## Cloning of the cDNA for human 5-lipoxygenase

(leukotrienes/transcriptional regulation/differentiation/HL60 cells)

Richard A. F. Dixon<sup>\*†</sup>, Raymond E. Jones<sup>\*</sup>, Ronald E. Diehl<sup>\*</sup>, Carl D. Bennett<sup>‡</sup>, Stacia Kargman<sup>§</sup>, and Carol A. Rouzer<sup>§</sup>

Departments of \*Virus and Cell Biology Research and <sup>‡</sup>Medicinal Chemistry, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486; and <sup>§</sup>Department of Pharmacology, Merck Frosst Canada, Inc., Dorval, PQ, Canada H9R 4P8

Communicated by L. L. Iversen, October 7, 1987

ABSTRACT The enzyme 5-lipoxygenase (5-LO) catalyzes the first two reactions in the pathway leading to the formation of leukotrienes from arachidonic acid. Leukotrienes are potent arachidonic acid metabolites possessing biological activities that suggest a role in the pathophysiology of allergic and inflammatory diseases. To obtain structural information about 5-LO for use in developing anti-inflammatory chemotherapeutic agents, the enzyme was purified from human polymorphonuclear leukocytes and the amino acid sequences were determined for several cyanogen bromide-derived peptides. A cDNA clone encoding a 674-amino acid protein containing all of the derived peptide sequences was isolated from a dimethyl sulfoxide differentiated HL60 cell cDNA library. The mRNA encoding 5-LO was detected only in differentiated HL60 cells and not in the undifferentiated cell line, indicating that the expression of 5-LO in this cell line is transcriptionally regulated. In addition, the human protein displays some amino acid sequence homology with several lipases and significant homology with the partial sequences of soybean and reticulocyte lipoxygenases. Thus, 5-LO appears to be a member of a larger family of related enzymes.

The enzyme 5-lipoxygenase (5-LO) catalyzes the first two reactions in the biosynthetic pathway leading to the formation of the leukotrienes from arachidonic acid. The first step is the addition of oxygen at carbon 5 of arachidonic acid, yielding (5S)-hydroperoxy-6,8,11,14-icosatetraenoic acid, which is transformed to leukotriene A<sub>4</sub> (5,6-oxido-7,9,11,14icosatetraenoic acid) by dehydration in the second step (1-5). Leukotriene biosynthesis is known to occur in leukocytes after exposure to inflammatory or immunologic stimuli (1). The biological activities of these compounds, including leukocyte chemotaxis, airway constriction, increased vascular permeability, arteriolar constriction, and dilation of postcapillary venules, suggest a role for the leukotrienes in the pathophysiology of immediate hypersensitivity reactions and inflammation (1). Consequently, the regulation of 5-LO activity has recently become a subject of considerable interest.

The purification of 5-LO from human and porcine peripheral blood leukocytes (PMN), rat basophilic leukemia cells, and mouse mastocytoma cells has now been accomplished (3–7). The human enzyme requires  $Ca^{2+}$  for activity and is stimulated by ATP and a number of nondialyzable cellular components whose identity and function remain undefined (6). Despite these advances, very little is known about the structure of the enzyme or its regulation at the genetic level. In this paper, we report the cloning of the cDNA for 5-LO from differentiated HL60 cells and provide the complete amino acid sequence of the enzyme deduced from the cDNA sequence.

# The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

#### **MATERIALS AND METHODS**

Protein Purification, Peptide Isolation, and Protein Sequencing. 5-LO was purified from human PMNs and the enzymatic activity was determined as described (2, 6). Protein determinations were performed by the method of Lowry (8). Proteins were analyzed by NaDodSO<sub>4</sub>/PAGE as described by Laemmli (9). For amino acid sequence analysis, the final enzyme fraction of 5-LO (1.9 nmol) was precipitated with 15% trichloroacetic acid, rinsed with diethyl ether, and lyophilized. Peptides were prepared by treating 0.5 nmol of the purified protein with 0.4 mM CNBr in 70% formic acid at 23°C for 20 hr. After lyophilization, the samples were resuspended in 20 mM trifluoroacetic acid and the soluble peptides were separated by reversed-phase HPLC on a Vydac C-4 column eluted with a 10-70% acetonitrile gradient containing 20 mM trifluoroacetic acid. Amino-terminal amino acid sequence was determined for each purified peptide and for the intact protein with an Applied Biosystems (Foster City, CA) gas-phase sequenator as described (10, 11).

Cell Culture. HL60 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum in slowly rotating roller bottles at 37°C. For differentiation, HL60 cells were diluted to  $2 \times 10^5$  cells per ml and dimethyl sulfoxide and dexamethasone (Me<sub>2</sub>SO/ Dex) were added to 1.3% and 10  $\mu$ M, respectively. Cells were harvested at various times by centrifugation at 500 × g for 10 min, washed once with phosphate-buffered saline, and frozen at  $-70^{\circ}$ C. 5-LO activity in the cells was assayed as described (2).

RNA Isolation and Blotting. Total cellular RNA was isolated from HL60 cells by the guanidinium isothiocyanate/ CsCl method (12). The  $poly(A)^+$  RNA fraction was separated by chromatography on oligo(dT)-cellulose as described (13). For RNA blot analysis, 10  $\mu$ g of total RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to nitrocellulose, and baked (14). The filter was prehybridized and hybridized in  $5 \times$  standard saline citrate (SCC; 1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 6.8)/10× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/50 mM NaPO<sub>4</sub>, pH 6.8/0.1% NaDod- $SO_4/50 \ \mu g$  of denatured salmon sperm DNA per ml at 65°C. The double-stranded DNA probe was labeled by nicktranslation with  $[\alpha^{-32}P]dCTP$  and used at 10<sup>6</sup> cpm/ml in the hybridization. The filters were washed four times in  $5 \times$ 

Abbreviations: LO, lipoxygenase; PMN, polymorphonuclear leukocyte; Me<sub>2</sub>SO/Dex, 1.3% dimethyl sulfoxide/10  $\mu$ M dexamethasone.

