Transcriptional activation of human 12-lipoxygenase gene promoter is mediated through Sp1 consensus sites in A431 cells

Yi-Wen LIU*, Toshiya ARAKAWA†, Shozo YAMAMOTO† and Wen-Chang CHANG*‡

*Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan, Taiwan 70101, Republic of China, and †Department of Biochemistry, Tokushima University School of Medicine, Tokushima 770, Japan

The functional 5' flanking region of the human 12-lipoxygenase in epidermoid carcinoma A431 cells was characterized. By a primer extension method, the transcription initiation sites were mapped at –47 adenosine, –48 guanosine and –55 guanosine upstream of the ATG translation start codon. Transient transfection with a series of 5' and 3' deletion constructs showed that the 5' flanking region spanning from –224 to –100 bp was important for the basal expression of 12-lipoxygenase gene. Gel mobility shift assays with antibodies of transcription factors showed that both Sp1 and Sp3 required highly GC-rich Sp1 sites within this region for binding. Disruption of two Sp1 recognition motifs residing at –158 to –150 bp and –123 to –114 bp by site-directed mutagenesis markedly reduced the basal 12-lipoxy-

genase promoter activity and abolished the retarded bands in a gel-shift assay, indicating that these two Sp1-binding sites were essential for gene expression. The same two Sp1-binding sites in this promoter region were also responsible for epidermal growth factor (EGF)-induced expression of 12-lipoxygenase gene. Moreover, EGF also induced the transcriptional activation of luciferase driven by SV40 early promoter, which contained rich Sp1-binding sites. Taken together, the results suggest that two specific Sp1 consensus sites are involved in the mediation of the basal promoter activity as well as EGF induction of the 12-lipoxygenase gene and that Sp1 and Sp3 transcription factors might have a role in their regulation.

INTRODUCTION

Arachidonate 12-lipoxygenase (arachidonate:oxygen 12-oxidoreductase, EC 1.13.11.31) in platelets was the first mammalian lipoxygenase discovered [1,2]. It catalyses the transformation of arachidonic acid into 12(*S*)-hydroperoxyeicosatetraenoic acid, which is subsequently converted into 12(*S*)-hydroxyeicosatetraenoic acid by a glutathione-dependent peroxidase [3]. In addition to human platelet 12-lipoxygenase, a second mammalian 12-lipoxygenase was found in the pig leucocyte [4]. These two 12-lipoxygenases differ in substrate specificity and are expressed from different genes [5]. The genomic DNAs of the platelet-type 12-lipoxygenase in human erythroleukaemia (HEL) cells [6,7] and mouse platelets [8] and of the leucocyte-type 12-lipoxygenase in pig [9] and mouse [8] leucocytes have been cloned and sequenced. The gene promoter of these two 12-lipoxygenases is an example of a housekeeping gene, which has neither a TATA nor a CCAAT box. However, these two housekeeping genes have a very GC-rich regulation region and a multiple Sp1-binding GC motif [6,9]. In addition to human platelets, the platelet-type 12-lipoxygenase was also found in human epidermoid carcinoma and epidermal cells [10,11] and mouse megakaryocytes and eosinophil cells [12].

In studying the regulation of 12-lipoxygenase expression in human epidermoid carcinoma A431 cells, we found that epidermal growth factor (EGF) approximately doubled the mRNA level with a lag period of 10 h, which was paralleled by the increases in enzyme activity [10,13] and protein [14]. A doubling of 12-lipoxygenase mRNA was also observed in human erythroleukaemia cells treated with PMA for 72 h [15]. The enzyme is overexpressed in epidermis of patients with inflammatory

psoriasis [16,17], indicating that 12-lipoxygenase might have a central role in the pathogenesis of some epidermal inflammation. These results have indicated that their gene expression can be up-regulated by extracellular stimulations, which required a minimum induction period of 10 h.

Analysis of human platelet 12-lipoxygenase gene promoter revealed the presence of five putative Sp1-binding sites, eight potential AP2-binding sites and one NFκB-binding site in the first 750 bp before the ATG start codon [6,18]. In the present study we analysed the functional promoter sequence in human 12-lipoxygenase gene for the basal and EGF-induced expression in A431 cells. With the aid of electrophoretic mobility-shift assay and site-directed mutagenesis, we demonstrated that two Sp1 consensus sites residing at –158 to –150 bp and –123 to –114 bp in the promoter region were essential for the transcriptional activation of the 12-lipoxygenase gene in A431 cells. This is the first demonstration of Sp1 consensus sites mediating the gene expression of mammalian lipoxygenases.

