Identification and Characterization of a Phorbol Ester-responsive Element in the Murine 8S-Lipoxygenase Gene*

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Murine 8S-lipoxygenase (8S-LOX) is a 12-O-tetradecanoylphorbol-13-acetate (TPA)-inducible lipoxygenase. That is, it is not detected in normal mouse skin, however, a significant increase in expression is detected in the skin of TPA promotion-sensitive strains of mice after TPA treatment. In this study, we found TPA-induced 8S-LOX mRNA expression is a result of increased transcription in SSIN primary keratinocytes and further investigated transcriptional regulation of 8S-LOX expression by cloning its promoter. The cloned 8S-LOX promoter (−2 kb) in which a transcription initiation site was mapped at −27 from the ATG has neither a TATA box nor a CCAAT box. However, the promoter was highly responsive to TPA in TPA-promotion-sensitive SSIN but not in TPA promotion-resistant C57BL/6J primary keratinocytes. We then identified a Sp1 binding site located −77 to −68 from the ATG that is a TPA-responsive element (TRE) of the promoter and that Sp1, Sp2, and Sp3 proteins bind to the TRE. We also found that the binding of these proteins to the TRE was significantly increased by TPA treatment and inhibition of the binding by mithramycin decreased TPA-induced promoter activity as well as 8S-LOX mRNA expression. These data suggest that increased binding of Sp1, Sp2, and Sp3 to the TRE of the 8S-LOX promoter is a mechanism by which TPA induces 8S-LOX expression in keratinocytes.

Tumor promotion is a critical step in two-stage skin carcinogenesis (1). Cells initiated with a subthreshold dose of carcinogen usually do not further develop into visible and detectable tumors without promotion. 12-O-tetradecanoylphorbol-13-acetate (TPA),† which is the most commonly used tumor promoter in skin carcinogenesis, has strong effects on mouse skin, causing such events as hyperplasia, inflammation, and ornithine decarboxylase induction (1–3). Substantial evidences suggest that some lipoxygenase (LOX) metabolites of arachidonic acid are involved in TPA-induced epidermal hyperplasia and tumor promotion and that LOX inhibitors can effectively inhibit skin carcinogenesis (2–6).

Lipoxygenases are non-heme iron proteins that dioxygenate polyunsaturated fatty acids such as arachidonic or linoleic acid to hydroperoxy derivatives, hydroxyeicosatetraenoic acid (HETE) or hydroxyoctadecadienoic acid (HODE) by glutathione peroxidase. Lipoxygenases are classified according to the position of oxygen insertion onto specific carbons. For example, 8S-LOX oxygenates arachidonic acid at carbon-8. At least five different LOXs exist in mouse skin, including 5S- (8), leukocyte-type (l), 12S- (9), platelet-type (p), 12S- (9), epidermal-type (e), 12S- (10), and 8S-LOX (11–13). However, 8S-LOX is the lipoxygenase most strongly induced by TPA treatment of mouse skin (11–13).

8S-Lipoxygenase is a recently cloned murine lipoxygenase that has 78% homology with human 15S-LOX-2 (13). It metabolizes arachidonic acid as its preferred substrate and produces 8S-HETE. In addition, it was shown that 8S-LOX can convert linoleic acid to 8S-HODE, although with a much lower efficiency (14). So far constitutive enzyme expression has been detected only in mouse brain, footsole, tail, forestomach, and hair follicles (12, 13, 15). In mouse skin, however, the level of 8S-LOX message, protein, as well as enzyme activity are highly induced by a single topical treatment of TPA (11–13). Notably, the protein expression was shown to be restricted to the postmitotic, terminally differentiated epidermal compartment, stratum granulosum (13). This characteristic of 8S-LOX protein expression implies a causal relationship between enzyme expression and terminal differentiation in keratinocytes. In fact, we have recently reported that 8S-LOX transgenic mice have a highly differentiated and keratinized epidermis along with abnormally high expression of a differentiation marker, keratin-1 (16). In addition, the ability of 8S-HETE to induce keratinocyte differentiation through peroxisome proliferator-activated receptor (PPARγ) was shown in vitro (16). On the other hand, increased 8S-LOX expression was also detected in the culture of calcium-induced differentiated keratinocytes.‡ Interestingly, this prominent TPA-induced 8S-LOX expression is observed only in TPA promotion-sensitive mice (SENCAR, NMRI, and CD-1) but not in the promotion-resistant C57BL/6J mice (Refs. 14 and 17 and data not shown). The responses of

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† The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; EMSA, electromobility shift assay; GSP, gene-specific primer; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LOX, lipoxygenase; MMA, mithramycin A; PKC, protein kinase C; RT, reverse transcriptase; TRE, TPA-responsive element; PPAR, peroxisome proliferator-activated receptor; RACE, rapid amplification of cDNA ends; CMV, cytomegalovirus.