<sup>&</sup>lt;sup>†</sup>To whom reprint requests should be addressed.

<sup>&</sup>lt;sup>¶</sup>This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03600).

SSC/0.1% NaDodSO<sub>4</sub> at 65°C, dried, and autoradiographed (14).

Oligonucleotide Synthesis. All oligonucleotides were synthesized using an Applied Biosystems model 380A DNA Synthesizer. Two overlapping complementary oligonucleotides, whose sequence was based on preferred codon usage, were synthesized corresponding to either the amino-terminal peptide (amino acids 12-22) or peptide-27 amino acid sequence. The oligonucleotide sequences corresponding to the amino-terminal peptide are 5'-pCAGTGGTTTGCTGGCA-CAGATGACTACATCTAC and 5'-pGTAGATGTAGTC. The oligonucleotide sequences corresponding to peptide 27 are 5'-pATGGCCCGGTTCCGGAAGAACCTGGAGGC-CATTGTCTCTGTGATTGCTGAGCGGAACAAGAAG and 5'-pGGAGTTGGGGGATCCGGTCAGGGGACAGG-TAGTAGTATGGCAGCTGCTTCTTCTTGTTCCGCTC. For peptide 18, a pool of oligonucleotides was synthesized corresponding to all possible coding choices for amino acids 3-10 of peptide 18. The oligonucleotide sequences of the pool are 5'-pGA $_{G}^{A}$ GA $_{G}^{A}$ CA $_{C}^{T}$ TT $_{C}^{T}$ ATNGA $_{G}^{A}$ AA $_{G}^{A}$ CC. cDNA Cloning. Double-stranded oligo(dT)-primed cDNA

**cDNA Cloning.** Double-stranded oligo(dT)-primed cDNA was synthesized from the HL60 cell  $poly(A)^+$  RNA by a modification of the method of Gubler and Hoffman (15) as



N-TERMINUS: PSYTVTVATGSQWFAGTDDYIYLSLVGSA

PEPTIDE 8: VQRA

PEPTIDE 18: YPEEHFIEKPVKEA

PEPTIDE 27: ARFRKNLEAIVSVIAERNKKKQLPYYYLSPDRIPNS

FIG. 1. Purification and amino acid sequence determination of human PMN 5-LO. (A) The final enzyme fraction (10  $\mu$ g) of 5-LO was electrophoresed on a 10% polyacrylamide gel containing NaDodSO<sub>4</sub> and silver-stained. Arrow indicates position and apparent molecular mass (in kDa) of the major polypeptide referred to in the text. (B) CNBr peptides were prepared from purified 5-LO as described. The peptides were resolved by reversed-phase HPLC using a Vydac C-4 column. A tracing of the absorbance profile at 214 nm of material eluting from the column is shown. The major peptide peaks are indicated by arrows. (C) Amino acid sequences obtained for the intact protein and the three CNBr-derived peptides indicated in B are shown. Single-letter amino acid code is used, beginning with the amino terminus of each peptide at the left. The first amino acid shown in all cases was determined in the first cycle of the sequenator and should represent the amino-terminal amino acid of that peptide.

described (16). The cDNA was size-selected by agarose gel electrophoresis (14), ligated to  $\lambda gt10$  arms (17), and packaged in vitro (Gigapack, Stratagene, San Diego, CA). The library was plated on Escherichia coli LE392 and screened unamplified (14). The phage plaques were lifted onto nitrocellulose filters and processed for hybridization as described (14). The filters were prehybridized and hybridized in  $5 \times$ SSC/10 × Denhardt's solution/50 mM NaPO<sub>4</sub>, pH 6.8/0.1% NaDodSO<sub>4</sub>/50  $\mu$ g of denatured salmon sperm DNA per ml/20% formamide (vol/vol) at 42°C. For hybridization, <sup>32</sup>P-labeled probes were used at 10<sup>6</sup> cpm/ml. The overlapping oligonucleotides were labeled with all four  $[\alpha$ -<sup>2</sup>P]dNTPs and Klenow DNA polymerase, the pool oligonucleotides were labeled with  $[\gamma^{-32}P]ATP$  and polynucleotide kinase, and double-stranded DNAs were labeled with  $\left[\alpha\right]$ <sup>32</sup>PldCTP by nick-translation. Filters were washed four times in  $5 \times SSC/0.1\%$  NaDodSO<sub>4</sub> at temperatures ranging from 37°C to 55°C depending on the probe, dried, and autoradiographed (14). Hybridizing phage were purified and phage DNA was isolated as described (14). All other recombinant DNA techniques have been described (14).

**DNA Sequence Analysis.** The *Eco*RI inserts from the  $\lambda$ gt10 5-LO clones were subcloned into both M13mp19 and pUC13 (18). The DNA sequence was determined by the dideoxynucleotide chain-termination method using either Klenow DNA polymerase or avian myeloblastosis virus reverse transcriptase and <sup>35</sup>S-labeled deoxyadenosine 5'-[ $\alpha$ -thio]triphosphate (19, 20). The entire DNA sequence for both strands of each clone was determined.

