promoter construct by PCR amplification using a previously reported method (26, 38). Site mutations were introduced based upon originally published sequence necessary to mediate DNA binding, as indicated for Site 1 (33), Site 2 (39), Site 3 (40), and Site 4 (24) (**Table 1**). Briefly, each individual site mutation (1–4) was introduced by parallel amplification of two smaller inserts (comprising the 203 bp region) using select pairs of wild-type and mutated primers, flanked by appropriate restriction enzyme sequences for ligation. Each pair of fragments, designed to

TABLE 1. Primer pairs used for introduction of site-specific mutations within the wild-type 203 bp LCAT promoter fragment by PCR

	Fragment 1		Fragment 2	
	5′ Primer (5′→ 3′)	3′ Primer (5′→ 3′)	5′ Primer (5′→ 3′)	3′ Primer (5′→ 3′)
Site 1	CGGGGTACCGAGAAGTCAGTGCCCTTTG TGGTTCAgaattcttgGGGTGGCAGGGTCAG[a,b]	CTAGCTAGCAGGAGATA GCCTTGGTCCCATA	—	—
Site 2	CGGGGTACCGAGAAGTCAGTGCCCTTTGT	GTAGcaagaattcaAGTCATT CTGATTCCTCCGCACC	GACTtgaattcttgCTACCCAAG CTAGTGCTCATTTC	CTAGCTAGCAGGAGAT AGCCTTGGTCCCATA
Site 3	CGGGGTACCGAGAAGTCAGTGCCCTTTGT	CCTCgaattccTGAGCACTA GCTTGGGTAGGGCTGC	CTCAggaattcGAGGCAAGGT GGTCACATTGGGCCA	See above
Site 4	CGGGGTACCGAGAAGTCAGTGCCCTTTGT	TGGCCagaattctaCACCTTG CCTCCGGGAAATGAGCACT	GTGtagaattctGGCCAGACAG GCCCCATCCCCCTTTT	See above

[a] Lower case denotes mutated bp sequence introduced by PCR amplification.
[b] Underlined sequence denotes a restriction enzyme site.

contain an internal *Eco*R1 site upon ligation (introduced within the cluster mutation) and an external 5′ *Kpn*1 and 3′ *Nhe*1 site, was subject to restriction enzyme digest and subsequently ligated within the *Kpn*1/*Nhe*1 sites of pGL3 promoter vector as described above. The integrity of all wild-type and mutated plasmid constructs was confirmed by restriction enzyme digest and DNA sequencing.

Cell culture and transient transfections

HepG2 human hepatoma and NIH/3T3 mouse fibroblast cells were purchased from ATCC (Rockville, MD). The rat hepatoma cell line, H35, was kindly provided by Drs. Judy Shigenaga and Carl Grunfeld (University of California, San Francisco). Both hepatoma cell lines were maintained in low glucose DMEM media supplemented with 10% FBS (Summit Biotechnology, Ft. Collins, CO), 2 mM/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES (GibcoBRL). NIH/3T3 cells were incubated in high glucose DMEM supplemented as listed above. All cells were maintained in humidified 95% air/5% CO_2 at 37°C. For transfection, cells were seeded at 1.5×10^5 cells/2 cm^2 in sterile 24-well plates (Corning, Inc.) 24 h prior to transfection. Cells were transfected in triplicate with 0.5 μg/well of pGL3-LCAT plasmid reporter DNA for 6 h using Lipofectamine™ 2000 (GibcoBRL) according to the manufacturer's instructions. For cotransfection experiments, an equal concentration (0.5 μg) of either prcCMV-STAT3 (WT or Y705F) (41) or pMSV/CEBP/β (42) overexpression plasmids, or pBluescript II® KS+ (Stratagene) empty vector control were included with the reporter constructs as described in the text. To demonstrate the specificity of prcCMV-STAT3 overexpression, a tandem repeat of the STAT3 DNA binding sequence (pSTAT3-TA-Luc) (Clontech Laboratories, Inc., Palo Alto, CA) containing four copies of the STAT3 enhancer element, and an empty vector control (pTA-Luc) were similarly cotransfected as described above. To normalize for variation in DNA uptake, samples were cotransfected with 0.05 μg pRSV-β-galactosidase (β-gal) in all experiments. After transfection, cells were allowed to recover for 24 h prior to either 4 h or 16 h cytokine treatment. Unless otherwise described, IL-6, IL-1β, TNF-α (10 ng/ml final concentration), and LPS (10 μg/ml) were included in phenol-red free DMEM (GibcoBRL) containing 5% FBS and 2 mM/l L-glutamine. To measure luciferase and β-gal enzymatic activities, cell lysates were prepared with lysis buffer (Promega) and subjected to a series of three freeze-thaw cycles. Measurements were obtained using the Dual-Light® Reporter Gene Assay System (Tropix, Bedford, MA) and a MicroLumat Plus microplate luminometer (EG&G Berthold). All relative luciferase activity is reported as the corrected luciferase/β-gal ratio.

