# Induction of 12-lipoxygenase expression by transforming growth factor-α in human epidermoid carcinoma A431 cells

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**Abstract Transforming growth factor-α (TGF-α) increased the expression of 12-lipoxygenase activity in a time-dependent manner in human epidermoid carcinoma A431 cells. The increase of 12-lipoxygenase activity was accompanied by an increase in 12-lipoxygenase mRNA. The effect of TGF-α on the promoter activation of 12-lipoxygenase gene was analyzed by using the luciferase fusion vectors. A dose-dependent effect of TGF-α on the reporter activity was observed, which paralleled with its effect on enzyme activity. Transient transfection with a series of 5′-deleted constructs showed that the 5′-flanking region spanning from −224 to −100 bp from translation starting site played an important role for TGF-α response. Site-directed mutagenesis and gel mobility shift assay indicated that two Sp1 binding sequences residing at −158 to −150 bp and −123 to −114 bp were responsible for the TGF-α in activation of human 12-lipoxygenase gene transcription. Expression of Sp1, but not Sp3, stimulated the promoter activity of 12-lipoxygenase in SL2 cells, indicating that the binding of Sp1 with Sp1 binding sequences played a significant role in the regulation of 12-lipoxygenase gene.**



*Key words:* TGF-α; 12-Lipoxygenase; Sp1

## 1. Introduction

Arachidonate 12-lipoxygenase (arachidonate: oxygen 12-oxidoreductase, EC 1.13.11.31) in the platelet is the first mammalian lipoxygenase discovered [1,2]. It catalyzes the transformation of arachidonic acid into 12(*S*)-hydroperoxyeicosatetraenoic acid, which is subsequently converted to 12(*S*)-hydroxyeicosatetraenoic acid (12(*S*)-HETE) by a glutathione-dependent peroxidase [3]. The human platelet-type 12-lipoxygenase is also found in human erythroleukemia cell [4,5], epidermal cell [6], epidermoid carcinoma A431 cell [7] and keratinocyte [8].

In comparison, the biological activities of 12(*S*)-HETE are less well understood than the metabolites formed by 5-lipoxygenase catalysis. However, 12(*S*)-HETE may play a significant role in the pathogenesis of some epidermal and epithelial inflammation. In the psoriatic lesions, a markedly elevated 12-HETE was found whereas the levels of prostaglandins $E_2$ and $F_{2\alpha}$ were only minimally elevated [9]. Although 12-HETE is less potent than leukotriene $B_4$ with regard to neutrophil activation, it has been shown to be a chemotactic stimulus for epidermal cells [10]. In vivo, moreover, in guinea pig skin, unequivocal growth promotion in addition to the inflammation reaction was observed upon 12-HETE activation [11]. Therefore, high tissue concentration of 12(*S*)-HETE contributes to the inflammatory change and the abnormal epidermal hyperproliferation in the development of a psoriatic plaque. Furthermore, Hussain et al. [8] recently found an overexpression of the human platelet-type 12-lipoxygenase in germinal layer keratinocyte in psoriasis.

Keratinocytes, in vitro, can be induced to synthesize transforming growth factor-α (TGF-α) [12], which is a natural ligand for epidermal growth factor (EGF) receptors. Both EGF receptors and TGF-α are overexpressed in psoriatic epidermis [13,14]. In the study of the regulation of 12-lipoxygenase expression in human epidermoid carcinoma A431 cells, which overexpress EGF receptors, we found that EGF approximately doubled the mRNA level and enzyme activity [7]. In the present study, the direct effect of TGF-α on the expression of 12-lipoxygenase in A431 cells was investigated. The results indicate that TGF-α induced the expression of 12-lipoxygenase, and activated the promoter of 12-lipoxygenase gene in the same fashion as EGF.