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‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF2556379.

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SSIN and C57BL/6J mice to TPA treatment have been reported to be similar with respect to the induction of ornithine decarboxylase and to the synthesis of other arachidonic acid metabolites from cyclooxygenases or lipoxigenases pathway. However, TPA does not induce hyperplasia, edema, or oxidant generation in C57BL/6J mice (17). Considering the amount of evidence showing an association of arachidonic acid metabolites with these events in TPA treated mouse skin (3–5, 18), SS-HETE, the only arachidonic acid metabolite that is different between SSIN and C57BL/6J mice, may be critical to these events and to tumor promotion.

Despite these potentially important roles of SS-LOX in mouse skin, the mechanism by which it is regulated by TPA has not been previously reported. In an early study, Furstemberger et al. (12) proposed that TPA-induced SS-LOX enzyme activity depends on protein biosynthesis, based on the observation that treatment of mouse skin with cycloheximide before or during TPA treatment prevented an increase in SS-LOX activity. Thereafter, Jisaka et al. (13) found that SS-LOX protein expression was restricted to the stratum granulosum compartment and that increased expression of SS-LOX by TPA was associated with an expansion of this compartment in TPA-induced hyperplastic skin. Given this observation, they suggested an increase in the number of cells which produce SS-LOX is one of the mechanisms of the TPA-induced enzyme activity. However, the fact that SS-LOX message induction occurs as quickly as 3 h after TPA treatment suggests that TPA regulates SS-LOX gene expression at the message level. TPA regulates SS-LOX gene expression at the message level. The region of the murine SS-LOX promoter identified in this report is a composite of two separate promoters downstream of the SS-LOX promoter region to which a non-complementary protruding XhoI site is linked at each 5′ end (5′-tcgatgAGGCGGCGGCGAATCATC-3′ and 5′-tcgatgAGGCGGCGGCGAATCATC-3′) as the nested GSP. The same GSP was used to amplify the resulting 5′-RACE-ready cDNA, and the desired fragment was further amplified by PCR using SS-LOXA297 (5′-AGTGAGCACTCCAGTCTGAAAC-3′) as the nested GSP. The nested products were gel-purified and cloned into pCR 2.1 for automated sequencing.

Site-directed Mutagenesis—Seven nucleotides, GGCGCGG, in the Sp1 binding motif in the −121 deletion construct was mutated to TTTATTT by PCR-based site-directed mutagenesis (21). The initial overlapping fragments were generated by PCR amplification of the wild type −121 deletion construct, using the upstream outer primer 5′-CAACATCAACCTATCTG-3′ and the mutant primer 5′-CTTCAAGGTTTTGACAGG-3′ using the downstream outer primer 5′-ATAGCCCTTTAAGTCCTC-3′ and the mutant primer 5′-CTTCAAAGGTTTTGACAGG-3′ (IDT). The product of annealing between the initial overlapping fragments was amplified by PCR using the upstream and downstream outer primers. The final product was digested with PvuI and XbaI and ligated into corresponding sites in pGL2 basic-m.

Cell Culture—Primary keratinocytes were isolated from 1- to 2-day-old SSIN or C57BL/6J mouse skin by trypsinization as described previously (22, 23) and grown in Eagle’s minimal essential medium supplemented with 5% chexeld fetal bovine serum at 37 °C under 5% CO₂.