Computer-aided mapping of regulatory binding sites within the LCAT promoter

A comprehensive map of putative transcription factor binding sites within the full-length of the human LCAT promoter was generated by the use of the following web-based search programs: TESS (http://www.cbil.upenn.edu/tess/) and Signal Scan (http://bimas.dcrt.nih.gov/molbio/matrixs/). Published regulatory sequences were identified for the following cytokine-inducible transcription factors and coactivators: NF-κB, CEBP/β, STAT3, STAT1, AP-1, SP-1, and YY-1. The GCG program consensus/fitconsensus was used to identify four specific, putative IL-6 response sites within the 203 bp distal LCAT promoter sequence for the functional mutation analysis.

Statistical analyses

Data values are reported as the mean ± SEM. Where indicated in the results, an unpaired two-tailed student's *t*-test was used to determine statistical significance. In some experiments, a one-way ANOVA was performed and used Dunnett's procedure to adjust for multiple comparisons among treatment groups.

RESULTS

IL-6 activates the LCAT promoter

Treatment with the inflammatory stimuli LPS, TNF-α, IL-1β, and IL-6 have been shown to regulate LCAT mRNA in cultured hepatic cells (8, 20, 21). To determine whether this effect is mediated at the level of transcription, the full-length (−2651/+6) human LCAT promoter was ligated to a luciferase reporter construct and transfected into HepG2 cells. Cells were incubated for 16 h with either LPS, TNF-α, IL-6, or IL-1β and processed for measurement of luciferase activity, as described in Materials and Methods. Treatment with IL-6 induced a consistent 2.5-fold increase in LCAT promoter activity when compared with the activity of the full-length, vehicle-treated control (**Fig. 1**). In contrast, LPS and TNF-α had no effect, while treatment with IL-1β caused only a moderate reduction in LCAT transcriptional activity. These results suggest the presence of an IL-6 responsive, upstream regulatory element within the human LCAT promoter capable of activating LCAT transcription.

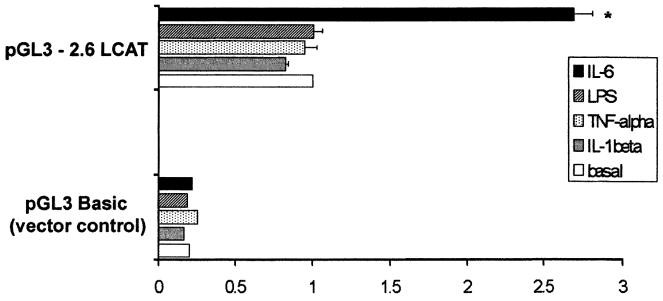

Fig. 1. Fold change in luciferase activity. Interleukin-6 (IL-6) induces a significant 2.5-fold increase in LCAT promoter activity. HepG2 cells were transfected with either the full length (−2651/+6 bp) LCAT promoter (pGL3 − 2.6 LCAT) or the pGL3-Basic vector alone and treated with either IL-6, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) (10 ng/ml), lipopolysaccharide (LPS) (10 μg/ml), or vehicle (PBS) for 16 h as described in Materials and Methods. Data presented is from three to five independent experiments performed in triplicate and represents normalized luciferase activity corrected for transfection efficiency. Error bars represent the standard error of the mean (mean ± SEM). Statistically significant differences between control (basal indicates control) and cytokine treatment groups are shown (*$P < 0.0001$).

The −2651 bp to −1464 bp promoter region mediates IL-6 regulation of the LCAT promoter

To identify upstream elements contributing to regulation of LCAT transcriptional activity, a series of sequential 5′ deletion reporter constructs were generated and similarly transiently transfected into HepG2 cells for measurement of promoter activity. Differential basal LCAT promoter activity was detected in untreated cells (**Fig. 2**). Maximal promoter activity was observed within the first 532 bp (−532/+6 bp) of upstream promoter sequence (Fig. 2), consistent with previously published results (36, 37). Basal expression of the full length 2.6 kb promoter (−2651/+6 bp) construct, however, was significantly reduced when compared with the activity of the truncated (−1464 bp, −847 bp, and −532 bp) promoter constructs. A strikingly similar pattern of full-length basal repression was observed upon parallel transfection of H35 rat hepatoma cells (data not shown). These findings suggest that proximal promoter elements (−1464 bp to +6 bp) contribute to constitutive expression, while distant elements (−2651 bp to −1464 bp) may be involved in regulation of this constitutive expression. Interestingly, only the −2651 bp, and not the −1464 bp, −847 bp, −532 bp, or −103 bp constructs, showed a robust 2.5-fold upregulation in response to treatment with IL-6 (Fig. 2). This further implied the presence of a *cis*-acting, IL-6 responsive DNA element located within the distal (−2651 bp to −1464 bp) promoter region that is involved in IL-6 mediated LCAT promoter activation.