MATERIALS AND METHODS

Materials

Mouse EGF (natural, culture grade) was purchased from Collaborative Research (Bedford, MA, U.S.A.). [α -³²P]dCTP (3000 Ci/mmol) and [γ -³²P]ATP (5000 Ci/mmol) were purchased from Amersham (Little Chalfont, Bucks, U.K.). Polyvinyl alcohol, Hepes and *o*-nitrophenyl β -galactopyranoside were from Sigma (St. Louis, MO, U.S.A.). The luciferase assay system was from Promega (Madison, WI, U.S.A.). Qiagen-tip 100 was from Qiagen (Hilden, Germany). Luciferase plasmid pXP-1 was a gift

Abbreviations used: EGF, epidermal growth factor; HEL, human erythroleukaemia; SPM, mutant at a site of Sp1.

‡ To whom correspondence should be addressed.

CTCTCTCAAAGGTCTGGCCCATGATATTGGGTGACATTGGGACCCAGC -701
 GATA AP2
 CCCCAGATATTGAAACGGGGTGGGAGCGGGTGTGAGCTGCTACATT -651
 GATA
 TCCAGGTTCTAGGTACCTGGGGAACCCCTGGTCGTTGACGGAAGCACAG -601
 CGTTCAAGGGCGGCAAGAGCTCAGGTACTGGAGGACACATGGGCGCGCCG -551
 Sp1 AP2
 GGTCTAGCCGCGGGACATCCCGAGACCCAGCACGGCCCTCCAGGCCGA -501
 NFkB AP2 AP2
 CCTTCCAGAAGCCGGCCCACTGCTCCCGGGTCCAGGCCAGAAAAGC -451
 AP2 AP2
 GGAGACCTTCCCTCCGTTTGGGAAGATGGACTCATCCCTACTTCTCTCT -401
 C/EBPβ
 GGCTCCCCAGAAGACCTGGAGACCTCGGGAAGTGTTCATCTATGTTCTG -351
 CTCCAACCCGGGGCTCAGGCCACCTCGATTCCGCTTCCCGGGAGGTCC -301
 AGGAAGGTCCCGCCGACCCACCCGTTGCGCTCACCTGGGCTCGGGCCC -251
 CACCC CACCC
 CTTTATCATCTGCAGCTACGCTCCGACCAGGAGGCACAGCCAGTCCCA -201
 AP2
 CAGCGGGGCTTACCGCTCTGCTCGCAGGTTGGGCTAGTCTGGGGCGGG -151
 Sp1
 GACTTGGGAGCGCCTAAACTTGCAGGAGGGCGGGGCCGAGACCGGTC -101
 Sp1
 CTTTAAAGGTTGGAAGTGGCCCCGAGGCGCCGGCAGCCCTGGGCGGGTCC -51
 Sp1
 GGGAATCGCACAGGACCCGGCTCCCTCGCCTAAGCTGCTGGGGGGCGCC -1

Figure 2 Regulatory elements in human 12-lipoxygenase gene

Each putative consensus sequence in the 5' flanking region of human 12-lipoxygenase gene [6] is enclosed in a box. Abbreviations: Sp1, Sp1-binding site; CACCC, CACCC box found in β -globin gene promoters; AP2, binding site of AP2; NFkB, binding site of NFkB; C/EBP β , binding site for CCAAT/enhancer-binding protein; GATA, GATA box.

pestle. The suspension was stirred for 1 h at 4 °C and then centrifuged at 16000 *g* for 60 min in a microcentrifuge. Supernatants were collected and dialysed for 16 h against 50 vol. of buffer C [20 mM Hepes (pH 7.9)/0.1 mM EDTA/75 mM NaCl/20 % (v/v) glycerol]. Dialysates were centrifuged at 7500 *g* for 10 min and the supernatants were stored at -70 °C until use.

Gel-shift assay

Double-stranded oligonucleotides were prepared by the PCR method in a solution containing 0.2 μ M [α -³²P]dCTP, 10 μ M

dCTP and 50 μ M d(A/G/T)TP. Labelled oligonucleotides were purified on a 5 % (w/v) polyacrylamide gel and used as probes. The binding reaction was performed in a 15 μ l reaction mixture containing 1 μ g of poly(dI-dC)·poly(dI-dC), 20 mM Hepes, pH 7.9, 0.1 mM KCl, 2 mM MgCl₂, 15 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 10 % (v/v) glycerol, 2 % (w/v) polyvinyl alcohol, 2–3 μ g of the cell nuclear extracts, and the radiolabelled probe (2×10^4 c.p.m.). The mixtures were incubated at room temperature for 30 min and loaded on a 4 % (w/v) polyacrylamide gel. Electrophoresis was performed at a constant 120 V for 3 h. The gel was dried and autoradiographed.