Transient Transfections and Luciferase Assays—The assembled SS-LOX promoter reporter constructs or corresponding parent vector were co-transfected with the pCMV-β-galactosidase expression vector (Clone) into primary keratinocytes, 24 h after plating at 1 × 10⁵ cells/35-mm dish, using FuGENE™6 transfection reagent (Roche Applied Science, Indianapolis, IN) as described by the manufacturer. After 16 h, transfected cells were treated for 24 h with either acetone vehicle or TPA (10 nM) as a set of experiments. Cells were treated with mithramycin A (SSIN, Sigma, St. Louis, MO) 1 h before acetone or TPA treatment. Luciferase activity was measured using the Luciferase Assay System (Promega), and β-galactosidase activity was measured using the Galacto-Light™2 assay kit (Tropix, Bedford, MA). Light from either assay was detected by a luminometer (Tropix). The protein concentration of each cell lysate was quantified by the BCA assay (Pierce, Rockford, IL). Luciferase activity was normalized to β-galactosidase activity and protein concentration and then expressed as relative luciferase activity. Luciferase Mobility Shift Assay—Nuclear extracts were prepared as previously described (24) from SSIN primary keratinocytes cultured in the presence of acetone or TPA (10 nM). For one set of experiments, cells were treated with 1 μM MMA (Sigma) 1 h before acetone or TPA treatment. A synthetic probe (IDT), 5′-CTTAAACTTGTGTGATGGCGGGGCATCGTCTGAGGAGG-3′ (−92/−58), 5′-CTTAAACTTGTGTGATGGCGGGGCATCGTCTGAGGAGG-3′ (−92/−58), or 5′-tcgatgAGGCGGCGGCGAATCATC-3′ as the nested GSP. The nested products were gel-purified and cloned into pCR 2.1 for automated sequencing.
GGCGCGGGCATC-3' (XhoI–8S–8S–65) was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Amersham Biosciences, Piscataway, NJ), and 15,000 cpm of the labeled probe was incubated with 2 μg of nuclear extracts and 1 μg of poly(dI-dC) in binding buffer (20 mM Tris-HCl, 60 mM HEPESS-KOH (pH 7.9), 300 mM KCl, 60% glycerol, 2.5 mM EDTA, and 5 mM dithiothreitol) for 25 min at room temperature. The probe was then electrophoresed on a 5% non-denaturing polyacrylamide gel and viewed by autoradiography. Binding specificity was tested in parallel assays through the addition of unlabeled probe or consensus oligonucleotides for Sp1, AP1, CREB, or NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) at 100-fold molar excess over the labeled probe. Superimposition was assayed by incubating 2-μg nuclear extracts on ice for 30 min with 2 μl of Sp1, Sp2, Sp3, or Sp4 antibody (Santa Cruz Biotechnology) before inclusion of the extracts in the binding mixture.

**Northern Analysis**—Total RNA was isolated from mouse whole skin or primary keratinocytes treated with acetone, TPA, or a combination of TPA and actinomycin D (Sigma) for various time points using Trizol (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. 10 μg of RNA was separated on a formaldehyde-containing 1% agarose gel, transferred onto nylon membrane (Micron Separation, Westboro, MA), and UV cross-linked onto the membrane with a Stratalinker (Stratagene). cDNAs for 8S-LOX, Sp1, and glyceraldehyde-3-phosphate dehydrogenase were labeled with [α-32P]dCTP by using a Random Primed DNA Labeling kit (Roche Applied Science) and hybridized to the blot by using the QuickHyb (Stratagene) solution. Specific bands were detected by autoradiography.

**Reverse Transcriptase-PCR—RT-PCR**—was performed with the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen) following the manufacturer's protocol. First strand cDNA was synthesized from total RNA extracted from SSIN primary keratinocytes after treatment with acetone, TPA (30 ng/ml) or a combination of MMA (1 μM) plus TPA (30 ng/ml) for 6 h using the oligo(dT) primer. mRNA was added 1 h before TPA treatment. Resulting cDNA was then amplified with the GSPs 8S-LOX33 (5'-GGCGGAAATGCAGGCGTGAGATGATCC-G-3') and 8S-LOXA483 (5'-GGCGGAAATGCAGGGTGAGAGTATCC-G-3') primers were used in the separate reaction mixtures to control for amplification.

**Western Analysis**—Nuclear extracts prepared from SSIN primary keratinocytes were separated on a 8% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Pierce). The blot was blocked with ratatinocytes were separated on a 8% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Pierce). The blot was blocked with ratat. (1 after treatment with acetone, TPA (30 ng/ml) or a combination of MMA (1 μM) plus TPA (30 ng/ml) for 6 h using the oligo(dT) primer. mRNA was added 1 h before TPA treatment. Resulting cDNA was then amplified with the GSPs 8S-LOX33 (5'-GGCGGAAATGCAGGCGTGAGATGATCC-G-3') and 8S-LOXA483 (5'-GGCGGAAATGCAGGGTGAGAGTATCC-G-3') primers were used in the separate reaction mixtures to control for amplification.