A 203 bp region (−1639 bp to −1437 bp) within the distal LCAT promoter is sufficient to confer the IL-6 response

To further delineate the location of the distal IL-6 response element(s), an additional series of 5′ truncation constructs were designed based upon computer-aided mapping of putative cytokine responsive transcription factor binding sites (see Materials and Methods). Sequential promoter segments ranging from −2650/−1437 bp to −1528/−1437 bp were linked upstream to the minimal SV40 promoter using the pGL3-promoter vector, as described (**Fig. 3A**). HepG2 cells were transfected with these constructs and treated with IL-6. Vectors containing the −1214 bp, −876 bp, −454 bp, −308 bp, and −203 bp distal promoter regions consistently showed a 3-fold activation in response to IL-6 (Fig. 3B). In contrast, the vector containing the −92 bp promoter region was not capable of mediating the IL-6 response. Similar results were also obtained in mouse fibroblast NIH/3T3 cells, suggesting that the IL-6 response is not dependent upon activation of liver-specific transcription factors in vitro (Fig. 3C). Collectively these data and those in Fig. 2 identify a minimal 203 bp region located within −1639 bp to −1437 bp of the distal LCAT promoter that is necessary and sufficient

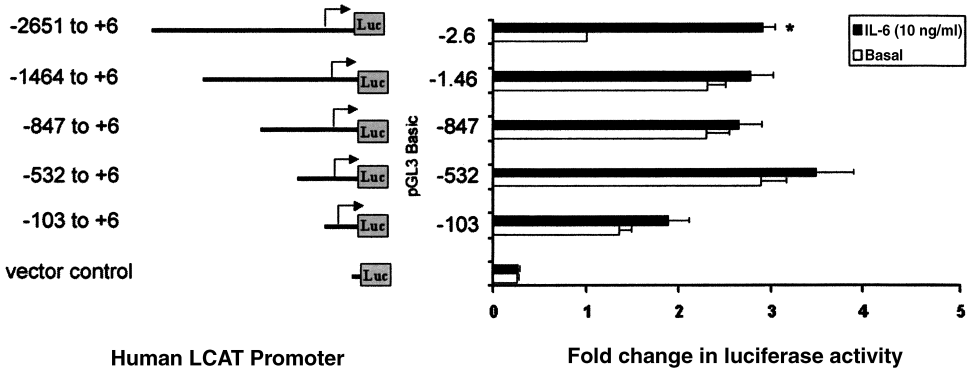

Fig. 2. An IL-6-responsive regulatory element is located within the distal (−2651 bp to −1464 bp) LCAT promoter region. The full length (−2651/+6 bp) and sequential deletion promoter constructs (−1464/+6 bp, −847/+6 bp, −532/+6 bp, and −103/+6 bp) were transfected in HepG2 cells as described and treated with IL-6 (10 ng/ml) or vehicle (PBS) for 16 h. All data presented represents the mean ± SEM for normalized luciferase activity from seven independent experiments performed in triplicate. Statistically significant differences within each individual length promoter construct (basal vs. IL-6 treatment) are shown (*$P < 0.0001$).