RESULTS

Identification of the transcription initiation site of the 12-lipoxygenase gene in A431 cells

The transcription initiation site of the 12-lipoxygenase mRNA in A431 cells was determined by primer extension analysis. As shown in Figure 1, three major transcription initiation sites at -47 adenosine, -48 guanosine and -55 guanosine counted from the translation starting codon were observed in the 12-lipoxygenase gene of both control and EGF-treated cells.

Expression of DNA constructs with 5' and 3' deletions of 12-lipoxygenase gene promoter in A431 cells

Several response elements are present in the 750 bp promoter region of the human 12-lipoxygenase gene (Figure 2). To study the transcriptional regulation of 12-lipoxygenase gene, the luciferase reporter vectors bearing various lengths of 5' flanking regions of 12-lipoxygenase were constructed as shown in Figure 3. Among these DNA constructs, pXLO-6D and pXLO-8D were the 3' deletion vectors prepared from the same length (-962 bp) of 5' flanking promoter region. Cells were transfected with various DNA constructs, and the basal expression of luciferase activity in these vectors was studied. The results are summarized in Figure 3. No obvious expression was observed in cells transfected with pXLO-8 (-100 bp) and pXLO-6D, and there

Figure 3 Transcriptional analysis of luciferase expression vectors bearing various lengths of 12-lipoxygenase gene promoter

Truncated promoter fragment was ligated into a luciferase plasmid as described in the Materials and methods section. Numbers indicate the distance in base pairs from the translation start site. A mixture of each pXLO luciferase plasmid (0.5 μ g) and β -galactosidase plasmid (0.1 μ g) was transfected into A431 cells by the lipofection method. The luciferase activity was normalized with the β -galactosidase activity. Values are means $\pm$ S.E.M. for three determinations.

Figure 4 Gel-shift analysis of the nuclear factors binding to the 12-lipoxygenase gene promoter

Two DNA probes, A-101 and B-63, were labelled with [α - 32 P]dCTP as described in the Materials and methods section. (A) Oligonucleotides covering the promoter region from -224 to -124 bp (A-101) and -145 to -83 bp (B-63) are shown. Arrows indicate the 5' truncated constructs of the luciferase vector. The three major transcription initiation sites in A431 cells are also indicated. (B) [α - 32 P]dCTP-labelled promoter fragment A-101 was used as a probe and incubated with the nuclear extracts prepared from A431 cells. Lane 1, three retarded bands (a, b and c) are indicated; lane 2, 50 ng of competitive oligonucleotide containing Sp1-binding site was added in the reaction mixture; lane 3, 50 ng of competitive oligonucleotide containing AP2-binding site was included; lane 4, no nuclear extract was included. (C) [α - 32 P]dCTP-labelled fragment A-101 was used as a probe. Lanes 1 and 5, nuclear extract only; lane 2, 0.1 μ g of antibodies against Sp1 was included, and the supershifted band is indicated by b*; lane 3, 0.1 μ g of antibodies against Sp3 was added. Lane 4, 0.1 μ g of each Sp1 and Sp3 antibodies was added simultaneously. Lanes 6 and 7, 0.1 μ g of antibodies against p53 and c-Fos respectively was included in the reaction mixture. (D) [α - 32 P]dCTP-labelled fragment B-63 was used as a probe. Lanes 1 and 6, nuclear extracts only; lanes 2 and 3, 50 ng of competitive oligonucleotides containing Sp1-binding site and AP2-binding site respectively were included; lanes 4, 5, 7, 8 and 9, antibodies against different proteins were added to the reaction mixture.