#### RESULTS

5-LO Protein Characterization. 5-LO was purified from human PMNs as described (2, 6). The final enzyme preparation, which was  $\approx 85\%$  pure as assessed by NaDodSO<sub>4</sub>/PAGE (Fig. 1A), contained a major polypeptide with an apparent molecular mass of 80 kDa and a minor component of 63 kDa. The size of the 80-kDa band is consistent with the previously published estimates for the rat, porcine, and human enzymes (3–7). To obtain peptide sequence for 5-LO, the protein was subjected to chemical cleavage with CNBr. The soluble peptides resulting from the cleavage were resolved by reversed-phase HPLC and purified (Fig. 1B). The three fractions containing the most UV absorbing material and the intact purified protein were subjected to amino acid sequence analysis yielding the sequences shown in Fig. 1C. It should be noted that the amino acid sequence determined for the amino terminus of intact human



FIG. 2. Time course for induction of 5-LO activity in HL60 cells after differentiation. HL60 cells were seeded at  $2 \times 10^5$  cells per ml in the presence (•) or absence ( $\odot$ ) of Me<sub>2</sub>SO/Dex as described. The cells were harvested at the times indicated and assayed for 5-LO activity. One unit of 5-LO activity is the amount of enzyme that will produce 1 nmol of (5S)-hydroperoxy-6,8,11,14-icosatetraenoic acid in 10 min at 37°C using 100  $\mu$ M arachidonic acid as the substrate.

5-LO is identical to the first 25 amino acids reported for the purified rat 5-LO (5). That peptide 27 is derived from the 80-kDa polypeptide is demonstrated by the observation that an antisera directed against a peptide with the same sequence reacts with the 80-kDa and not the 63-kDa polypeptide by immunoblotting (data not shown).

HL60 Cell Expression of 5-LO Activity. Although an adequate source of protein, circulating PMNs proved to be a poor source of mRNA for use in cDNA library construction. Consequently, alternative cell lines were examined for 5-LO expression. Upon exposure to dimethyl sulfoxide, the hu-