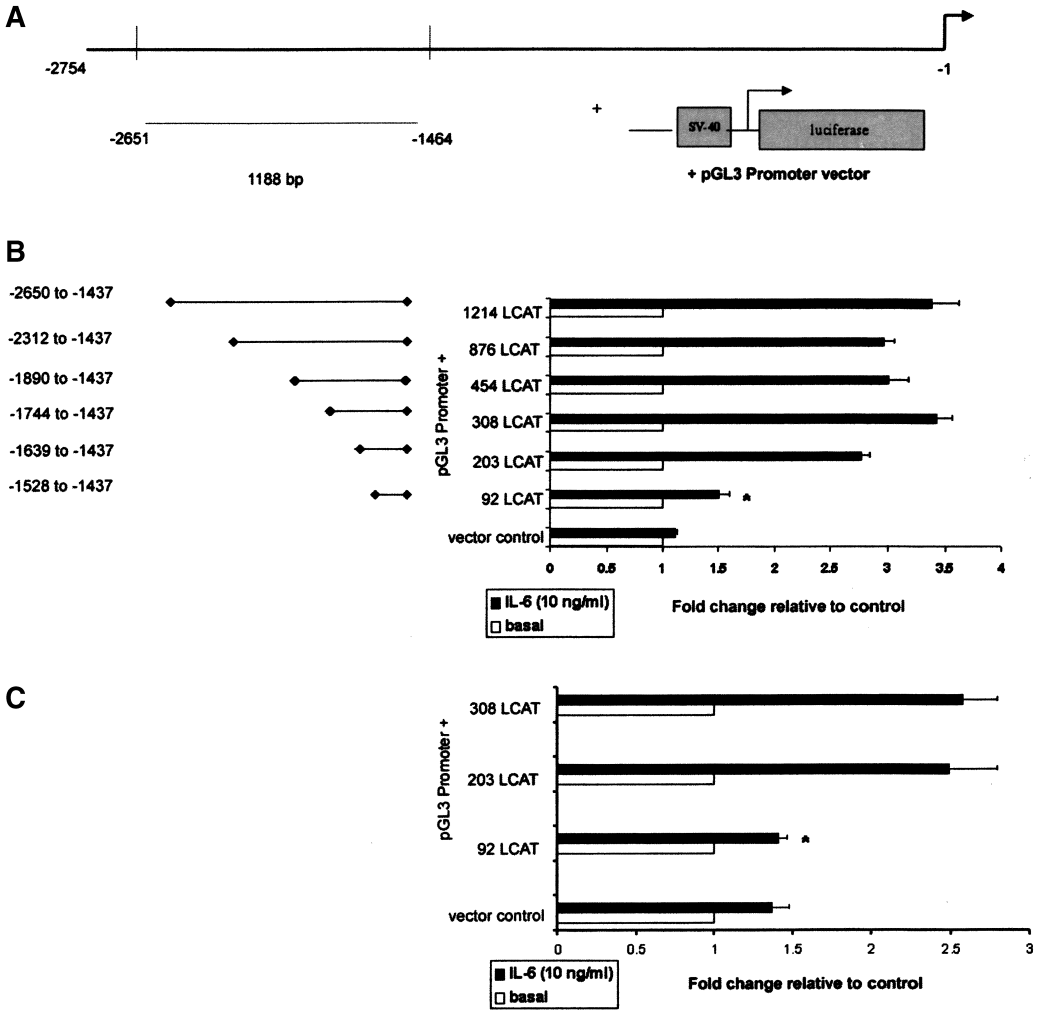

Fig. 3. A 203 bp (−1639 bp to −1437 bp) region is sufficient to mediate the IL-6 induced increase in LCAT promoter activity independent of the downstream promoter sequence. A: Sequential promoter segments within the −2651 bp to −1464 bp promoter region were linked to a minimal SV40 promoter construct (pGL3-promoter) as described in Materials and Methods. B: HepG2 cells were transfected with the −1214 bp, −876 bp, −454 bp, −308 bp, −203 bp and −92 bp distal constructs. Cells were treated with either IL-6 (10 ng/ml) or vehicle (PBS) for either 4 or 16 h. C: Mouse fibroblast NIH/3T3 cells were similarly transfected with the −308 bp, −203 bp, and −92 bp promoter constructs to determine whether the IL-6 response conferred by the 203 bp region was liver specific. Data presented represents results from three independent experiments performed in triplicate. The mean ± SEM for normalized luciferase activity is shown. Statistically significant differences between the longest (B, 1214 bp and C, 308 bp) and the respective shorter constructs within each figure are shown (*$P < 0.001$).

to mediate the IL-6 response independent of the remaining downstream promoter sequence.

A STAT3 binding site is mapped within a minimal IL-6 response element (−1514 bp to −1508 bp)

To identify the minimal DNA element(s) necessary to mediate the IL-6 response, putative IL-6-responsive transcription factor binding sites were mapped within the 203 bp (−1639 bp to −1437 bp) promoter region using both GCG and web-based transcription factor search programs (see Materials and Methods). This analysis indicated the presence of four potentially relevant transcription factor binding sites: Site 1 (−1616 bp to −1598 bp) for a sterol-response element (SRE)/Sp-1 (33), Site 2 (−1548 bp to −1527 bp) for AP-1/Sp-1 (39), Site 3 (−1514 bp to −1505 bp) for STAT3 (25, 40, 43, 44), and Site 4 (−1491 bp to −1483 bp) for CEBP/β (24) (**Fig. 4A**). To determine the contributions of each of these sites to the IL-6 response, independent cluster mutations (mut 1–4) were introduced within each respective site within the context of the 203 bp segment and cloned into the PGL3 promoter vector. The resulting constructs were used for transient transfection. The results show that constructs containing mutations within Sites 1 and 4 retained the full IL-6 response, while the construct containing the mutation within Site 2 consistently reduced promoter activity by approximately 30% (relative to control) (Fig. 4B). In contrast, mutation within Site 3 completely abolished the IL-6 response. Al-