**RESULTS**

**Transcriptional Regulation of 8S-Lipoxygenase by TPA**—TPA-induced 8S-LOX expression in SSIN primary keratinocytes occurred quickly and with a pattern that is similar to many immediate early genes, i.e. increased message was observed by 3 h, peaked at 9 h and started to decline thereafter (Fig. 1A). We then treated cultures of the cells with acetone, TPA (30 ng/ml) or the combination of TPA (30 ng/ml) plus actinomycin D (5 μg or 10 μg/ml, a transcription inhibitor) for 9 h to see whether TPA induces 8S-LOX transcriptionally. 8S-LOX mRNA was readily detected after treatment with TPA but not detected after treatment with acetone or after a combined treatment of actinomycin D and TPA (Fig. 1B). This data strongly suggest that TPA-induced elevation of the 8S-LOX message in SSIN primary keratinocytes occurs through transcriptional activation.

**Cloning and Characterization of the Murine 8S-LOX Promoter**—To better understand the transcriptional regulation of 8S-LOX by TPA treatment, we cloned the 2248 bp of genomic sequence immediately upstream of the translation start site, using a PCR-based method. Fig. 2 shows the cloned sequence. Unlike regions that have been shown to serve as the promoter for other lipoxygenases (25–28), this sequence was not G+C-rich. The mononucleotide frequencies were T (28.1%), C (25.8%), A (27.5%), and G (18.5%), and these frequencies were observed even near the downstream end of the fragment. The sequence presented in Fig. 2 has neither a TATA box nor a CCAAT box, however, this sequence contains several putative binding sites for transcription factors such as AP1 (five sites), C/EBP (two sites), GATA (three sites), NF-kB (one site), Oct-1 (three sites), and Sp1 (one site).

**Mapping the Transcription Start Site**—The previously described 8S-LOX cDNA contains 27 bp of 5'-untranslated region (13), and this was confirmed in our hands by 5'-RACE. Performing 5'-RACE with a 30-bp adaptor oligonucleotide and an antisense primer that binds from 483 to 456 bp downstream of the transcription start codon abundantly produced a 540-bp fragment containing only the previously reported sequence. This result was verified by 5'-RACE with a nested primer that binds 186 bp closer to the predicted cDNA end (data not shown).

**TPA Responsiveness of the 8S-LOX Promoter**—Because the transcription start site for the 8S-LOX gene appears to fall within the region we cloned, we wanted to determine whether the cloned region contains elements responsive to TPA. A reporter construct containing the entire cloned fragment (−2248) was transfected into SSIN primary keratinocytes, and the level of luciferase activity after treatment with various concentrations of TPA was measured. Basal luciferase activity was detected from the −2248 construct and the activity was clearly increased by TPA, whereas luciferase activity from a promoterless control vector was not (Fig. 3A). Induction of the reporter increased with increasing doses of TPA up to 30 ng/ml, however, it decreased thereafter, likely due to the observed toxicity of TPA to SSIN primary keratinocytes.

**TPA-induced 8S-LOX mRNA expression was very strong in TPA promotion-sensitive SSIN mouse skin, whereas the expression was quite weak in TPA promotion-resistant C57BL/6J mouse skin even when higher doses of TPA were used (Fig. 3B). To determine whether this relationship was retained in cultured keratinocytes, we transfected the −2248 construct into both SSIN and C57BL/6J primary keratinocytes and compared the level of luciferase activity after TPA treatment.** Consistent with the expression profile, TPA-induced luciferase activity was also much lower in C57BL/6J primary keratinocytes compared with that in SSIN primary keratinocytes (Fig. 3B). Taken together, these data suggest that the cloned region of 8S-LOX promoter contains at least one TPA-responsive element (TRE), and it mimics, at least in part, the normal regulation of the endogenous gene.

**Isolation of a TPA-responsive Region in the 8S-LOX Promoter**—To locate TPA-responsive elements in the isolated re-
region of the 8S-LOX promoter, we inserted progressively shortened segments of the cloned promoter in front of a luciferase gene and transfected the resulting constructs into SSIN primary keratinocytes. Fig. 4 shows the structure of the deletion constructs and the corresponding luciferase activities with or without TPA treatment. Although fold induction of the luciferase activity by TPA was variable to some extent, deletions of the promoter up to 93 bp from the translation start site did not affect the TPA response of the promoter. However, deletion from 92 to 70 led to the complete loss of both basal and TPA-induced activity similar to that seen with a promoterless control vector (pGL2m). It therefore appears that the response of the 8S-LOX promoter to TPA is mediated within a 92 to 70 segment of the promoter that is also critical to basic promoter activity.

