

Cholesterol sulfate stimulates involucrin transcription in keratinocytes by increasing Fra-1, Fra-2, and Jun D

Karen Hanley,* Ladonna Wood,* Dean C. Ng,** Shan Shan He,* Peggy Lau,* Arthur Moser,^{†,††} Peter M. Elias,** Daniel D. Bikle,**,†† Mary L. Williams,*[§] and Kenneth R. Feingold^{1,*†,*,*,††}

Departments of Dermatology,* Medicine,[†] and Pediatrics,[§] University of California, San Francisco, San Francisco, CA 94143; and Dermatology Service** and Medical Service,^{††} Department of Veterans Affairs Medical Center, San Francisco, CA 94121

Abstract Lipids that are synthesized *de novo* in the epidermis, including fatty acids, oxysterols, 1,25-dihydroxyvitamin D₃, and farnesol, can regulate the differentiation of normal human keratinocytes (NHK). Cholesterol sulfate (CS), an epidermal lipid that is produced in the upper nucleated layers of the epidermis coincident with terminal differentiation, has been shown to play a role in the regulation of the late stages of keratinocyte differentiation, including formation of the cornified envelope. In the present study, we determined *i*) whether CS regulates involucrin (INV), an early keratinocyte differentiation marker, and *ii*) the mechanism by which CS regulates differentiation. mRNA and protein levels of INV, a precursor protein of the cornified envelope, increased 2- to 3-fold in NHK incubated in the presence of CS. In contrast, cholesterol had no effect on INV protein or mRNA levels. Transcriptional regulation was assessed in NHK transfected with INV promoter-luciferase constructs. CS increased luciferase reporter activity approximately 2- to 3-fold in NHK transfected with a 3.7-kb INV promoter construct. Deletional analysis revealed a CS-responsive region of the INV promoter located between bp -2452 and -1880. A 5-base pair (bp) mutation of the AP-1 site (bp -2117 to -2111) within this responsive region abolished CS responsiveness, suggesting a role for the AP-1 complex in the regulation of INV transcription by CS. Electrophoretic mobility shift analysis demonstrated increased binding of nuclear extracts isolated from CS-treated NHK to AP-1 DNA as compared with vehicle-treated controls. Incubation of the nuclear extract with the appropriate antibodies showed that the AP-1 DNA-binding complex contained Fra-1, Fra-2, and Jun D. Western blots demonstrated that CS treatment increased the levels of Fra-1, Fra-2, and Jun D, and Northern analyses revealed that CS increased mRNA levels for these same AP-1 factors. These data indicate that CS, an endogenous lipid synthesized by keratinocytes, regulates the early stages of keratinocyte differentiation, and may do so through its ability to modulate levels of AP-1 proteins. —Hanley, K., L. Wood, D. C. Ng, S. S. He, P. Lau, A. Moser, P. M. Elias, D. D. Bikle, M. L. Williams, and K. R. Feingold. Cholesterol sulfate stimulates involucrin transcription in keratinocytes by increasing Fra-1, Fra-2, and Jun D. *J. Lipid Res.* 2001. 42: 390–398.

Supplementary key words transglutaminase • cornified envelope • differentiation • AP-1

The differentiation of epidermal keratinocytes includes reorganization of intermediate filaments, production of selected lipids, and expression of a variety of structural proteins, culminating in the formation of structurally rigid corneocytes in the outermost layers of the epidermis (1–3). The corneocyte is an anucleate cell consisting of a filament network surrounded by an insoluble envelope of extensively cross-linked proteins. Proteins such as involucrin (INV), which is expressed relatively early in the program of differentiation, and loricrin, are incorporated into the cornified envelope by the calcium-sensitive membrane-bound enzyme transglutaminase (TGase) during the final stages of differentiation (1–7). The regulation of differentiation-specific gene expression is complex and not yet fully understood, and the early signals that initiate differentiation are largely unknown. Increased extracellular calcium, resulting in elevated intracellular calcium, is the most widely studied signal for the induction of normal human keratinocyte (NHK) differentiation (8, 9). Calcium stimulates the activity of the protein kinase C (PKC) pathway, which is known to influence gene expression through AP-1, a transcriptional complex consisting of Fos and Jun family proteins (10, 11). Phorbol esters such as phorbol 12-myristate 13-acetate (PMA), which activate AP-1 proteins by stimulating PKC activity, also induce NHK differentiation (3, 12, 13).