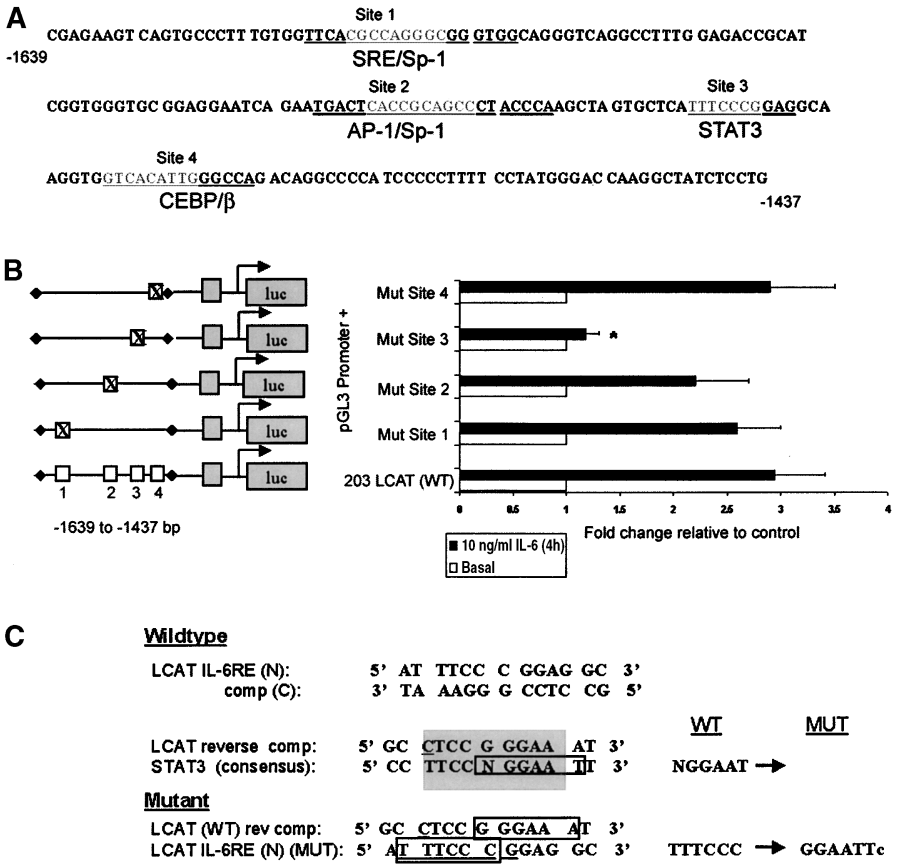

Fig. 4. Functional mutation analysis of the 203 bp distal promoter region identifies a minimal IL-6 response element. A: Computer-aided database analysis of the human LCAT promoter (Acc. no: X51966) identified four putative binding sites for IL-6 responsive transcription factors (underlined), including sterol response element (SRE)/Sp-1, AP-1/Sp-1, STAT3, and CEBP/β (Sites 1–4, respectively). Individual cluster mutations (shaded in gray) were introduced within the 203 bp LCAT promoter construct by PCR amplification as described in Materials and Methods. B: HepG2 cells were transfected with either the wild-type 203 bp LCAT promoter construct (pGL3 Promoter + 203 LCAT) or one of a series of four individual cluster mutation constructs (pGL3 + 203 Mut Site 1–4). Results represent normalized luciferase activity (mean ± SEM) from three independent experiments performed in triplicate. The statistical differences between reporter activity of the wild-type control (203 LCAT) and the four individual mutated constructs (Mut Site 1–4) are shown ($*P < 0.05$). C: Comparison of human LCAT promoter Site 3 (−1514 bp to −1508 bp) to the wild-type STAT3 consensus sequence. The 9 bp STAT3 palindrome sequence is highlighted in gray. The GGGAA(A/T) sequence (enclosed box) is necessary to mediate STAT3-DNA binding, as referenced in the text. A comparable mutation was introduced within the putative LCAT IL-6 response element (Site 3).

though contribution of the upstream promoter sequence within Site 2 cannot be discounted, the LCAT promoter sequence within Site 3 (−1514 bp to −1508 bp) appears to play a dominant role in the IL-6 mediated response.

Further examination of the LCAT IL-6 response element (IL-6RE) within Site 3 revealed high sequence homology to the well characterized mediator of IL-6 signal transduction and STAT family member STAT3 (25, 44) (Fig. 4C). The binding of STAT3 is mediated through a highly conserved TTC(C/T)GGGAA consensus motif (40, 43). A one base pair difference is noted upon comparison of the STAT3 wild-type sequence and the minimal LCAT IL-6RE (Fig. 4C). These data raise the possibility that IL-6-induced STAT3 activation contributes to IL-6 mediated regulation of the LCAT promoter.

Overexpression of STAT3 upregulates LCAT promoter activity in HepG2 cells in the presence of IL-6

To determine whether STAT3 is involved in IL-6 mediated regulation of the LCAT promoter, HepG2 cells were cotransfected with either wild-type STAT3 (WT), mutant STAT3 (Y705F), or CEBP/β, and either the full-length LCAT construct (pGL3 − 2.6 LCAT) or the 203 bp (pGL3 Promoter + 203 LCAT) wild-type or mutant Site 3 constructs. Cells were treated with IL-6 (10 ng/ml) for 4 h and collected for quantitation of luciferase activity. In the presence of IL-6, overexpression of wild-type STAT3 (WT) consistently enhanced the activity of the both the full-length LCAT construct and the shorter 203 bp WT fragment from an average of 2.5- to 6.5-fold, and 2.8- to 5.9-fold, respectively (**Fig. 5A**). In contrast, overexpression of the dominant negative mutant of STAT3 (Y705F) (45) actually inhibited