was very little expression in pXLO-7-2 (-145 bp). However, significant expression was observed in luciferase vectors with a promoter longer than -224 bp. The luciferase activity apparently

decreased in two regions covering -224 to -100 bp (pXLO-7-1 to pXLO-8) and -951 to -754 bp (pXLO-1 to pXLO-3). No well-defined consensus binding sequence of transcription factor was observed in the region from -951 to -754 bp. In contrast, two potential binding sites for Sp1 and one for AP2 were present in the region from -224 to -145 bp (pXLO-7-1 to pXLO-7-2), and another Sp1 consensus sequence was located in the region spanning from -145 to -100 bp (pXLO-7-2 to pXLO-8). The important role of the 12-lipoxygenase gene promoter region covering from -224 to -100 bp in the regulation of luciferase expression was further confirmed by vectors with a 3' deletion region covering from -1 to -290 bp (pXLO-6D) and from -1 to -83 bp (pXLO-8D). pXLO-6D, with a deletion from -1 to -290 bp, had no significant expression; pXLO-8D, with a deletion from -1 to -83 bp, induced a significant expression of luciferase activity to the approximate extent of pXLO-1. The region from -1 to -83 bp, which was deleted in pXLO-8D, covered the transcriptional initiation sites detected in A431 cells. These results from the 3' deletion vectors indicated that the 5' flanking region covering from -290 to -83 bp instead of transcriptional initiation sites has an essential role in the expression of 12-lipoxygenase.

Identification of the nuclear factors involved in the expression of 12-lipoxygenase gene

Sequence analysis of the promoter region from -224 to -100 bp indicated the presence of three putative binding sites for Sp1 (-169 to -161 bp, -158 to -150 bp and -123 to -114 bp) and one potential binding site for AP2 (-204 to -197 bp). DNA mobility shift and supershift assays were performed to examine whether any of the above-mentioned nuclear factors bound the promoter sequence from -224 to -100 bp. Oligonucleotides covering the regions from -224 to -124 bp (A-101) and from -145 to -83 bp (B-63) were synthesized and labelled with [α - 32 P]dCTP by the PCR method. As indicated in Figure 4(A), oligonucleotide A101 covered one AP2 and two Sp1 sites, and oligonucleotide B-63 covered one Sp1 site. When nuclear extracts from A431 cells were allowed to react with A-101 probe, three retarded bands (a, b and c) were observed (Figure 4B, lane 1). These three bands were competed out by a double-strand DNA of SV40 early promoter with Sp1 consensus sequence (5'-GCGGAAGTGGGCGGAGTTAGGGGCG-3'), but not by a DNA of SV40 enhancer with AP2 consensus sequence (5'-GGTGTGGAAAGTCCCCAGGCTCCCCAGCA-3'). These results suggested that the three retarded bands might be due to Sp1 and other related proteins that interacted with the DNA probe. For identifying whether Sp1 and Sp3 bound the DNA probe, polyclonal antibodies that specifically recognize human Sp1 and Sp3 proteins were used for the gel-shift assay. The retarded band b was shifted by antibodies against Sp1 protein but not by antibodies against p53 and c-Fos, as determined with oligonucleotide A-101 as a probe (Figure 4C). The retarded bands a and c were not shifted by Sp1 antibodies, but were completely abolished by Sp3 antibodies. The same pattern of DNA-shifted bands were observed with oligonucleotide B-63 as a probe (Figure 4D). These results indicated that both Sp1 and Sp3 were the major nuclear factors that bound the sequences from -224 to -145 bp and from -145 to -83 bp in the 12-lipoxygenase gene promoter.

Effect of mutations at the consensus Sp1 sites

To determine which of these Sp1 sites in the promoter region from -224 to -100 bp is functionally relevant in the expression

Figure 5 Transcriptional analysis of Sp1 site-mutated vectors

(A) Site-directed mutagenesis of Sp1 consensus sequences in the 5' flanking region from -170 to -110 bp of 12-lipoxygenase gene. The sequence of wild-type (WT) promoter and the mutated nucleotides in mutant constructs are indicated. (B) A mixture of luciferase plasmid-bearing 12-lipoxygenase gene promoter ($0.5 \mu\text{g}$) and β -galactosidase plasmid ($0.1 \mu\text{g}$) was transfected into A431 cells by the lipofection method. The luciferase activity was normalized with the β -galactosidase activity. Values are means $\pm$ S.E.M. for three determinations.