## 2. Materials and methods

### 2.1. Materials

Human TGF-α was purchased from Collaborative Research (Bedford, MA, USA). [1-$^{14}$C]arachidonic acid (58 mCi/mmol), [α-$^{32}$P]dCTP (3000 Ci/mmol) and [γ-$^{32}$P]ATP (5000 Ci/mmol), megaprime DNA labeling system, and nylon membrane (Hybond-N) were purchased from Amersham (Little Chalfond, Bucks, UK). Bovine serum albumin, polyvinyl alcohol, HEPES and *o*-nitrophenyl β-galactosidase were from Sigma (St. Louis, MO, USA). The luciferase assay system was from Promega (Madison, WI, USA). Qiagen-tip 100 was from Qiagen (Hilden, Germany). Luciferase plasmid pXP-1 was a gift of Dr. T. Sakai (Kyoto Prefecture University of Medicine, Kyoto, Japan). β-Galactosidase plasmid (CMVβ) was from Clontech Laboratories (Palo Alto, CA, USA). pCMV-Sp1 and pCMV-Sp3 were provided by Dr. Gunstram Suske (University of Marburg, Germany). Lipofectamine, Dulbecco's modified Eagle's medium, Opti-MEM medium and Schneider's *Drosophila* medium were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was from Hyclone Laboratories (Logan, UT, USA).

### 2.2. Cell culture

Human epidermoid carcinoma A431 cells were grown at 37°C in air/$CO_2$ (19:1) in 10-cm plastic dishes containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin and 100 i.u./ml penicillin. Schneider line 2 (SL2) cells were grown at 25°C in a 10-cm plastic dish containing 10 ml of Schneider's *Drosophila* medium supplemented with 10% (v/v) fetal bovine serum.

### 2.3. Treatment of cells with TGF-α

The confluent cells were treated with 50 ng/ml TGF-α in serum-free medium. After 30 min of TGF-α treatment, the medium was removed. The cells were further cultured in fresh serum-free medium up to 18 h unless stated otherwise. No significant change in cell via-

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bility and morphology was observed under this experimental condition.

*2.4. Assay of the microsomal 12-lipoxygenase activity*

The preparation of microsome and analysis of 12-lipoxygenase activity were performed as previously described [15]. The assay mixture contained 8.5 μM [1-$^{14}$C]arachidonic acid (0.1 μCi) and an appropriate amount of microsomes in a final volume of 0.2 ml. After extraction with 1 ml of ethyl acetate, the organic layer was evaporated. Residues were dissolved in ethanol and applied to thin-layer chromatography plates. The formation of [1-$^{14}$C]12(*S*)-HETE was quantitated by a System 2000 Imaging Scanner (BIOSCAN).

*2.5. RNA blot analysis*

RNA blot analysis was performed as previously described [15]. Total RNA was isolated from A431 cells by the guanidine thiocyanate-phenol-chloroform method [16]. Twenty μg per lane of total RNA was used for electrophoresis and transferred to a nylon membrane as previously described [7]. The cDNA probes used were the *Hin*dIII-*Bam*HI fragment (2.3 kb) of human platelet 12-lipoxygenase cDNA [18] and the human 18S rRNA [17]. Probes were labeled with [α-$^{32}$P]dCTP using a megaprime DNA labeling system. The mRNA levels were calculated on the basis of hybridization signal measured by Fujix Bio-imaging Analyzer BAS1000 (Fuji Photo Film Co., Tokyo, Japan), and the mRNA ratio of 12-lipoxygenase to 18S rRNA in each sample was obtained.

*2.6. Construction of luciferase reporter vector*

The human 12-lipoxygenase promoter regions of various lengths were prepared either by restriction enzyme digestion of the genomic clone for the preparation of pXLO-01, 1 and 3 or by the PCR amplification method for the preparation of pXLO-4-2, 4-3, 5-2, 7-1 and 8, as described previously [19]. The mutants at Sp1 site (SPM) were constructed by the site-directed mutagenesis method as described [19]. All the DNA fragments were ligated into a luciferase plasmid pXP-1. SV40 early promoter was obtained from pGL2 control vector (Promega) digested with *Bgl*II and *Hin*dIII and ligated with pXP-1 to form vector SV40-LUC. Vector SV40-pXLO-7-1 was produced by the ligation of SV40 early promoter and vector pXLO-7-1, with the insertion of SV40 early promoter between the luciferase gene and 12-lipoxygenase promoter region. All the plasmids for transfection were purified by the use of Qiagen-tip 100.