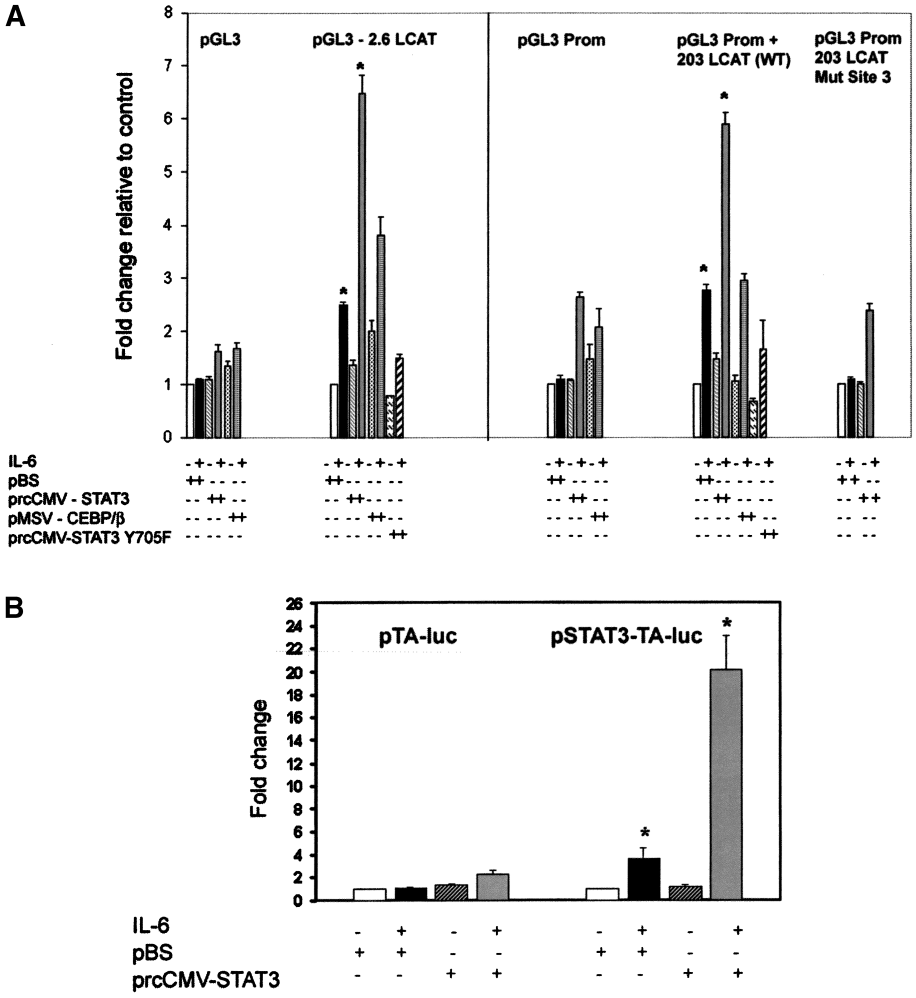

Fig. 5. Overexpression of STAT3 significantly enhances the IL-6 effect on LCAT promoter activity in HepG2 cells. A: HepG2 cells were cotransfected with either the full length (pGL3 − 2.6 LCAT), wild-type 203 bp (pGL3 Promoter + 203 LCAT), or mutant (Site 3) 203 bp LCAT (pGL3 Promoter + 203 LCAT Mut Site 3) promoter constructs and either prcCMV-STAT3 (wild-type), STAT3 Y705F (mutant), pMSV-CEBP/β expression plasmids, or pBluescript (pBS) to maintain equal concentrations of transfected DNA in each sample. Cells were incubated in IL-6 (10 ng/ml) for 4 h and collected for quantitation of luciferase activity. B: HepG2 cells were similarly cotransfected with either the pSTAT3 − TA-luc (positive control) construct, containing four sequential copies of a STAT3 response element, or pTA vector alone and either prcCMV-STAT3 or pBluescript (pBS). Cells were treated with IL-6 (100 ng/ml) for 4 h. The mean ± SEM for normalized luciferase activity from three independent experiments is shown for each panel (A and B). Statistical differences in reporter activity between control (pBS without IL-6) and the various overexpression treatment groups (±IL-6) for each construct are shown (A, $*P < 0.001$, B, $*P < 0.05$).

the IL-6 response by ~40% upon double transfection with either length wild-type promoter construct.

Previously published data indicates that a functional JAK-STAT signaling pathway is activated in IL-6 treated HepG2 (46) and NIH/3T3 (47) cells. In this study, HepG2 cells were transfected with a positive control STAT3 tandem response element construct (pSTAT3-TA-Luc) and exposed to IL-6 as described. Treatment with IL-6 (100 ng/ml) alone was sufficient to significantly increase pSTAT3-TA luciferase activity in these cells (Fig. 5B). This effect was dramatically enhanced by cotransfection with wild-type STAT3 (WT). Collectively these data confirm the presence of the STAT3 signaling pathway within HepG2 cells.

As illustrated in Fig. 4B, although mutation of a putative CEBP/β site (Site 4) had no effect on the IL-6 response, further confirmation was sought by overexpression of wild-type CEBP/β in these cells. Consistent with the functional mutation analysis, CEBP/β overexpression did not affect IL-6 mediated activation of the 203 bp LCAT promoter fragment (wild-type) (Fig. 5A), indicating that CEBP/β does not bind within the 203 bp region. CEBP/β did have a moderate effect on the full-length promoter construct in the presence of IL-6, increasing the IL-6 response from 2.5- to 3.8-fold (Fig. 5A). This later observation indicates that additional CEBP/β binding sites may be located outside of the distal 203 bp region described here. These data do however, provide evidence

that STAT3 is a contributing IL-6-responsive transcription factor binding within the LCAT IL-6RE (−1514 bp to −1508 bp) described above, suggesting that in these cells the increase in LCAT promoter activity is activated, at least in part, via the JAK-STAT3 pathway.

IL-1β inhibits the IL-6 mediated upregulation of LCAT promoter activity

The final outcome of cytokine-mediated transcriptional regulation is largely influenced by simultaneous activation of multiple signal transduction pathways during the inflammatory response (27, 28, 48). To distinguish whether LCAT promoter activity is differentially regulated in the presence of more than one cytokine, transfected cells were simultaneously exposed to either IL-6, IL-6 + IL-1β, or IL-6 + TNF-α. The addition of IL-1β inhibited the significant IL-6 mediated upregulation of LCAT promoter activity (**Fig. 6**). A similar inhibition of the IL-6 response was observed in the presence of TNF-α (data not shown). Involvement of upstream signal transduction crosstalk is suggested.