of 12-lipoxygenase gene, the three consensus Sp1 sites were modified by site-directed mutagenesis (Figure 5A). All the mutants comprised the same length (-224 bp) of 5' flanking promoter region as pXLO-7-1. SPM4 contained a 2 bp mutation in the middle Sp1 site (-156 to -155 bp). SPM5 contained a 3 bp mutation in the upstream Sp1 site (-167 to -165 bp), whereas a 6 bp mutation was introduced into the downstream Sp1 site (-120 to -115 bp) to generate SPM6. Furthermore SPM45, SPM8 and SPM9 were composed of two different Sp1 mutation sites and SPM7 was mutated at all three Sp1 sites. Except for SPM5, all the mutants significantly decreased the luciferase activity (Figure 5B). Plasmids with mutations at the downstream Sp1 site and also at either the middle or upstream Sp1 sites (SPM7, SPM8 and SPM9) almost eliminated the luciferase reporter activity. Binding of the Sp1-directed mutant DNA to cell nuclear proteins was then studied by gel-shift assay. Oligonucleotide A-101 with Sp1 site-directed mutation was used as a probe to study the binding of cell nuclear proteins to the middle and upstream Sp1 sites, and oligonucleotide B-63 with Sp1 site-directed mutation was used for binding to the downstream Sp1 site. Nuclear protein binding was almost eliminated in SPM45 DNA probe with mutations at the middle and upstream Sp1 sites and also in SPM4 DNA probe with a mutation at the middle Sp1 site, whereas no significant elimination was observed in SPM5 DNA probe with a mutation at the upstream Sp1 site (Figure 6A). A complete elimination of nuclear protein binding was also observed in SPM6 DNA probe with a mutation at the downstream Sp1 site (Figure 6B). These results indicated that the downstream and middle Sp1 sites were the major DNA sequences for binding cell nuclear proteins including Sp1 and Sp3.

Effect of EGF on transcriptional up-regulation of the 12-lipoxygenase gene

We recently reported that EGF up-regulated the transcriptional

Figure 6 Gel-shift analysis of the nuclear factors interacting with the mutated gene promoter

(A) Wild-type and mutated A-101 probes were labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ by the PCR method and incubated with cell nuclear extracts in the presence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5 and 7) of $0.1 \mu\text{g}$ of Sp1 antibodies (Ab). Three retarded bands (a, b and c) and one supershifted band (b*) are indicated. (B) Wild-type and mutated B-63 probes labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ were used. The probes were incubated with cell nuclear extracts in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of $0.1 \mu\text{g}$ of Sp1 antibodies.

regulation of 12-lipoxygenase gene in A431 cells [14]. To identify the promoter elements responsive to the EGF induction, DNA constructs with 5' and 3' deletions and with an Sp1-directed

Figure 7 Analysis of EGF-responsive sequence in the 5' flanking region of the 12-lipoxygenase gene

Plasmid transfection in A431 cells was performed as described in the Materials and methods section. After the change of Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then treated with 50 ng/ml EGF. After 30 min of EGF treatment, the medium was removed and then the cells were further cultured in fresh medium up to 18 h. The expressions of luciferase activity and β -galactosidase activity were determined. The expression ratio of EGF-treated cells to control cells is indicated. Each value is from one experiment, and three determinations in control and EGF-treated cells were performed in each experiment. Values are means $\pm$ S.E.M.

mutation were transiently transfected into A431 cells. The effect of EGF on the luciferase reporter activity was then studied. The results are summarized in Figure 7. The transcription activities of luciferase-bearing vectors pXLO-1, pXLO-4-1, pXLO-4-4, pXLO-5-3, pXLO-7-1 pXLO-8D and SPM5 were stimulated by EGF treatment. The stimulation was in the range 5–6-fold, obtained by comparing the luciferase activity in EGF-treated cells with that in control cells. An apparent decrease in the stimulatory response of EGF was observed in vectors bearing a promoter with a deletion from –224 (pXLO-7-1) to –100 bp (pXLO-8). A 6-fold stimulation of EGF response was observed in pXLO-7-1, but the stimulatory response of EGF was drastically decreased in pXLO-7-2 (–145 bp) and completely disappeared in pXLO-8, indicating that a promoter region ranging from –224 to –100 bp was important for the EGF-stimulated response of 12-lipoxygenase expression. This observation was further confirmed with vectors with the 3' deletion. A 6-fold stimulation of EGF response was also observed in pXLO-8D with a deletion of –1 to –83 bp, but no response to EGF was observed in pXLO-6D with a deletion of –1 to –290 bp. A significant decrease in EGF-stimulated response was also observed in luciferase-bearing vectors SPM6 and SPM9, and a complete elimination of EGF response was detected in vectors SPM7 and SPM8. These results indicated that the downstream and middle Sp1 sites residing at –123 to –114 bp and –158 to –150 bp respectively have a critical role in EGF-induced transcription of the human 12-lipoxygenase gene.