*2.7. Transfection with lipofectamine and reporter gene assays*

A431 cells were replated 36 h before transfection at a density of $3\times10^5$ cells in 2 ml of fresh culture medium in a 3.5-cm plastic dish. For use in transfection, 12.5 μl of lipofectamine was incubated with 0.5 μg of pXLO luciferase plasmid and 0.1 μg of β-galactosidase plasmid in 1 ml of Opti-MEM medium for 30 min at room temperature, followed by incubation at 37°C for 24 h. 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 TGF-α. The luciferase and β-galactosidase activities in cell lysate were determined as described previously [19].

*2.8. Transfection by calcium phosphate method*

One day prior to transfection, SL2 cells were plated onto 6-cm plastic dish at a density of $4.5\times10^6$ cells, and transfected by the calcium phosphate method as described [20]. Every plate received 5 μg of DNA including 0.5 μg of pXLO-7-1 luciferase plasmid, 0.5 μg of β-galactosidase plasmid, and CMV-Sp1 or CMV-Sp3 plasmid. Variable amounts of expression plasmids were compensated with the empty vector pBSSK. Twenty-four h after addition of DNA, the medium was changed. After another 24 h, cells were washed twice with phosphate-buffered saline and harvested.

*2.9. Gel-shift assays*

Nuclear protein extraction and gel-shift assay were performed as previously described [19]. The binding reaction was performed with end-labeled oligonucleotide from the 12-lipoxygenase promoter region from −224 to −124 bp and 6 μg of nuclear extract. Electrophoresis was performed at a constant 120 V for 3 h. The gel was dried and autoradiographed.

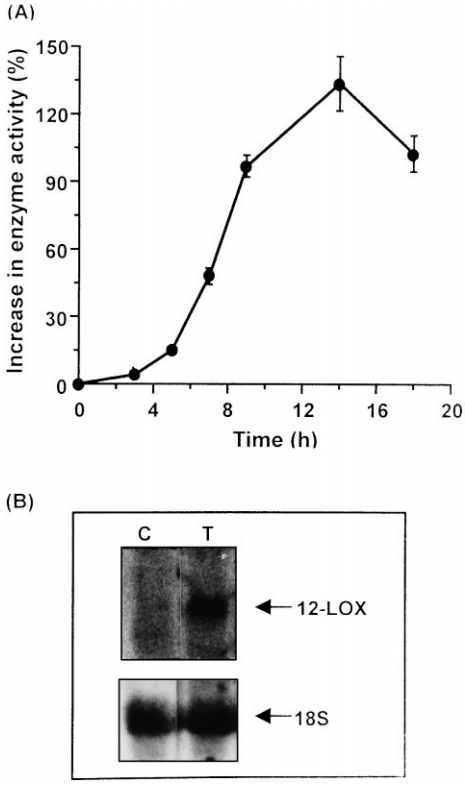


Fig. 1. Time-dependent effect of TGF-α on 12-lipoxygenase activity and mRNA expression. A: Confluent cells were treated with 50 ng/ml TGF-α in serum-free medium for 30 min, which were then switched to TGF-α-free medium for 3–18 h of incubation as indicated. Percentage of the change in 12-lipoxygenase activity induced by TGF-α is indicated at various time intervals. Results shown represent the mean ± S.E.M. of three determinations. B: For the determination of 12-lipoxygenase mRNA expression, cells were treated with (T) or without (C) 50 ng/ml TGF-α for 30 min, which were then switched to TGF-α-free medium for 9 h. Expression of 12-lipoxygenase mRNA was then determined.