Interaction between Nuclear Proteins and the TPA-responsive Region of the 8S-LOX Promoter—Because the segment between 92 and 70 seems to be critically important to the 8S-LOX promoter function, we looked for the binding of factors within that segment (Fig. 5). The nuclear extracts prepared from TPA-treated SSIN primary keratinocytes were incubated with radiolabeled oligonucleotides spanning from 92 to 58 of the promoter. This binding reaction generated three retarded protein-DNA complexes (complexes I, II, and III, lane 2). To determine the specificity of these binding complexes, we added 100-fold molar excess of unlabeled 8S-LOX oligonucleotide (92/58) to the binding reaction. Complexes I and II were entirely competed away, however, complex III was not significantly affected (lane 3). Based on the putative transcription factor binding site information presented in Fig. 2, an Sp1 binding motif located between 77 and 68 of the promoter was the only predicted site in the fragment between 92 and 70. We therefore tried to determine if this putative Sp1 binding motif was responsible for producing these protein-DNA complexes. Interestingly, when nuclear extracts were incubated with radiolabeled 92/58 probe in which the putative Sp1 binding motif was point mutated, complexes I and II were no longer detected (lane 10). Moreover, when 100-fold molar excess of the unlabeled 92/58 probe was added to the binding reaction, it could not compete away complexes I and II (lane 4). The critical role of the putative Sp1 binding site to generate complexes I and II was further confirmed when we chased labeled 92/58 probe with unlabeled Sp1, AP1, CREB, and NF-I consensus oligonucleotides. Only Sp1 consensus oligonucleotide competed away complexes I and II (lane 5), whereas AP1, CREB, and NF-I consensus oligonucleotides did not reduce complex formation (lanes 6–8). These data collectively demonstrate that a specific interaction between at least
one nuclear protein and the Sp1 binding motif generated complexes I and II, whereas non-specific binding gave rise to complex III.

Identification of a Sp1 Binding Site as a TRE in the 8S-LOX Promoter—To verify that this Sp1 binding site plays a critical role in promoter activity, we mutated the Sp1 binding site in the −121 construct and transfected the resulting mutant construct (−121m) into SSIN primary keratinocytes. In these cells, the −121m construct retained basal activity and responded to TPA as previously shown in Fig. 4. However, the −121m construct could not generate either basal or TPA-induced luciferase activity (Fig. 6A). These data distinctly prove that the Sp1 binding site encompasses a functionally essential segment of the 8S-LOX promoter.

The inability of TPA to induce luciferase activity from the −121m construct presents a strong possibility that the Sp1 binding site may also mediate the TPA responsiveness of the promoter, however, we could not further test it in the absence of basal transcription activity. We therefore generated a new reporter construct based on a TATA box containing reporter vector (pLuc-MCS). In the construct, a single copy of the 8S-LOX promoter segment (−81/−65), which includes just the Sp1 binding motif (−77/−68), was inserted in front of the TATA box in the reporter vector. We then transfected the resulting construct, pLuc-8S-LOX(−81/−65), into the SSIN primary keratinocytes and treated the cells with vehicle or TPA to see if the insertion of the Sp1 binding motif could exert TPA responsiveness on the reporter vector. As shown in Fig. 6B, basal luciferase activity from the pLuc-8S-LOX(−81/−65) construct was significantly increased compared with that of the control vector and the activity was further increased by TPA treatment (about 2- to 3-fold induction), whereas the activity from control vector was not increased by TPA treatment. This observation clearly demonstrates that the single Sp1 binding site alone can mediate TPA response of the promoter and further suggests that this Sp1 binding site is a TRE of the 8S-LOX promoter.

A Mechanism for TPA-induced 8S-LOX Gene Transcription—To understand how a single Sp1 binding site can mediate TPA responsiveness of the promoter, we first compared nuclear protein binding to the Sp1 binding site between acetone- and TPA-treated SSIN primary keratinocytes. We therefore prepared nuclear extracts from 6 h acetone- or TPA-treated cells and incubated the extracts with radiolabeled oligonucleotides spanning −81 to −65 of the 8S-LOX promoter. From this reaction, three retarded protein-DNA complexes were generated, as shown in Fig. 5, and the migration pattern of these complexes was not different between acetone- and TPA-treated cells (Fig. 7A). However, it was obvious that the formation of these binding complexes, especially complexes I and II, were clearly increased by TPA treatment (Fig. 7A). This experiment was repeated with more than five independent nuclear extract preparations, all yielding the same results.