To examine the specific role of Sp1 in EGF induction, an SV40 early promoter was used. The SV40 early promoter contains a TATA box and six Sp1-binding sites located in the 21 bp repeat region [21]; the presence of Sp1 sites, not a TATA box, is absolutely necessary for gene expression [22]. The Sp1 sequence of SV40 early promoter could compete with all nuclear protein binding to the 12-lipoxygenase promoter DNA ranging from –224 to –100 bp, which was critical for transcription of the 12-lipoxygenase gene (Figures 4B and 4D). Plasmid SV40-LUC bearing the SV40 early promoter-driven luciferase gene was transiently transfected into A431 cells, and the effect of EGF on

the expression of luciferase activity was studied. Expression of the luciferase activity driven by SV40 early promoter was approximately equal to that of pXLO-7-1, and an approx. 9-fold increase in EGF response was obtained in both vectors SV40-LUC and pXLO-7-1. If these two promoters were ligated to form a vector SV40-pXLO-7-1, the basal luciferase activity in cells was to be the sum of SV40-LUC and pXLO-7-1. EGF also induced a 6.5-fold increase in the luciferase expression of this vector. These results indicated that the Sp1-binding sites in the promoter region of 12-lipoxygenase and SV40 have a critical role in the gene expression of EGF response.

Binding of the cell nuclear proteins prepared from EGF-treated cells to 12-lipoxygenase gene promoter was then analysed by gel-shift assay. As the binding depends mainly on the Sp1 and Sp3 proteins in control cells, three retarded bands (a, b and c) were observed in EGF-treated cells. No change in these two nuclear protein–DNA complexes was found in cells treated with EGF for 4, 7 or 16 h.

DISCUSSION

Several pieces of evidence were provided in this study to indicate that the two Sp1-binding sites from –158 to –150 bp and –123 to –114 bp in the promoter region have a critical role in the transcription of the human 12-lipoxygenase gene in A431 cells. First, the promoter region, which was essential for the expression of luciferase activity, was narrowed down to 125 bp ranging from –224 to –100 bp with the aid of 5' and 3' deletion constructs of 12-lipoxygenase gene promoter. Secondly, site-directed mutagenesis at both these two Sp1-binding sites in the essential promoter region (SPM8) almost abolished the luciferase reporter activity of basal expression and EGF response. Thirdly, Sp1 and Sp3 were the major nuclear proteins bound to the essential promoter region for gene transcription. Lastly, site-directed mutagenesis at these two Sp1-binding sites blocked the binding of Sp proteins to the promoter DNA. The critical role of these two Sp1 consensus sites in the transcriptional regulation of 12-lipoxygenase gene promoter in HEL cells was not found,

because no obvious expression was observed in cells transfected with pXLO-7 (−218 bp), in which these two Sp1 consensus sites were conserved in the promoter [18]. Instead, two kinds of NF κ B heterodimer (p50/p50 and p50/c-Rel) could bind the NF κ B-binding site (−539 to −530 bp) and repressed the expression of luciferase reporter activity in HEL cells. The presence of multiple transcription initiation sites is characteristic of housekeeping genes. At promoters without a TATA box, an activator protein such as Sp1 can direct site-specific initiation near its binding site by tethering transcription factor IID to the DNA and by stabilizing the preinitiation complex through protein–protein interactions [23]. For example, Lu et al. [24] reported that the multiple initiation sites were spread over a 90 bp region in the promoter of the human Ha-ras gene, and the start sites were strongest when put into an Sp1-selected locus 36–53 bp downstream from the nearest GC box. Three major transcription initiation sites were mapped at −47 adenosine, −48 guanosine and −55 guanosine in A431 cells, which were 59–67 bp downstream from the nearest Sp1-binding site (−123 to −114 bp) in the promoter. However, one major transcription initiation site at −306 adenosine and two minor start sites at −302 cytosine and −300 adenosine in HEL cell were reported [6], which was 142–148 bp upstream from the nearest essential Sp1-binding site (−158 to −150 bp). A tissue- or cell-specific selection of start sites has been reported in the transcriptional regulation of certain housekeeping gene promoters. In the transcriptional initiation of rat phospholipid-hydroperoxidase glutathione peroxidase, the predominant initiation site in testis was approx. 100 bp longer at the 5′ terminus than the sites in somatic tissue including kidney, liver and heart [25]. The major transcription initiation site in aspartate aminotransferase gene promoter was also different in rat testis and liver [26]. The difference in the transcription initiation sites in the 12-lipoxygenase gene promoter between A431 and HEL cells provides evidence to explain that these two Sp1 consensus sites residing from −158 to −150 bp and −123 to −114 bp in the promoter have a positively regulatory role in the transcriptional regulation of the 12-lipoxygenase gene in A431 cells, whereas they were inert in HEL cells. Mechanisms for the selection of transcription initiation sites by Sp1 in the 12-lipoxygenase gene promoter of A431 cells await further investigation.