## 3. Results

*3.1. Stimulation of 12-lipoxygenase expression*

TGF-α stimulated the expression of 12-lipoxygenase activity in a time-dependent manner. The percentages of increase in the enzyme activity induced by 50 ng/ml TGF-α were 15, 47, 94, 130 and 100% for 5, 7, 9, 14 and 18 h of treatments, respectively (Fig. 1A). As shown in Fig. 1B, the expression mRNA was induced by TGF-α. The maximum effect of TGF-α on the expression of 12-lipoxygenase was approximately half of the EGF response under the same experimental condition (data not shown).

*3.2. 12-Lipoxygenase promoter analysis*

Using cells transfected with vector pXLO-01 (−2 kb), the dose-response effect of TGF-α on 12-lipoxygenase gene transcription was studied. A dose-dependent response of TGF-α was observed, which paralleled with its effect on the enzyme activity (Fig. 2). In order to study the transcriptional regulation of 12-lipoxygenase gene, cells were transfected with various pXLO DNA constructs. The effect of TGF-α on the luciferase activity of these pXLO vectors was studied. The results are summarized in Fig. 3. The transcriptional activities of luciferase-bearing vectors pXLO-01 (−951 bp), pXLO-3

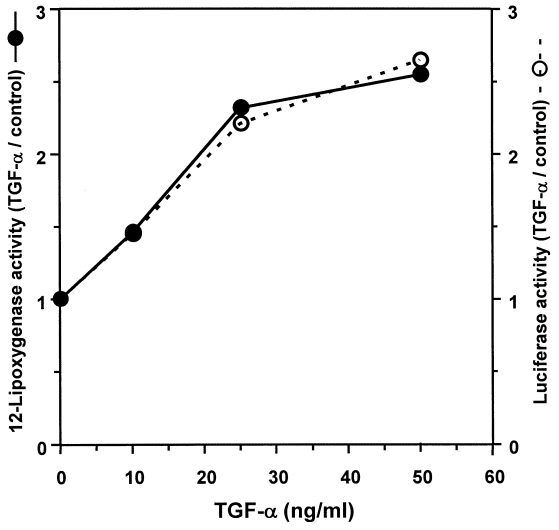


Fig. 2. Dose-dependent effect of TGF-α on 12-lipoxygenase activity and luciferase reporter activity. For studying the effect of TGF-α on 12-lipoxygenase activity (closed circles), confluent cells were treated with various concentrations of TGF-α in serum-free medium for 30 min. They were then switched to TGF-α-free medium up to 18 h. 12-Lipoxygenase activity in microsomes of cells was assayed. For studying the effect of TGF-α on luciferase reporter activity (open circles), a mixture of 0.5 μg of pXLO-01 and 0.1 μg of β-galactosidase plasmid was transfected into A431 cells by the lipofection method. The transfected cells were treated with various concentrations of TGF-α for 30 min. They were then switched to fresh serum-free medium up to 18 h. The expressions of luciferase and β-galactosidase activities of cell lysate were determined and normalized. The expression ratio of TGF-α-treated cells to control cells is indicated. The results shown represent the mean of three determinations.

(−754 bp), pXLO-4-2 (−550 bp), pXLO-5-2 (−378 bp), pXLO-7-1 (−224 bp), were stimulated by the treatment of the corresponding transfected cells with 50 ng/ml TGF-α. The stimulation ratio ranging from 2.8 to 4.6 was obtained by comparing the luciferase activity in TGF-α-treated cells to that of the control cells. The stimulation response of TGF-α was drastically reduced in pXLO-7-2 (−145 bp) and completely disappeared in pXLO-8 (−100 bp), indicating that a promoter region ranging from −224 bp to −100 bp was important for the TGF-α-stimulated response of 12-lipoxygenase expression. Three Sp1 binding sites (−169 to −161 bp, −158 to −150 bp and −123 to −114 bp) exist in this region. Plasmids with mutated Sp1 binding sites in the promoter region were constructed by site-directed mutagenesis (Fig. 4A). The effect of TGF-α on the reporter activity of these constructs is summarized in Fig. 4B. Among the vectors with single mutation, 0%, 63%, and 43% decrease in TGF-α response were observed in SPM5, SPM4 and SPM6, respectively. A 73% decrease was observed in SPM9 with mutation at both upstream and downstream of Sp1 site. Plasmids with mutation at downstream and at the middle of Sp1 site (SPM7 and SPM8) almost eliminated the luciferase reporter activity induced by TGF-α treatment. These results indicate that the nucleotide sequences residing at −123 to −114 bp and −158 to −150 bp, played a critical role in TGF-α-induced transcription of human 12-lipoxygenase gene.