DISCUSSION

Increased concentrations of inflammatory cytokines, including IL-6, have been associated with abnormalities in lipid metabolism observed during inflammatory conditions (2). In particular, several components of the reverse cholesterol transport system are adversely affected by proinflammatory cytokines (4–8). The data presented here suggest that the IL-6 mediated upregulation of the human LCAT promoter in human hepatoma HepG2 cells involves, at least in part, activation of the STAT3 pathway.

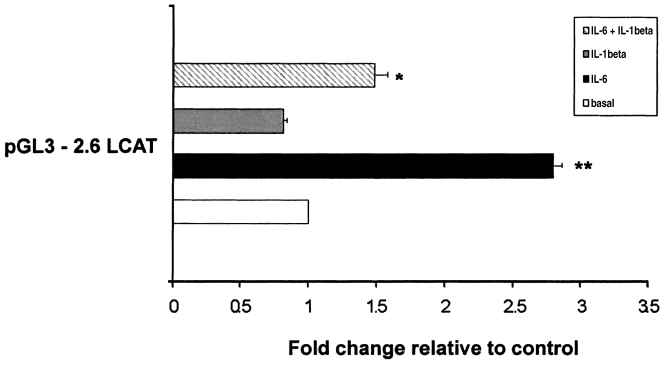

Fig. 6. IL-1β inhibits the IL-6 mediated upregulation of LCAT promoter activity. HepG2 cells were transfected with the full length (−2651/+6 bp) LCAT promoter construct (pGL3 − 2.6 LCAT) and treated with either IL-6 (10 ng/ml), IL-1β (10 ng/ml), IL-6 and IL-1β, or vehicle (PBS) for 16 h as described in the Materials and Methods. Data presented is from three independent experiments performed in triplicate and represents normalized luciferase activity corrected for transfection efficiency. Error bars represent the standard error of the mean. Statistically significant differences in luciferase activity between the full-length untreated control (basal) and the three cytokine treatment groups are shown (*$P < 0.01$, **$P < 0.0001$).

A minimal IL-6 response element, located approximately 1.5 kb upstream of the LCAT gene start site, was identified.

In this analysis, treatment with IL-6 induced a consistent 2.5-fold activation of the human LCAT promoter in transiently transfected HepG2 cells. In contrast, neither LPS, TNF-α, nor IL-1β significantly altered LCAT transcriptional activity. This selective activation of the LCAT promoter by IL-6 is consistent with a previous report indicating that endogenous LCAT transcriptional activity is modestly increased by IL-6 in HepG2 cells (21).

Deletion mapping analysis of the full length LCAT promoter indicated the presence of a *cis*-acting regulatory element located within a 203 bp distal (−1639 bp to −1437 bp) promoter region. This region alone is sufficient to mediate the IL-6 induced upregulation of LCAT promoter activity observed within the full-length construct. Further promoter mapping studies revealed a minimal IL-6 response element within the −1514 bp to −1508 bp segment (Site 3) of the distal LCAT promoter. Comparison of the minimal LCAT IL-6RE sequence, CTCCGGGAA, indicated high sequence homology to the consensus STAT3 DNA binding motif TTC(C/T)GGGAA (25, 40, 43, 44), suggesting that Site 3 is a STAT3 binding site. Mutagenesis of Site 3 suppressed the IL-6 mediated upregulation of LCAT promoter activity. Furthermore, cotransfection experiments showed that overexpression of wild-type STAT3 (WT) with the wild-type 203 bp LCAT promoter construct amplified the IL-6 response, while similar studies with the mutant Site 3 reporter showed no response to IL-6. In contrast, cotransfection with a mutant form of STAT3 (Y705F) inhibited the ability of IL-6 to increase promoter activity, consistent with previous reports indicating that this dominant negative form of STAT3 inhibits STAT3-dependent induction of target genes (45). These data suggest that the JAK-STAT3 signaling pathway contributes to IL-6 regulation of the LCAT promoter.

IL-6 initiates several distinct signaling pathways that converge upon defined motifs within the promoters of target genes (28, 43). Although novel, nonconsensus IL-6 response elements have recently been described (26, 33). IL-6 signal transduction generally involves either the MAP-kinase/CEBP/β (NF-IL6) signaling pathway (24, 49) or the JAK-STAT signaling pathway (25, 44). In hepatocytes, activation of JAK-STAT pathway predominantly involves phosphorylation of STAT3, although minor activation of STAT1 has been shown (1, 50). The recent generation of STAT3 conditional knockout mice conclusively identified STAT3 as the primary mediator of IL-6 during the hepatic acute phase response (51). In this study, further experiments involving mutagenesis of the putative CEBP/β site (Site 4) within the 203 bp region, and overexpression of CEBP/β, indicated that this particular site does not play a role in the IL-6 response. Although it is likely that other IL-6 responsive signaling pathways contribute to LCAT transcriptional regulation, these data provide evidence that the JAK-STAT, but not the MAP kinase/CEBPβ pathway, is involved in regulation of LCAT transcription in response to IL-6.