Interestingly, not only the basal expression of 12-lipoxygenase but also EGF induction was regulated by Sp1-binding sites in the promoter region. The same specific Sp1 consensus sites residing at −158 to −150 bp and −123 to −114 bp were involved in the mediation of EGF induction of the 12-lipoxygenase gene as those responsible for the basal promoter activity. Stimulation of SV40 early promoter-driven luciferase reporter activity by EGF provided additional evidence to support the requirement of the Sp1 site in the EGF response because the SV40 early promoter contains multiple Sp1 sites. Mediation of promoter activation by Sp1 consensus sites in basal transcription and induction of the cyclin-dependent kinase inhibitor p15^{INK4B} expression by transforming growth factor β in human keratinocyte HaCaT cells was also reported [27]. It is still unclear how Sp1 sites mediate the induction of EGF-induced 12-lipoxygenase expression because no increase in binding between nuclear proteins including Sp1 and promoter DNA was observed in gel-shift analyses. Growth factor induction without significant change in nuclear factor binding also has been observed for Sp1 binding to the EGF response element in the human gastrin promoter [28]. Evidence is accumulating that suggests that Sp1 and its family of proteins regulate the transcriptional machinery through the action of TATA box-binding protein-associated factors [29]. It is possible that some TATA box-binding protein-associated factors could

relay the signals of EGF, which lead to the activation of 12-lipoxygenase gene expression in A431 cells. Post-translation modification of Sp1 such as dephosphorylation [30] and O-linked glycosylation [31], resulting in the transactivation of Sp1, is also possible. Daniel et al. [32] recently reported that dephosphorylation of Sp1 by protein phosphatase 1 was involved in the glucose-mediated activation of the acetyl-CoA carboxylase gene in mouse 30A5 preadipocytes, and a phosphatase with a molecular mass of 38 kDa was induced by glucose treatment. We previously reported that protein biosynthesis *de novo* was essential for EGF-induced 12-lipoxygenase mRNA expression in A431 cells [10]. It will be interesting to study the EGF effect along this line including the induction of phosphatase and the dephosphorylation of Sp1.

With our results we are now able to give a partial explanation of the activated expression of the human 12-lipoxygenase gene by demonstrating the functional integration of Sp1 and Sp3 with GC-box motifs within the 12-lipoxygenase promoter. More detailed understanding awaits the characterization of other co-operating elements and factors in the transcriptional machinery. Characterization of Sp1-binding sites and its interactions with Sp1 and Sp3 will help us to define molecular interplay among multiple *cis*-acting elements and *trans*-acting factors in the regulation of 12-lipoxygenase transcription under various conditions. It will be through a better understanding of the mechanism of activated 12-lipoxygenase transcription in A431 cells that we hope to elucidate the role of 12(*S*)-hydroxy-eicosatetraenoic acid in some epidermal inflammations, including psoriasis.

We thank Dr. Ming-Derg Lai, Dr. Bei-Chang Yang and Dr. Lee-Yang Chau for valuable discussions; Dr. Shen K. Yang and Dr. Kun-yen Huang for a critical review of this manuscript; and Y. L. Chang for secretarial assistance. This work was supported in part by grants NSC 85-2331-B006-092 and NSC 85-2331-B006-001-M09 from the National Science Council of the Republic of China (W.C.C.), and by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (S.Y.).