### 3.3. Activation of 12-lipoxygenase promoter by Sp1

In an earlier study [19], results of gel mobility shift assay indicated that proteins extracted from A431 cell nucleus interacting with highly GC-rich Sp1 sites within the promoter region ranging from −224 to −100 bp were mostly due to Sp1 and Sp3. To verify whether Sp1 and Sp3 activate the promoter activity of 12-lipoxygenase gene, vector pXLO-7-1 was transfected into the Sp1-deficient *Drosophila* Schneider

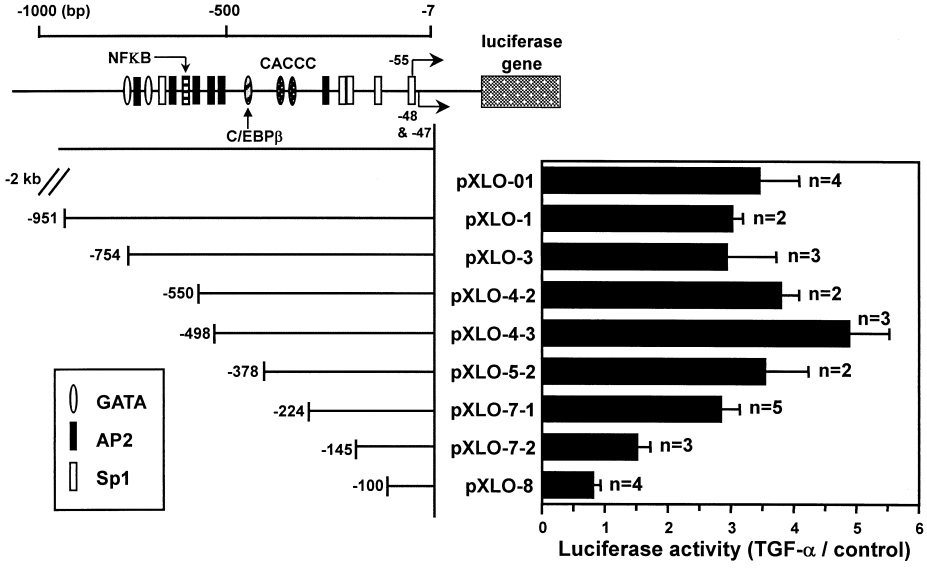


Fig. 3. Effect of TGF-α on the expression of luciferase vectors bearing various lengths of 12-lipoxygenase gene promoter. Luciferase vectors bearing various lengths of 12-lipoxygenase gene promoter were constructed as indicated. The potential consensus sequences in the 5′-flanking region are indicated. Abbreviations used are Sp1, Sp1 binding sites; CACCC, CACCC box found in β-globin gene promoters; AP2, AP2 binding sites; NFκB, binding sites of NFκB; C/EBPβ, binding sites for CCAAT/enhancer binding protein; and GATA, GATA box. The transcription initiation sites at −55G, −48G and −47A in A431 cells are also indicated. Plasmid transfection was performed as described in Section 2. The expression ratio of TGF-α-treated cells to control cells is indicated. The results shown represent the mean ± S.E.M. of two to five determinations.