GGGCC	GCCGA	G GI	стосс	CGCC	GCT	CGCTG	CT	CCCCG	GCCCG	CG	CC	ATG	CCC	TCC	TAC	ACG The	62 GTC Val
													INT	361	191		116
104	GTG	222	ACT	000	AGC	CAG	TGG	TTC	600	GGC	ACT	GAC	GAC	TAC	ATC	TAC	CTC
Thr	Val	Ala	Thr	61v	Ser	Gln	Tro	Phe	Ala	Glv	Thr	Asp	Asp	Tyr	Ile	Tyr	Leu
7																	_
																	170
AGC	CTC	GTG	GGC	TCG	GCG	GGC	TGC	AGC	GAG	AAG	CAC	CTG	CTG	GAC	AAG	222	TTC
Ser	Leu	Val	Gly	Ser	Ala	Gly	Cys	Ser	Glu	Lys	His	Leu	Leu	Asp	Lys	Pro	Phe
25																	
			***						C 1 T	104	TAC		CTC	ACT	OTC	CAC	224 CAC
Tue	AAC	GAU	Dha	6A6	4.001	000	41.	V-1	Aco	Sen	Tue	Aco	Va1	The	Val	Asn	61
43	ASI	ASP	rne	610	vi.R	uly	AIG	Val	vsh	361	.,,,	Ash	101		101	пар	010
43																	278
GAA	CTG	GGC	GAG	ATC	CAG	CTG	GTC	AGA	ATC	GAG	AAG	CGC	AAG	TAC	TGG	CTG	AAT
Glu	Leu	Gly	Glu	Ile	Gln	Leu	Val	Arg	Ile	Glu	Lys	Arg	Lys	Tyr	Trp	Leu	Asn
61																	
																	332
GAC	GAC	TGG	TAC	CTG	AAG	TAC	ATC	ACG	CIG	AAG	ACG	000	CAC	666	GAC	TAC	AIL
Asp	Asp	Irp	lyr	Leu	Lys	lyr	116	Inr	Leu	Lys	100	Pro	m15	619	ASP	IYE	116
/9																	386
GAG	TTC	000	TGC	TAC	CGC	TGG	ATC	ACC	GGC	GAT	GTC	GAG	GTT	GTC	CTG	AGG	GAT
Glu	Phe	Pro	Cys	Tyr	Arg	Trp	Ile	Thr	Gly	Asp	Val	Glu	Val	Val	Leu	Arg	Asp
97			•	·	-	-			-								
																	440
GGA	CCC	GCA	AAG	TTG	GCC	CGA	GAT	GAC	CAA	ATT	CAC	ATT	CTC	AAG	CAA	CAC	CGA
Gly	Arg	Ala	Lys	Leu	Ala	Arg	Asp	Asp	Gln	Ile	His	Ile	Leu	Lys	Gln	His	Arg
115																	404
001		***	CTC						***	TAT		TCC	ATC	CAC	TCC		00T
Arg	Ive.	61	1.00	Glu	The	Arg	Gln	1 vs	Gln	Tvr	Arg	Tro	MET	Glu	Tra	Asn	Pro
133	-,,,	010		010				-,-	••••	. <b>.</b> .				••••			
																	548
GGC	TTC	CCC	TTG	AGC	ATC	GAT	GCC	AAA	TGC	CAC	AAG	GAT	TTA	CCC	CGT	GAT	ATC
Gly	Phe	Pro	Leu	Ser	Ile	Asp	Ala	Lys	Cys	His	Lys	Asp	Leu	Pro	Arg	Asp	Ile
151																	
																	602
CAG	m	GAT	AGI	GAA	AAA	GGA	GIG	GAC	111	611	016	AAI	TAU	ILL For	AAA	606	AIG MET
100	rne	ASP	Ser	610	Lys	619	Val	ASP	rne	vai	Leu	ASI	iyr.	Ser	Lys	ALS	HIC 1
103																	656
GAG	AAC	CTG	TTC	ATC	AAC	CGC	TTC	ATG	CAC	ATG	TTC	CAG	TCT	TCT	TGG	AAT	GAC
Glu	Asn	Leu	Phe	Ile	Asn	Arg	Phe	MET	His	MET	Phe	Gln	Ser	Ser	Trp	Asn	Asp
187						-											
																	710
TTC	GCC	GAC	ш	GAG	AAA	ATC	III	GTC	AAG	ATC	AGC	AAC	ACT	ATT	TCT	GAG	CGG
Phe	Ala	Asp	Phe	Glu	Lys	Ile	Phe	Val	Lys	Ile	Ser	Asn	Thr	He	Ser	Glu	Arg
205																	764
010	ATC	AAT	CAC	TGC	CAG	GAA	GAC	CTG	ATG	TTT	000	TAC	CAG	TTC	CTG	AAT	04
Val	MET	Asn	His	Tro	Gln	Glu	Asp	Leu	MET	Phe	Glv	Tvr	Gln	Phe	Leu	Asn	Glv
223					••••						•••,	.,.	••••				•,
																	818
TGC	AAC	CCT	GTG	TTG	ATC	CGG	CGC	TGC	ACA	GAG	CTG	CCC	GAG	AAG	CTC	CCG	GTG
Cys	Asn	Pro	Val	Leu	Ile	Arg	Arg	Cys	Thr	Glu	Leu	Pro	Glu	Lys	Leu	Pro	Val
241																	
					~~~	100									~~~	~~~	8/2
The	The	C1	MET	U1A Vo1	Clu	Cue	Son		6AG	Aca	Cla	1.00	Sec	110	6AG	Cla	GAG C1
259		910		441	010	cy s	361	Leu	010		010	Leu	Jei	Leu	010	910	910
																	926
GTC	CAG	CAA	GGG	AAC	ATT	TTC	ATC	GTG	GAC	III	GAG	CTG	CTG	GAT	GGC	ATC	GAT
Val	Gln	Gln	Gly	Asn	Ile	Phe	Ile	Val	Asp	Phe	Glu	Leu	Leu	Asp	Gly	Ile	Asp
277																	
						100			~ ~ ~				007			100	980
412	ARG	AAA Lue	The	GAL Aco	Pro	Cue	The	1.00	Cla	Pha	1.00	41-	410	Pag	AIC II.	Cue	116
295		L y 3		w2h		Uy S		Leu	910	1116	Leu	A14	M10		116	cys	Leu
																	1034
CTG	TAT	AAG	AAC	CTG	GCC	AAC	AAG	ATT	GTC	CCC	ATT	GCC	ATC	CAG	CTC	AAC	CAA
Leu	Tyr	Lys	Asn	Leu	Ala	Asn	Lys	Ile	Val	Pro	Ile	Ala	Ile	Gln	Leu	Asn	Gln
313																	
470			C 47			<b>CCT</b>		TTO	CTC	007	100						1088
TIA	Pro	66A	GAI	6A6	AAC	Peo	110	Pho	1.00	Bee	100	GAL	GCA	AAA	TAG	GAC	166
331	110	913	w2b	910	420	11.0	116	rne	Leu	F (10	Ser	ASP	A13	LYS	iyr	лsр	irp
																	1142
CTT	TTG	GCC	<b>A</b> A A	ATC	TGG	GTG	CGT	TCC	AGT	GAC	TTC	CAC	GTC	CAC	CAG	ACC	ATC
Leu	Leu	Ala	Lys	Ile	Trp	Val	Arg	Ser	Ser	Asp	Phe	His	Val	His	Gln	Thr	Ile
349																	
	<b></b>					C+7		070	101		<b>67</b> -						1196
The	LAC Hie	1	رال الما	Ace.	The	UAI Hie	LIG	616 Vs)	Ser	GAG	- G   ] - V-1	Ph-	GGC	ALL T1-	GCA	AIG	THE
367									961	910			319	116		112.1	· <b>·</b> ·

man promyelocytic leukemia cell line HL60 can be induced to differentiate into cells that closely resemble mature granulocytes and that are capable of synthesizing leukotrienes (21–23). HL60 cells were seeded in the presence or absence of Me<sub>2</sub>SO/Dex, allowed to grow, and tested for 5-LO activity. As shown in Fig. 2, little 5-LO activity was detected in untreated HL60 cells. However, cells grown for 7 days in the presence of Me<sub>2</sub>SO/Dex contained 6–8 times the amount of 5-LO activity found in the untreated cells. The increase in 5-LO activity was continuous and linear throughout the time course of the experiment.