We then tried to identify the nuclear protein(s) composing these complexes in acetone- or TPA-treated SSIN primary keratinocytes. Because the segment −81/−65 encodes the consensus binding site for Sp1, the effect of antibodies against Sp1, Sp2, Sp3, and Sp4 upon the complexes was first tested (Fig. 7B). Incubating the Sp1 antibody with keratinocyte nuclear extracts led to the disappearance of most of complex I (lanes 3 and 7), the Sp2 antibody supershifted a portion of complex I (lanes 4 and 8), and the Sp3 antibody supershifted some of complex II (lanes 5 and 9). However, incubating the Sp4 antibody with the same extracts did not supershift any of the complexes but rather generated even stronger binding complexes than the original complexes in the absence of the antibody (compare lanes 1 and 6 with lanes 2 and 10). This phenomenon can occur with some antibodies that stabilize protein-DNA interactions (29). Because complex II was not significantly affected by Sp1, Sp2, or Sp3 antibody in our supershift assay conditions, we explored the possible existence of other transcription factor(s) in this complex. Antibodies against c-Jun, c-Fos, JunB, FRA1, FRA2, Ap2, TFIIF, ets-1/ets-2, CBP, CREB, c-myc, C/EBPα, C/EBPβ, C/EBPδ, PPARα, PPARβ, PPARγ, Smad2/3, USP1, or USP2 were incubated with the nuclear extracts, and none of the antibodies could supershift either complex I or complex II (data not shown). However, as we increased the amount of antibody against Sp1, Sp2, or Sp3 in the binding mixture, a supershifted band grew more intense and most of complex II disappeared (data not shown). These data demonstrate that complexes I and II include Sp1, Sp2, and Sp3, and that complex II, in particular, includes these factors in a stable, tightly bound state. On the other hand, we did not detect any differences in the level of Sp1, Sp2, and Sp3 protein expression between acetone- and TPA-treated cells (Fig. 7C).
To identify the transcription start site of the 8S-LOX gene, several deletion constructs of the proximal region of the 8S-LOX promoter were transfected into SSIN primary keratinocytes. After 16 h of transfection, the cells were subsequently treated with acetone or TPA (30 ng/ml) for 24 h. Shown above on the right is the relative luciferase activity from each construct in response to acetone or TPA. Luciferase activity was normalized to both β-galactosidase activity and protein concentration and then standardized to the normalized activity from pGL2m-8S-LOX (−2248) after acetone treatment. Each value is the mean ± S.D. of at least three independent experiments.

**DISCUSSION**

The most notable difference between murine 8S-LOX and other lipoxigenases is that the levels of 8S-LOX message, protein, and activity are very weak in normal mouse skin but are strongly increased after a single topical treatment of TPA (11–13). In NMRI mouse skin, for example, the activity of 8S-LOX is dramatically increased by TPA treatment, yet the activity of 12S-LOX, which is constitutively expressed, is not, and the activity of 5S-LOX is increased only slightly (11). Recent studies have proposed that the induction of 8S-LOX activity by TPA is a result of protein biosynthesis (12, 13), however, the specific mechanism through which TPA induces 8S-LOX expression has not been previously demonstrated.

Since we found that TPA-induced expression of 8S-LOX mRNA in SSIN primary keratinocytes was completely blocked by actinomycin D, we pursued the idea that TPA induction of 8S-LOX expression occurs at the transcription level. We began by cloning the 2248-bp region immediately upstream of the 8S-LOX translation start codon. This region contains a major transcription start site 27 bp upstream from the translation start site, and when inserted into a luciferase reporter construct, it promotes luciferase activity. It thus appears to include the 8S-LOX proximal promoter region. To date, the promoter of three human lipoxigenases (5S-LOX (31), 12S-LOX (26), and 15S-LOX-1 (27)) and the promoter of one mouse lipoxigenase (5S-LOX (28)) have been cloned. Each of these promoters also has a predominant transcription start site.
within 100 bp of the translation start site, and, like the cloned region from 8S-LOX, each lacks TATA and CCAAT boxes.

The 8S-LOX promoter not only drives activity of a reporter gene but also displays strong induction by TPA. From deletion and mutation analyses of the 8S-LOX promoter in SSIN primary keratinocytes, we identified the Sp1 binding motif between positions -77 and -68 of the promoter as being critical for basal and TPA-induced transcription. We then presented strong evidence that this site is a functional Sp1, Sp2, and Sp3 binding element.