REFERENCES

- Hamberg, M. and Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3400–3404
- Nugteren, D. H. (1975) *Biochim. Biophys. Acta* **380**, 299–307
- Chang, W. C., Nakao, J., Orimo, H. and Murota, S. (1982) *Biochem. J.* **202**, 771–776
- Yoshimoto, T., Miyamoto, Y., Ochi, K. and Yamamoto, S. (1982) *Biochim. Biophys. Acta* **713**, 638–646
- Yamamoto, S. (1992) *Biochim. Biophys. Acta* **1128**, 117–131
- Yoshimoto, T., Arakawa, T., Hada, T., Yamamoto, S. and Takahashi, E. (1992) *J. Biol. Chem.* **267**, 24805–24809
- Funk, C. D., Funk, L. B., FitzGerald, G. A. and Samuelsson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3962–3966
- Chen, X. S., Kurre, U., Jenkins, N. A., Copeland, N. G. and Funk, C. D. (1994) *J. Biol. Chem.* **269**, 13979–13987
- Arakawa, T., Oshima, T., Kishimoto, K., Yoshimoto, T. and Yamamoto, S. (1992) *J. Biol. Chem.* **267**, 12188–12191
- Chang, W. C., Liu, Y. W., Ning, C. C., Suzuki, H., Yoshimoto, T. and Yamamoto, S. (1993) *J. Biol. Chem.* **268**, 18734–18739
- Takahashi, Y., Reddy, G. R., Ueda, N., Yamamoto, S. and Arase, S. (1993) *J. Biol. Chem.* **268**, 16443–16448
- Nakamura, M., Ueda, N., Kishimoto, K., Yoshimoto, T., Yamamoto, S. and Ishimura, K. (1995) *J. Histochem. Cytochem.* **43**, 237–244
- Chang, W. C., Ning, C. C., Lin, M. T. and Huang, J. D. (1992) *J. Biol. Chem.* **267**, 3657–3666
- Liu, Y. W., Chen, B. K., Chen, C. J., Arakawa, T., Yoshimoto, T., Yamamoto, S. and Chang, W. C. (1997) *Biochim. Biophys. Acta* **1344**, 38–46
- Izumi, T., Hoshiko, S., Rådmark, O. and Samuelsson, B. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7477–7481
- Hussain, H., Shornick, L. P., Shannon, V. R., Wilson, J. D., Funk, C. D., Pentland, A. P. and Holtzman, M. J. (1994) *Am. J. Physiol.* **266**, C243–C253

- 17 Hammarström, S., Hamberg, M., Samuelsson, B., Duell, E. A., Stawiski, M. and Voorhees, J. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5130–5134
- 18 Arakawa, T., Nakamura, M., Yoshimoto, T. and Yamamoto, S. (1995) *FEBS Lett.* **363**, 105–110
- 19 Chen, B. K., Liu, Y. W., Yamamoto, S. and Chang, W. C. (1997) *Biochim. Biophys. Acta* **1344**, 270–277
- 20 Higuchi, R., Krummel, B. and Saiki, R. K. (1988) *Nucleic Acids Res.* **16**, 7351–7367
- 21 Dynan, W. S. and Tjian, R. (1983) *Cell* **35**, 79–87
- 22 Benoist, C. and Chambon, P. (1981) *Nature (London)* **290**, 304–310
- 23 Kollmar, R. and Farnham, P. J. (1993) *Proc. Soc. Exp. Biol. Med.* **203**, 127–139
- 24 Lu, J., Lee, W., Jiang, C. and Keller, E. B. (1994) *J. Biol. Chem.* **269**, 5391–5402
- 25 Pushpa-Rekha, T. R., Burdsall, A. L., Oleksa, L. M., Chisolm, G. M. and Driscoll, D. M. (1995) *J. Biol. Chem.* **270**, 26993–26999
- 26 Toussaint, C., Bousquet-Lemericiwe, B., Garlatti, M., Hanoune, J. and Barouki, R. (1994) *J. Biol. Chem.* **269**, 13318–13324
- 27 Li, J. M., Nichols, M. A., Chandrasekharan, S., Xiong, Y. and Wang, X. F. (1995) *J. Biol. Chem.* **270**, 26750–26753
- 28 Merchant, J. L., Shiotani, A., Mortensen, E. R., Shumaker, D. K. and Abraczinskas, D. R. (1995) *J. Biol. Chem.* **270**, 6314–6319
- 29 Gill, G., Pascal, E., Tseng, Z. H. and Tjian, R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 192–196
- 30 Leggett, R. W., Armstrong, S. A., Barry, D. and Mueller, C. R. (1995) *J. Biol. Chem.* **270**, 25879–25884
- 31 Jackson, S. P. and Tjian, R. (1988) *Cell* **55**, 125–133
- 32 Daniel, S., Zhang, S., DePaoli-Roach, A. A. and Kim, K. H. (1996) *J. Biol. Chem.* **271**, 14692–14697

Received 8 November 1996/3 January 1997; accepted 16 January 1997