The STAT3/IL-6 response element identified in this study represents the first description of a cytokine-inducible regulatory element within the human LCAT promoter. Previous studies identified two functional Sp1/Sp3 binding sites within the −72 bp region of the human LCAT promoter, and demonstrated that they are essential for basal transcription of the LCAT gene (37). Our studies indicate that these proximal sites do not play a role in IL-6 regulation of the LCAT promoter. It remains to be determined, however, whether interactions between these proximal promoter elements and the distal IL-6 response element identified here play a role in fine tuning the activity of the LCAT promoter in response to multiple signals. It is, however, conceivable that regulation of LCAT transcription may involve functional interplay between constitutive and highly inducible *trans*-acting regulatory factors that bind within the far upstream promoter region.

Although the molecular mechanisms contributing to transcriptional regulation of LCAT remain to be fully understood, LCAT mRNA and activity levels have been shown to be regulated under both pro- and anti-inflammatory conditions. Previous observations indicated that LCAT mRNA and activity are downregulated by LPS and TNF-α in vivo (6–8, 19). IL-1β and IL-6 also decreased LCAT activity, while IL-6 only moderately reduced LCAT mRNA levels in HepG2 cells (20, 21). The effect of IL-6 on LCAT mRNA did not involve post-transcriptional modification (21), suggesting that IL-6 acts directly to influence transcriptional regulation. Although here we clearly demonstrate that IL-6 activates LCAT transcription, these findings are in direct contrast to the changes in LCAT expression previously reported in IL-6-treated HepG2 cells.

There are several possible explanations for these contrasting observations. It is possible that, in addition to IL-6, the human LCAT promoter responds to other inflammatory mediators present in vivo, but not in the in vitro context. This interpretation implies that LCAT may be either up- or downregulated in response to temporal inflammatory conditions, and further may depend upon the nature of the dominant cytokine present during the sequential stages of the inflammatory response. More specifically, IL-6 may play a dual role during the evolution of the inflammatory response: a pro-inflammatory role during the early stages and an anti-inflammatory role during inflammation resolution, as recently suggested (52, 53). The early stages of the acute phase response are characteristically marked by the release of the pro-inflammatory cytokines TNF-α and IL-1β, while IL-6 subsequently drives the late stage of the acute phase response (52). Indeed, the induction of IL-6 is thought to be involved in maintenance of homeostasis during the inflammatory reaction, consistent with recent studies that demonstrate an anti-inflammatory role for this pleiotrophic cytokine in vivo (53, 54). Prolonged induction of the pro-inflammatory cytokines TNF-α and IL-1β was noted in STAT3 conditional mutants (51) and sustained levels of TNF-α were observed in IL-6$^{-/-}$ mice (54). IL-6 may therefore contribute to an inhibitory feedback loop capable of shifting between pro- and anti-inflammatory actions in a cell type-dependent manner (55). From this perspective, it is conceivable that the isolated in vitro cell system used for the current studies represents the physiological effects of IL-6 during inflammation resolution, where the previously repressed LCAT gene expression recovers under the influence of IL-6.

Cytokine crosstalk initiated during concurrent signal transduction has a significant impact on the ability of individual cytokines to complete their function, including regulation of gene transcription (48, 56). Accordingly, additional experiments were designed to determine whether concurrent signal transduction influenced the IL-6-mediated transactivation of the LCAT promoter in transiently transfected HepG2 cells. Preliminary data indicated that the IL-6 response is markedly inhibited in the presence of either IL-1β or TNF-α. It therefore appears that integration of the IL-6 signal with signaling pathways induced by other cytokines influence the LCAT promoter via synergistic or antagonistic interactions. Cytokine synergy, as well as antagonism, has recently been documented. For example, cooperative interaction between the IL-6 and TGF-β signaling pathways was recently shown to augment STAT3 mediated gene expression via physical interactions between STAT3 and SMAD3 (Sma- and MAD-related protein) in Hep3B liver cells (57). In contrast, the potential of IL-6 to activate γ-fibrinogen and α-macroglobulin transcription was compromised in the presence of IL-1β, attributed to competitive STAT3/NF-κB DNA binding (32, 48, 58). Alternatively, IL-1β was also recently shown to strongly inhibit IL-6 mediated STAT3 activation and DNA binding through direct activation of the p38 stress kinase pathway in HepG2 cells (55). The identification of STAT3 as a contributing activator of LCAT gene transcription will be useful for deciphering additional upstream cofactors that may influence the IL-6 signaling pathway(s) in the presence of other cytokines.

Accumulating evidence suggests that inflammation may play an important role in regulation of reverse cholesterol transport and plasma HDL-C levels (2). The IFN-γ induced JAK-STAT1 signaling pathway was recently implicated in the regulation of cholesterol trafficking in cultured macrophages (34, 59). The JAK-STAT pathway was also recently shown to be an upstream target of peroxisome proliferator-activated receptors, which are known regulators of lipid metabolism (60). Here we describe a distal IL-6RE, with high sequence homology to STAT3, capable of mediating the IL-6 upregulation of LCAT promoter activity. The opposing effects of IL-6 and other pro-inflammatory cytokines in the regulation of the LCAT promoter may help explain the variation of plasma HDL-C levels observed during progression and resolution of inflammation in vivo. In summary, our data suggests that IL-6 mediates regulation of LCAT transcription at least in part, via the JAK-STAT pathway, and further raises the possibility that this pathway may influence lipid metabolism.

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