	CGC Arg	CAG Gln	CTG Leu	CCT Pro	GCT Ala	GTG Val	CAC His	CCC Pre	ATT Ile	TTC Phe	AAG Lys	CTG Leu	CTG Leu	GTG Val	GCA Ala	CAC His	GTG Val	AGA Arg
	385 TTC	ACC	ATT	GCA	ATC	AAC	ACC	AAG	GCC	CGT	GAG	CAG	стс	ATC	TGC	GAG	TGT	1304 GGC
	403 CTC	111F	GAC	AAG	GCC	AAC	GCC	ACA	GGG	GGC	GGT	GCG	CAC	GTG	CAG	ATG	GTG	1358 CAG
	Leu 421	Phe	Asp	Lys	Ala	Asn	Ala	Thr	Gly	Gly	Gly	Gly	His	Val	Gln	HET	Val 8	<u>Gln</u> 1412
	AGG Arg 439	GCC Ala	ATG Met	AAG Lys	GAC Asp	CTG Leu	ACC Thr	TAT Tyr	GCC Ala	TCC Ser	CTG Leu	TGC Cys	TTT Phe	CCC Pro	GAG Glu	GCC Ala	ATC Ile	AAG Lys
	GCC Ala 457	CGG Arg	GGC Gly	ATG Net	GAG Glu	AGC Ser	AAA Lys	GAA Glu	GAC Asp	ATC Ile	CCC Pro	TAC Tyr	TAC Tyr	TTC Phe	TAC Tyr	CGG Arg	GAC Asp	GAC Asp
	GGG Gly	CTC Leu	CTG Leu	GTG Val	TGG Trp	GAA Glu	GCC Ala	ATC Ile	AGG Arg	ACG Thr	TTC Phe	ACG Thr	GCC Ala	GAG Glu	GTG Val	GTA Val	GAC Asp	1520 ATC Ile
	TAC Tyr	TAC Tyr	GAG Glu	GGC Gly	GAC Asp	CAG Gln	GTG Val	GTG Val	GAG Glu	GAG Glu	GAC Asp	CCG Pro	GAG Glu	CTG Leu	CAG Gln	GAC Asp	TTC Phe	1574 GTG Val
	493 AAC	GAT	GTC Val	TAC	GTG Val	TAC Tyr	66C 61 v	ATG MFT	CGG	GGC G1v	CGC	AAG Lys	TCC Ser	TCA Ser	GGC Glv	TTC Phe	CCC Pro	1628 AAG Lys
	511 TCG	GTC	AAG	AGC	CGG	GAG	CAG	CTG	TCG	GAG	TAC	CTG	ACC	GTG	GTG	ATC	ттс	1682 ACC
	Ser 529	Val	Lys	Ser	Arg	Glu	Gln	Leu	Ser	Glu	Tyr	Leu	Thr TAC	Val GAC	Val	Ile	Phe	Thr 1736 TCC
	Ala 547	Ser	Ala	Gln	His	Ala	Ala	Val	Asn	Phe	Gly	Gln	Tyr	Asp	Trp	Cys	Ser	Trp 1790
	ATC Ile 565	CCC Pro	AAT Asn	GCG Ala	CCC Pro	CCA Pro	ACC Thr	ATG Met	CGA Arg	GCC Ala	CCG Pro	CCA Pro	CCG Pro	ACT Thr	GCC Ala	AAG Lys	GGC Gly	GTG Val
	GTG Val 583	ACC Thr	ATT Ile	GAG Glu	CAG Gln	ATC Ile	GTG Val	GAC Asp	ACG Thr	CTG Leu	CCC Pro	GAC Asp	CGC Arg	GGC Gly	CGC Arg	TCC Ser	TGC Cys	TGG Trp
	CAT His	CTG Leu	GGT Gly	GCA Ala	GTG Val	TGG Trp	GCG Ala	CTG Leu	AGC Ser	CAG Gln	TTC Phe	CAG Gln	GAA Glu	AAC Asn	GAG Glu	CTG Leu	TTC Phe	1898 CTG Leu
	GGC Gly	ATG Met	TAC Tyr	CCA Pro	GAA Glu	GAG Glu	CAT His	TTT Phe	ATC Ile	GAG Glu	AAG Lys	CCT Pro	GTG Val	AAG Lys	GAA Glu	GCC Ala	ATG Net	1952 GCC Ala
	619 CGA	TTC	18 CGC	AAG	AAC	СТС	GAG	GCC	ATT	GTC	AGC	GTG	ATT	GCT	GAG Glu	CGC	AAC	2006 AAG
	637	AAG	CAG	CTG	CCA	TAT	TAC	TAC	TTG	TCC	CCA	GAC	CGG	ATT	CCG	AAC	AGT	2060 GTG
	<u>Lys</u> 655	Lys	Gln	Leu	Pro	Tyr	Tyr	Tyr	Leu	Ser	Pro	Asp	Arg	Ile	Pro	Asn	Ser	Va1 2126
GCC ATC TGAGGACACT GCCAGTCTCA CTGTGGGAAG GCCAGCTGCC CCAGCCAGAT GGACTCCAGC Ala Ile 673													2196					
CTGCCTGCCA GCCTGTCTGG CCAGGCCTCT TGGCAGTCAC ATCTCTTCCT CCGAGGCCAG T										TACC	111CCA							
	тп	ATTCT	π	GATCI	TCAG	6 G/	ACTG	CATA	GATI	GTAT	<b>X</b> /	AGTG	TAAAC	ACC	CATAGO	GA	CCCA	TTCTAC
	ACA	GAGCJ	NGG	ACTG	ACAG	6 C6	TCCT	STCC	ACAG	CCAG	ст (	CAGCAT	TTCC	AC	CCAN	<b>ca</b>	GCAA	CAGCAA
ATCACGACCA CTGATAGATG					5 TC	TCTATTCTTG			TTGGAGACAT			GGGATGATTA			TC	TATTTGTGCI		
	TAG	TCCA	ΛT	CCTTO	GCACA	T A6	TAGG	FACC	CAAT	TCAA	п	ACTAT	<b>GAA</b> T	GA	ATTAAG	AA	TTGG	Z476 TTGCCA
	TA	AAATA		TCAG	TCAT	T TA		2506 VAAA										

FIG. 3. DNA sequence for the *Eco*RI insert of  $\lambda$ 5LO-6 is shown. The amino acids predicted to be encoded by the DNA sequence are presented beginning at the first ATG contained within the clone. The positions of the derived peptide sequences shown in Fig. 1*C* are underlined and numbered accordingly. The nucleotides are numbered on the right of each line and the amino acids are numbered on the left.