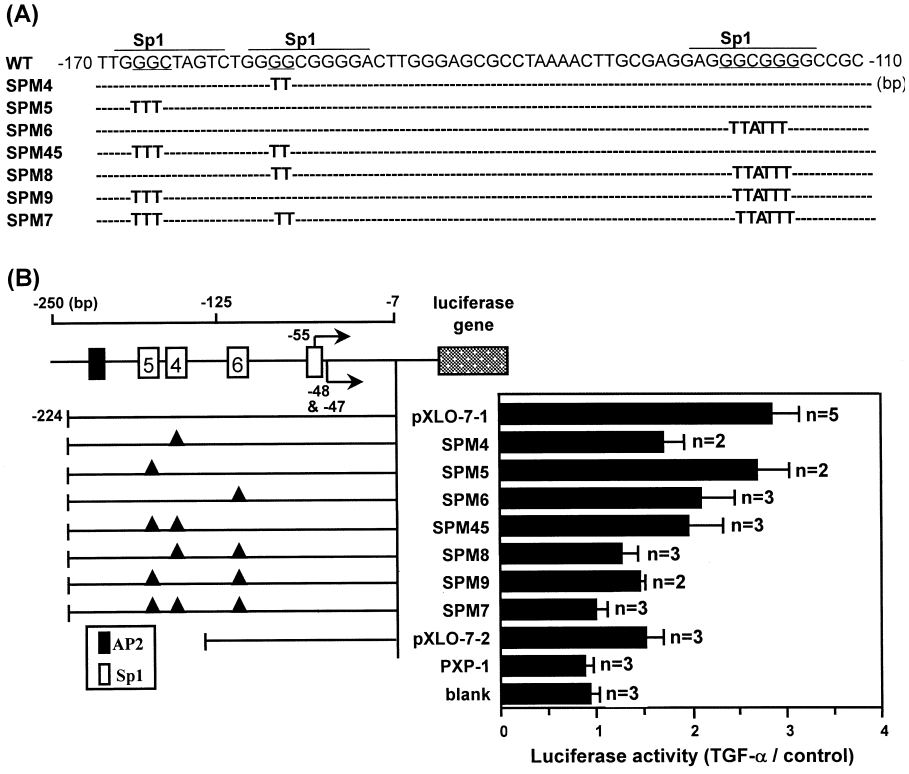


Fig. 4. Analysis of TGF-α-responsive sequence in the 5′-flanking region of 12-lipoxygenase gene. A: Site-directed mutagenesis of Sp1 consensus sequences in the 5′-flanking region ranging from −170 to −110 of 12-lipoxygenase gene. The sequence of wild-type (WT) promoter and mutated nucleotides in mutant constructs are indicated. B: The expression ratio of TGF-α-treated cell to control cells is indicated. The results shown represent the mean ± S.E.M. of two to five determinations.

SL2 cell line either in the presence or in the absence of Sp1 and Sp3 expression vectors. As shown in Fig. 5, expression of Sp1 stimulated the activity of 12-lipoxygenase promoter in a dose-dependent manner, while Sp3 did not show any significant effect. To examine the role of Sp1 binding sequence in the gene promoter activation by Sp1, an SV40 early promoter, which contains six Sp1 binding sites located in the 21 bp repeat region [21], was used. Expression of luciferase activity driven by SV40 early promoter was close to that of pXLO-1 upon stimulation by the expression of Sp1 (Fig. 6). If these two promoters were ligated to form a vector SV40-pXLO-7-1, an addition effect of these two promoters by the Sp1 expression was observed on the luciferase activity. These results indicate that the Sp1 binding sites in the promoter of 12-lipoxygenase and the SV40 early promoter responded equally well to Sp1.