At least one Sp1 binding site has been identified in the promoters of other lipoxygenases, and Sp1 has been shown to play a critical role in the basal or induced expression of such promoters in various cell types. The mouse 5S-LOX promoter has one Sp1 site at -189 to -184, and the human 5S-LOX promoter has five Sp1 binding sites located at -179 to -145 from ATG (25). Disrupting the single Sp1 binding site in the mouse 5S-LOX promoter dramatically reduces basal activity from a luciferase reporter plasmid transfected into mouse monocyte-macrophage cells (28). Adding or deleting Sp1 sites within the human 5S-LOX promoter greatly affects transcription from a CAT reporter in Schneider cells (32), and the Sp1 binding sites in the human promoter are important regulatory regions for TPA-induced expression (25). Notably, mutations in the Sp1 sites in the human 5S-LOX promoter are related to development of asthma (32) and breast cancer (33). Sp1 binding sites are also important in the human 12S-LOX promoter. That promoter has five Sp1 binding sites, and two of them (located at -158 to -150 and -123 to -114) are essential for basal and epidermal growth factor (EGF)-induced transcription in human epidermal carcinoma A431 cells (34).

If Sp1, Sp2, and Sp3 participate in the induction of 8S-LOX transcription by TPA, it will obviously be important to understand how this occurs. TPA treatment has been observed to increase the mRNA and protein expression of Sp1, as well as to enhance the binding of Sp1, in Chinese hamster ovary cells (35) and chronic myelogenous leukemia cells (36). So, we examined whether Sp1, Sp2, or Sp3 might participate in TPA induction of the 8S-LOX promoter through one of these mechanisms. In fact, we found a significant increase of these proteins binding to

Fig. 6. Identification of a Sp1 binding site as a TRE in the 8S-LOX promoter. Shown in A are relative luciferase activities from deletion constructs and the pGL2 basic-m in response to acetone or TPA. The seven nucleotides of Sp1 binding motif (GGGCGGG) in the -121 construct were mutated to TTTATTT in the -121m. Shown in B is a structure of the pLuc-8S-LOX(-81/-65) construct and the relative luciferase activities from the construct and from its parent vector, pLuc-MCS, in response to acetone or TPA. A 8S-LOX promoter segment between -81 and -65 inserted in front of the TATA box (open box) in the pLuc-MCS vector is illustrated as a hatched box, and the Sp1 binding motif located -77 to -68 is depicted as a solid box. The relative luciferase activities in A and B were obtained from SSIN primary keratinocytes transfected with 2 μg of each construct along with its parent vector for 16 h and thereafter treated with acetone or TPA (30 ng/ml) for another 24 h. The data are the mean ± S.D. of at least three independent experiments.
were resolved on a 5% non-denaturing polyacrylamide gel. The positions of three protein-DNA complexes (* or **). Shown in C is a Western blot of nuclear proteins (20 μg) prepared from SSIN primary keratinocytes after treatment with acetone or TPA (50 ng/ml) for various time periods. The blot was hybridized with an antibody against Sp1, Sp2, or Sp3 and thereafter re-hybridized with a β-actin antibody to control for loading.

the Sp1 binding site of the 8S-LOX promoter in the SSIN primary keratinocytes after TPA treatment. We did not measure a significant change in the endogenous level of Sp1, Sp2, or Sp3 protein, however, when keratinocytes were treated with TPA. Therefore, it appears likely that TPA transduces its effect on 8S-LOX expression through increased binding of Sp1, Sp2, and Sp3 to the Sp1 binding site of the promoter rather than through increased expression of these proteins in SSIN primary keratinocytes. The data, which show TPA-induced 8S-LOX gene transcription is inhibited by treating cells with Sp1 binding inhibitor, MMA, further support this conclusion. However, this is not in agreement with related observations of others. Binding of Sp1 to the human 5S-LOX promoter in HL-60 cells and binding of Sp1 to the rat ornithine decarboxylase promoter in Reuber H35 rat hepatoma cells are both unaffected by TPA treatment, although TPA activates both promoters (25, 37). Sp1 binding to the 12S-LOX promoter in human epidermal carcinoma A431 cells is also unchanged after EGF treatment, although EGF induces 12S-LOX promoter activity through Sp1 sites (34). Thus, it appears that an induction of gene transcription by Sp1 occurs in a gene-specific as well as cell-type-specific manner through different mechanisms.