#### Biochemistry: Dixon et al.

cDNA Cloning. Poly(A)<sup>+</sup> RNA was isolated from HL60 cells grown for 5 days in the presence of Me<sub>2</sub>SO/Dex and used to construct a cDNA library in  $\lambda$ gt10. Oligonucleotide probes were synthesized corresponding to the aminoterminal peptide sequence and the sequences of peptides 18 and 27. These oligonucleotides were used to screen  $2 \times 10^6$ recombinants from the HL60 cDNA library. A total of 35 clones were isolated that hybridized to probes for both peptide-18 and -27 sequences. No clones were isolated that hybridized to the oligonucleotides corresponding to the amino-terminal peptide sequence. Restriction analysis of the hybridizing clones revealed that all contained inserts ranging from 1.0 to 1.3 kilobases whose restriction maps overlapped. The inserts from the three longest clones ( $\lambda$ 5LO-10,  $\lambda$ 5LO-28, and  $\lambda$ 5LO-30) were sequenced and shown to contain identical overlapping DNA sequences. The cDNA sequence contained an open reading frame capable of encoding 211 amino acids, including the amino acid sequences of peptides 18 and 27, but not of the amino-terminal peptide or peptide 8. The DNA sequences encoding peptides 18 and 27 were at the extreme 3' end of the open reading frame. All clones contained a stretch of poly(A) residues 428 bases 3' of the open reading frame. These clones, therefore, probably correspond to the 3' end of the 5-LO mRNA.

To obtain full-length cDNA clones for 5-LO another cDNA library was made using poly(A)<sup>+</sup> RNA from 7-day differentiated HL60 cells. The double-stranded cDNA was sizeselected for those cDNAs >2.2 kilobases prior to ligation into the  $\lambda$ gt10 vector arms. This library contained 500,000 recombinants, which were screened unamplified with both a restriction fragment from  $\lambda$ 5LO-30 (see Fig. 3, nucleotides 1430– 1740) and with the oligonucleotides corresponding to the amino-terminal peptide. Of the 32 clones that hybridized to the  $\lambda$ 5LO-30 probe, 8 also hybridized to the probe for the amino-terminal sequence. These 8 clones contained inserts between 2.4 and 2.6 kilobases long and had restriction maps that overlapped the clones corresponding to the 3' end of the 5-LO mRNA. DNA sequencing of two of the clones ( $\lambda$ 5LO-4 and  $\lambda$ 5LO-6) revealed that both contained an identical open reading frame of 674 amino acids following the first ATG in the sequence (Fig. 3). This open reading frame would encode a polypeptide of 78 kDa, containing all of the 5-LO-derived peptide sequences (see Figs. 1C and 3). The first ATG contained in the cDNA clones immediately precedes the DNA sequence that encodes the amino-terminal peptide sequence derived from the mature protein. The peptide sequence that would be encoded by the DNA sequence preceding this ATG does not resemble any known leader or signal sequence. In addition, the DNA sequence surrounding this ATG is similar to the consensus sequence for eukaryotic initiation codons (24). Therefore, while the possibility of upstream initiation at an ATG not contained in this cDNA cannot be ruled out, it is likely that this codon serves as the start site for translation of 5-LO. As in the partial 5-LO clones, the termination codon is followed by 428 bases of 3' untranslated sequence. All of the clones contain a stretch of poly(A) residues at their 3' end, which is preceded by the consensus polyadenylylation signal sequence AATAAA.

**Expression of 5-LO Specific RNA in HL60 Cells.** The size of the mRNA corresponding to the cDNA clones was determined by RNA blot analysis using total RNA from differentiated and undifferentiated HL60 cells (Fig. 4). The cDNA hybridized to a single RNA species of 2700 nucleotides that was detected in the differentiated cell RNA and not in the undifferentiated cell RNA. The detection of mRNA homologous to  $\lambda$ 5LO-30 only in cells expressing 5-LO activity indicated that the induction of 5-LO activity in HL60 was regulated at the transcriptional level. In addition, the size of the mRNA suggests that the 5-LO clones that were obtained represent almost the entire message.

28S► ► 18S►

FIG. 4. RNA blot analysis of 5-LO mRNA expression in differentiated and undifferentiated HL60 cells. Total cellular RNA was isolated from HL60 cells grown in the presence (+) or absence (-) of Me<sub>2</sub>SO/ Dex for 5 days. The RNA (10  $\mu$ g) was electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to nitrocellulose, and processed as described. The EcoRI insert from  $\lambda$ 5LO-30, which encompasses the 3' third (nucleotides 1433-2506 in Fig. 3) of the 5-LO coding region, was nicktranslated and used as the hybridization probe. The positions of 28S and 18S ribosomal RNA are indicated. Arrowhead designates the location of the 2700-nucleotide species referred to in the text.

### DISCUSSION

This report describes the cloning of the cDNA for human 5-LO based on partial protein sequence. The availability of a clone for 5-LO should prove useful for studying the structure of the enzyme and the regulation of 5-LO gene expression. Expression of 5-LO is restricted to leukocytes and a few other tissues. Stimulation of cells containing 5-LO with agents that increase Ca<sup>2+</sup> activates the enzyme and allows the cells to produce leukotrienes, provided that free arachidonic acid is available. In HL60 cells, the ability to synthesize leukotrienes in response to  $Ca^{2+}$  ionophores requires that the cells be matured to granulocytes. The induction of leukotriene synthetic capability appears to involve an increase in both 5-LO mRNA and 5-LO protein. Thus, it is suggested that 5-LO gene expression is induced when the cells undergo differentiation. This system could, therefore, prove useful for defining the factors that control 5-LO gene regulation as well as granulocyte differentiation.