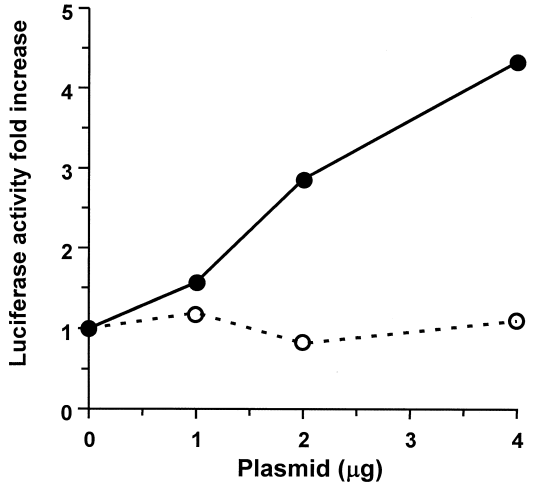


Fig. 5. Dose-response effect of Sp1 and Sp3 on luciferase reporter activity in SL2 cells. SL2 cells were transfected with 0.5 μg of pXLO-7-1 and 0.5 μg of CMV-β-gal with various amounts of CMV-Sp1 (close circles) or CMV-Sp3 (open circles) by calcium phosphate method. The total amount of DNA added was maintained at 5 μg with pBSSK. Cells were harvested 48 h after transfection. The expression of luciferase and β-galactosidase activity of cell lysate was determined and normalized. The results shown represent the mean of three determinations.

### 3.4. Gel-shift analysis

Binding of the cell nuclear proteins prepared form TGF-α-treated cells to 12-lipoxygenase gene promoter was analyzed by gel-shift assay. Since the three major retarded bands depend mainly on Sp1 and Sp3 proteins [19], the binding of the Sp1 and Sp3 of the cell nuclear extract to the promoter probe was studied. No change in these two protein-DNA complexes was found in cells treated with TGF-α for 5 and 9 h, compared with its respective control cells (Fig. 7).

## 4. Discussion

Several pieces of evidence were provided in this study to indicate that TGF-α stimulated the expression of 12-lipoxygenase mRNA and enzyme activity. TGF-α activated the promoter activity of 12-lipoxygenase gene in the same fashion as EGF in human epidermoid carcinoma A431 cells. Induction of 12-lipoxygenase expression by TGF-α was a slow and sustained reaction, which required a lag period of 5–7 h (Fig.

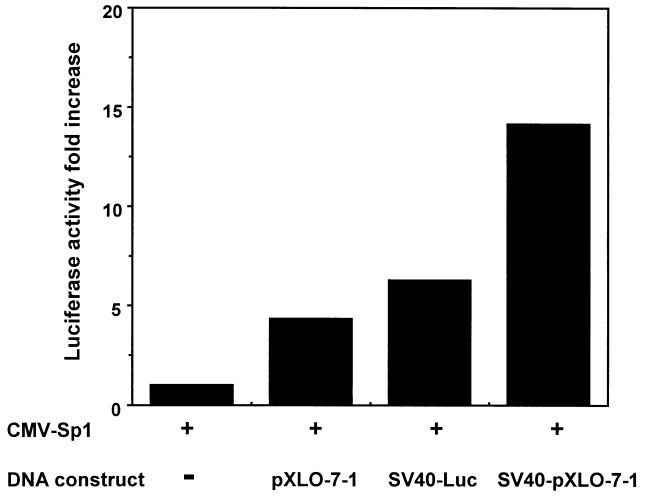


Fig. 6. Effect of Sp1 on the expression of vectors bearing Sp1 consensus sites in SL2 cells. SL2 cells were cotransfected with 0.5 μg of DNA construct bearing Sp1 consensus sites as indicated as well as with 0.5 μg of CMV-β-gal and 4 μg of CMV-Sp1 by calcium phosphate method. The total amount of DNA added was maintained at 5 μg with pBSSK. Cells were harvested 48 h after transfection. The expression of luciferase and β-galactosidase activity of cell lysate was determined and normalized. The results shown represent the mean of three determinations.