The functional consequences of elevated 8S-LOX expression by TPA in mouse skin are still not known. Considering previous studies, which suggest 8S-LOX is associated with differentiation (13, 16), however, our finding of transcriptional activation of 8S-LOX by TPA provides a deeper insight into a function of this gene, at least in part, in the process of keratinocyte differentiation. That is, because the protein expression was found prominently in differentiated keratinocytes, it has been an unresolved question as to whether 8S-LOX induces keratinocyte differentiation or differentiated keratinocytes produce 8S-LOX. However, the fact that 8S-LOX is transcriptionally activated by TPA in a proliferating basal keratinocyte population indicates a more active participation of this gene in the process of keratinocyte differentiation. Interestingly, forced overexpression of 8S-LOX in C57BL/6J mice caused a highly differentiated as well as thinner epidermis and, moreover, resulted in fewer tumors than in wild type mice in a two-stage skin carcinogenesis protocol. Considering that the failure to fully differentiate is a defining characteristic of malignant cells, it is possible that the 8S-LOX gene could be a novel target for skin cancer prevention by modulating its expression.

In summary, our results demonstrate that TPA regulates 8S-LOX expression at the transcriptional level through an increased Sp1, Sp2, and Sp3 binding to the Sp1 binding site in the 8S-LOX promoter. However, this finding raises another question of how TPA alters DNA binding ability of those factors. Considering recent reports showing that protein kinase C (PKC, a cellular receptor for TPA)-mediated Sp1 phosphorylation increases Sp1 binding to a Sp1 binding site (38–40), it is possible that phosphorylation of Sp1, Sp2, and Sp3 following...
Keratinocytes were transiently transfected with 2/H9262 lane 1 relative to glyceraldehyde-3-phosphate dehydrogenase expression. cDNA synthesis was carried out on total RNA extracted from SSIN primary ng/ml), MMA (1 H9262 lane 2 with MMA 1 h before acetone or TPA treatment. Two micrograms of extracts was incubated with a 32P-end-labeled oligonucleotide spanning the (lane 3), TPA (30 ng/ml; lane 4), or MMA (1 µM) plus acetone (lane 5) for 6 h. Keratinocytes were treated with MMA 1 h before acetone or TPA treatment. Two micrograms of extracts was incubated with a 32P-end-labeled oligonucleotide spanning the 8-LOX promoter segment from 81 to 86. Labeled probe in the absence of nuclear extract migrated as shown in lane 1. The positions of three protein-DNA complexes (Complexes I, II, and III) are noted. Shown in B is the effect of MMA on TPA-induced 8-LOX promoter activity. Keratinocytes were transiently transfected with 2 µg of –121 reporter construct for 16 h and then treated for another 24 h with acetone, TPA (30 ng/ml), MMA (1 µM) plus acetone, or MMA (1 µM) plus TPA (30 ng/ml). TPA was treated 1 h before acetone or TPA treatment. Data are expressed as -fold induction of the luciferase activity by TPA relative to acetone treatment. The data are a representative of three independent experiments with similar results. Shown in C is an assay by RT-PCR to measure the inhibitory effect of MMA on TPA-induced 8-LOX mRNA expression relative to glyceraldehyde-3-phosphate dehydrogenase expression. cDNA synthesis was carried out on total RNA extracted from SSIN primary keratinocytes after treatment with acetone (lane 1), TPA (30 ng/ml; lane 2), or MMA (1 µM) plus TPA (30 ng/ml; lane 3) for 6 h. MMA was added 1 h before TPA treatment.

the activation of PKC may enhance their DNA binding activity to the 8-LOX promoter. On the other hand, we can not exclude a possibility of PKC-independent post-translational activation of Sp1. Torgeman et al. (41) reported that TPA-stimulated Sp1 DNA binding activity was not diminished by a PKC-specific inhibitor. Furthermore, they showed the Sp1 binding stimulation was mediated by formation of a Sp1-p53 protein complex following TPA treatment (42). This observation suggests TPA may modulate DNA binding activity of Sp1 by regulating its interaction with other transcription factors or cofactors. Therefore, pursuit of this question will require extensive work in the future and may well provide significant insight into how TPA promotes tumorigenesis on a global level.

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Identification and Characterization of a Phorbol Ester-responsive Element in the Murine 8 S-Lipoxygenase Gene

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