The predicted amino acid sequence of 5-LO was examined by the methods of Kyte and Doolittle (25) and Hopp and Woods (26) to determine the distribution of hydrophobic residues in the protein. While the central portion of the sequence is moderately hydrophobic, the protein is overall hydrophilic (average hydrophobicity, -0.28). There are no signal sequences or membrane-spanning domains predicted from the sequence, in agreement with the fact that 5-LO is only peripherally associated with the membrane when activated with  $Ca^{2+}$  (27). Interestingly, we note the presence of a sequence between amino acids 368 and 382 (His-Leu-Leu-Arg-Thr-His-Leu-Val-Ser-Glu-Val-Phe-Glv-Ile-Ala) that is related to the interface-binding domain of the human lipoprotein lipase (His-Leu-Leu-Gly-Tyr-Ser-Leu-Gly-Ala-His-Ala-Ala-Gly-Ile-Ala) (28) and the rat hepatic lipase (His-Leu-Ile-Gly-Tyr-Ser-Leu-Gly-Ala-His-Val-Ser-Gly-Phe-Ala) (29). As in the lipases, this peptide segment is localized within a region of overall hydrophobicity. The presence of this sequence suggests that the mechanism for 5-LO enzyme-substrate interaction might be similar to that of the lipases. As 5-LO activity is affected by Ca<sup>2+</sup> and ATP, the amino acid sequence was examined for the presence of consensus binding sites for these cofactors. No strong homology was observed in the sequence to any ATP binding sites. Two regions of the sequence (amino acids 16-28 and 488–512) exhibited weak homology to the consensus  $Ca^{2+}$ binding sites from lipocortin (30, 31) and calmodulin (32), respectively. The position of the actual Ca<sup>2+</sup>-binding site will have to be determined by direct biochemical and genetic analysis, however.

Comparison of the complete amino acid sequence for human 5-LO with the sequences for other cloned lipoxygenases revealed a significant amount of homology to the soybean *Lox1* gene product and the rabbit reticulocyte Α

manuscript. In addition, we would like to thank Drs. E. Scolnick, E. Cordes, R. Gerety, J. Rokach, and T. Ford-Hutchinson for their Lox 1 aa 481 SKAYVIVNDSCYHQLMSHWLNTHAAMEPFVIATHRHLSVLHPIYKLLTPH continuing support in this project. Samuelsson, B. (1983) Science 20, 568-575. 1.

- 2. Rouzer, C. A., Matsumoto, T. & Samuelsson, B. (1986) Proc. Natl. Acad. Sci. USA 83, 857–861.
- 3. Ueda, N., Kaneko, S., Yoskimoto, T. & Yamamoto, S. (1986) J. Biol. Chem. 261, 7982-7988.
- Shimizu, T., Izumi, T., Seyama, Y., Tadokora, K., Radmark, 4. O. & Samuelsson, B. (1986) Proc. Natl. Acad. Sci. USA 83, 4175-4179.
- 5. Hogaboom, G. K., Cook, M., Newton, J. F., Varrichio, A., Shorr, R. G. L., Sarau, H. M. & Crooke, S. T. (1986) Mol. Pharmacol. 30, 510-519.
- 6. Rouzer, C. A. & Samuelsson, B. (1985) Proc. Natl. Acad. Sci. USA 82, 6040-6044.
- 7. Goetze, A. M., Fayer, L., Bouska, J., Bornemeier, D. & Carter, G. W. (1985) Prostaglandins 29, 689-701.
- 8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
- 9 Laemmli, U. K. (1970) Nature (London) 227, 680-685.
- Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, 10. W. J. (1986) J. Biol. Chem. 256, 7790-7797.
- 11. Spiess, J., Rivier, J. E., Rodkey, J. A., Bennett, C. D. & Vale, W. (1979) Proc. Natl. Acad. Sci. USA 76, 2974–2978.
- 12. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Biochemistry 18, 5294-5299.
- Aviv, H. & Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 13. 1408-1412.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular 14. Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
- 15. Gubler, U. & Hoffman, B. J. (1983) Gene 25, 263-269.
- 16. Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J. & Strader, C. D. (1986) Nature (London) 321, 75-79.
- 17. Huynh, T., Young, R. & Davis, R. (1985) in DNA Cloning, A Practical Approach, ed. Glover, D. (IRL, Oxford), Vol. 1, pp. 49-78.
- 18. Vieira, J. & Messing, J. (1982) Gene 19, 259-268.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467. 19.
- 20. Hattori, M. & Sakaki, Y. (1983) Anal. Biochem. 152, 232-238.
- Collins, S. J., Ruscetti, F. W., Gallagher, R. E. & Gallo, R. C. 21. (1978) Proc. Natl. Acad. Sci. USA 75, 2458-2462.
- 22. Bonser, R. W., Siegel, M. I., McConnell, R. T. & Cuatrecasas, P. (1981) Biochem. Biophys. Res. Commun. 102, 1269-1275.
- Anthes, J. C., Bryant, R. W., Musch, M. W., Ng, K. & Siegel, M. I. (1986) *Inflammation* 10, 145–156. 23.
  - Kozak, M. (1984) Nucleic Acids Res. 12, 857-872. 24.
  - Kyte, M. & Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132. 25.
  - 26. Hopp, T. P. & Woods, K. R. (1981) Proc. Natl. Acad. Sci.
- USA 78, 3824-3828.
- 27. Rouzer, C. A. & Samuelsson, B. (1987) Proc. Natl. Acad. Sci. USA 84, 7393-7397.
- 28. Wion, K. L., Kirchgessner, T. G., Lusis, A. L., Schotz, M. C. & Lawn, R. M. (1987) Science 235, 1638-1641.
- Komaromy, M. C. & Schotz, M. C. (1987) Proc. Natl. Acad. 29. Sci. USA 84, 1526–1530.
- 30. Saris, C. J. M., Tack, B. F., Kristensen, T., Glenney, J. R. & Hunter, T. (1986) Cell 46, 201-212.
- 31. Geisow, M. J., Fritche, U., Hexham, J. M., Dash, B. & Johnson, T. (1986) Nature (London) 320, 636-638.
- Takahashi, N., Takahashi, Y. & Putnam, F. W. (1986) Proc. 32. Natl. Acad. Sci. USA 83, 8019-8023.
- Shibata, D., Steczko, J., Dixon, J. E., Hermodson, M., Yaz-33. danparost, R. & Axelrod, B. (1987) J. Biol. Chem. 262, 10080-10085.
- 34. Chou, P. Y. & Fasman, G. D. (1978) Annu. Rev. Biochem. 47, 251-276.
- Thiele, B. J., Black, E., Fleming, J., Nack, B., Rapoport, 35. S. M. & Harrison, P. R. (1987) Biomed. Biochim. Acta 46, 120-123.