1A). The stimulatory effect of TGF-α on cellular accumulation of 12-lipoxygenase mRNA was at least partly due to the increase in transcription of 12-lipoxygenase gene. This was verified by the luciferase reporter assay. The dose-response of TGF-α-induced expression of luciferase vector pXLO-7-1 was parallel to that of TGF-α-induced stimulation of 12-lipoxygenase activity. In the analysis of promoter regulation, we identified previously that the same specific Sp1 consensus sites residing at −158 to −150 bp and −123 to −114 bp in the promoter region were involved in the mediation of EGF induction of the 12-lipoxygenase gene are also responsible for the basal promoter activity [19]. The same result was also observed in TGF-α-induced activation of 12-lipoxygenase promoter in the present study (Figs. 3 and 4).

Sp1 and Sp3 were two major nuclear proteins in A431 cells which bind to the middle and downstream of Sp1 consensus sequences in the promoter region of the 12-lipoxygenase gene [19]. After transient transfection of the expression vectors containing Sp1 and Sp3 in SL2 cells, only Sp1 activated the promoter activity of 12-lipoxygenase gene. Sp3 did not have any direct effect (Fig. 5). In addition to the activation of the 12-lipoxygenase promoter, Sp1 also activated an SV40 early promoter containing rich Sp1 binding sites (Fig. 6), which was in accordance with the EGF effect on the same promoter in A431 cells [19]. Therefore, nuclear protein Sp1 played a critical role not only in the basal expression of 12-lipoxygenase gene, but also in the transcriptional regulation of 12-lipoxygenase induced by TGF-α and EGF in A431 cells. 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 has also been reported [22]. It is still unclear how Sp1 mediates the induction of 12-lipoxygenase gene transcription by TGF-α and EGF because no increase in binding between Sp1 and promoter DNA was observed in gel-shift analysis (Fig. 7). Accumulating evidence suggests that Sp1 and its protein family regulate the transcriptional machinery through the action of TATA box binding protein-associated factors [23]. It is possible that some TATA box binding protein-associated factors could relay the signal of TGF-α and EGF, which leads to the activation of 12-lipoxygenase gene expression in A431 cells.

TGF-α shares about 30% structure similarity with EGF, including the conservation of all six cysteines [24]. This sequence relationship and the presumed formation of three similar disulfide bridges in both molecules provide a molecular explanation for the interaction of the two growth factors with the same cellular EGF receptor [25]. They exert similar mitogenic effect in many cells types [25] including A431 cells [26]. While most biological actions of these factors are in common, certain differences, usually quantitative, between their effects have been reported. For the induction of 12-lipoxygenase expression in A431 cells, the present results suggest that TGF-α acted similarly as EGF, with the exception that the maximum stimulatory effect which was only half of that of EGF as we previously reported [19]. A smaller maximal effect of TGF-α on 12-lipoxygenase expression could be explained by a lower affinity to EGF receptor since the affinity of TGF-α to EGF receptor of A431 cells is about half of that of EGF [26]. Stimulation of the expression of 12-lipoxygenase by TGF-α in human epidermoid carcinoma A431 cells with an overexpression of EGF receptors may provide a possible mechanism for the explanation of the overexpression of platelet 12-lipoxygenase in psoriatic lesion [8].

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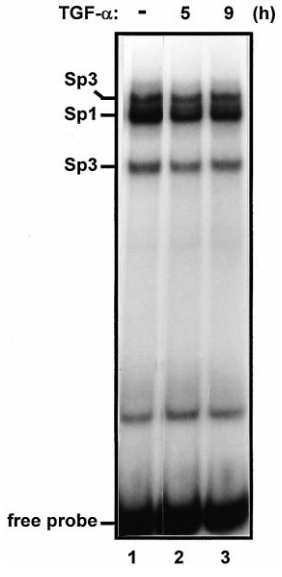


Fig. 7. Gel-shift analysis of the nuclear Sp proteins binding to the 12-lipoxygenase gene promoter. Oligonucleotide covering the promoter region from −224 to −124 bp was radiolabeled with [α-$^{32}$P]dCTP and used as a probe. The probe was incubated with the nuclear extracts prepared from control cells (lane 1) and cells treated with 50 ng/ml TGF-α for 5 and 9 h (lanes 2–3).

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