5-L0 aa 351 AKIWVRSSDFHVHQTITHLLRTHLVSEVFGIAHYRQLPAVHPIFKLLVAH Lox 1 aa 531 YRNNMNINALARQSLINANGIIETTFLPSKYS-VEMSSAVYKNWVFTNQA 11 11 11 5-L0 aa 401 VRFTIAINTKAREQLICECGLFDKANATGGGGHVOMVORAMKDLTYASLC Lox 1 aa 580 LPADLIK-RGVAIKDPSTPHGVRLLIEDYPYAADGLEIWAAIKTWVQEYV 5-L0 aa 451 FPEA-IKARGMESKED--IPYYFYRDDGLLVWEAIRTFTAEVV Lox 1 aa 629 PLY YARDDDVKNDSELQHWWKEAVEKGHGDLKDKPWWPK-LQTLEDLVEV 11 5-L0 aa 491 DIYYEGDQVVEEDPELQDFVNDVYVYGMRGRKSSGF-PKSVKSREOLSEY Lox 1 aa 678 CLIIIWIASALHAAVNFGQYPYGGLIMNRPTASRRLLPE-KGTPEYEEMI 5-LO aa 540 LTVVIFTASAQHAAVNFGQYDWCSWIPNAPPTMRAPPPTAKGVVTIEQ-I в 1 MPSYTVTVATGSQWFAGTDDYIYLSLVGSAGCSEKHLLDKPF YN 5-LO aa 1 MGVYRVCVSTGASIYAGSKNKVELWLVGQHGEVELGSCLRSH EH R-LO aa 5-L0 aa 550 QHAAVNFGQYDWCSWIPNAPPTMRAPPPTAKGVVTIEQIVDTLPD R-LO aa 45 QHSSIHLGQLDWFTWPNAPCTHRLPPPTTK-DATLETVHATLPS

FIG. 5. Comparison of the amino acid sequence of 5-LO with those of (A) the soybean Lox1 protein and (B) the rabbit reticulocyte LO. All sequences are aligned for maximum homology with identical matches indicated. Only those regions of each protein exhibiting significant amino acid homology are shown. Each amino acid sequence is numbered individually on the left of each line. Amino acids are identified by the single-letter code.

15-LO (Fig. 5). The Lox1 protein catalyzes an enzymatic reaction similar to 5-LO, but with differences in substrate preference and positional specificity (33). The greatest amino acid homology was observed between the central portions of both 5-LO and the Lox1 protein. In one region of 5-LO, 12 of 13 amino acids (including one serine, one histidine, and one tyrosine, amino acids likely to be present within the active site of the enzyme) are shared with the Lox1 protein. A comparison of the hydrophobicity profiles (25, 26) of the two proteins indicated a similar distribution of hydrophobic and hydrophilic residues throughout both proteins. Likewise, both proteins had similar overall secondary structures as predicted by the method of Chou and Fasman (34). The high degree of sequence conservation between an enzyme from plants and one from animals suggests that the conserved regions may play a role in enzymatic functions. The amino-terminal 88 amino acids of the rabbit reticulocyte 15-LO have been reported (35). While it is difficult to draw functional conclusions from the limited amount of sequence data, two regions of significant similarity were observed. The first region of similarity is at amino acids 1-34 of 15-LO and amino acids 1-34 of 5-LO, while the second is at amino acids 550-594 of 5-LO and amino acids 45-88 of 15-LO. The division of the similar regions in 5-LO may reflect structural or functional differences between the two enzymes. Thus, all of these enzymes that catalyze similar reactions may have diverged from a common ancestral gene product and represent members of a large family of related proteins. Taking the structural similarities into account, it should be possible to use molecular modeling studies combined with biochemical and genetic analysis to determine the structure-function relationship of 5-LO and design more effective chemotherapeutic agents that use 5-LO as a target.

The authors wish to express their appreciation to the staff of the Canadian Red Cross of Montreal for their generous cooperation in supplying human leukocyte concentrates. We wish to thank Dr. Irving Sigal for his helpful suggestions and critical reading of this