The epidermis is an active site of lipid synthesis, and studies have demonstrated the importance of lipids in regulating gene expression and keratinocyte differentiation (14). Moreover, studies indicate that intracellular endogenous lipid metabolites, such as fatty acids, oxysterols, and

Abbreviations: CS, cholesterol sulfate; DMSO, dimethyl sulfoxide; INV, involucrin; LXR, liver X receptor; NHK, normal human keratinocytes; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PPAR α , peroxisome proliferator-activated receptor α ; SREBP, sterol regulatory element-binding protein; TGase, transglutaminase.

¹ To whom correspondence should be addressed at the Metabolism Section (111F), Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121.

isoprenoids, regulate epidermal differentiation and development (15–18). Fatty acids, and the isoprenoid farnesol, induce NHK differentiation by activating the peroxisome proliferator-activated receptor α (PPAR α) (16, 18), a nuclear hormone receptor, whereas oxysterols appear to induce differentiation by activating the liver X receptor (LXR) (17). In addition to oxidation, cholesterol can also be modified by sulfation. In the epidermis, cholesterol sulfate (CS) is a membrane lipid generated from cholesterol by the enzyme cholesterol sulfotransferase in the stratum spinosum during the process of differentiation (19, 20). CS levels are highest in the granular layer and progressively decline from inner to outer stratum corneum, where steroid sulfatase catalyzes its hydrolysis (17, 19–22). A primary role for CS in desquamation has been demonstrated in X-linked ichthyosis, where a defect in the steroid sulfatase gene allows CS levels to reach levels 10-fold higher than normal, resulting in abnormal corneocyte retention and a thickened stratum corneum (19, 23, 24).

A role for CS in the regulation of the late stages of keratinocyte differentiation has been demonstrated. Late markers of differentiation, which normally occur in granular cells, such as expression of TGase 1, filaggrin, and loricrin, and the formation of cornified envelopes, increase in NHK after incubation with CS (25, 26). In the present study, we determined whether CS also stimulates earlier stages of differentiation, and explored the mechanism by which the CS effect is mediated. We report here that CS increases both protein and mRNA levels of INV, and that CS functions as a transcriptional regulator of INV. An AP-1 site in the CS-responsive region on the INV gene appears to be important for the CS effect. Furthermore, CS increases nuclear protein AP-1-DNA binding and increases protein and mRNA levels of several members of the AP-1 family of transcription factors. Thus, the modification of cholesterol by sulfation to form CS is another mechanism by which sterols can regulate gene expression.

MATERIALS AND METHODS

Cell culture

Human epidermis was isolated from newborn foreskins, and keratinocytes were plated in serum-free keratinocyte growth medium (KGM; Clonetics, San Diego, CA), as described (27). CS, cholesterol, PMA, and mevalonate were from Sigma (St. Louis, MO). CS and cholesterol were dissolved in dimethyl sulfoxide (DMSO) and stored at -70°C . PMA was dissolved in ethanol, and mevalonate was solubilized in sterile water.

RNA isolation, Northern blotting, and cDNA probes

Total RNA was isolated by using Trizol reagent (Sigma) according to the manufacturer protocol, and Northern analysis was performed as described previously (16–18). Blots were hybridized with ^{32}P -labeled INV cDNA probe (a gift from H. Greene, Harvard University, Boston, MA) overnight at 65°C and washed as described previously (16). The cDNA clone for human Fra-2 was obtained from the American Type Culture Collection (Manassas, VA). The cDNA clones for rat Fra-1 and mouse Jun D were gifts from D. Gardner (University of California, San Francisco). Autoradiography was performed at -70°C . Blots were

probed with β -actin or cyclophilin to confirm equal loading. Appropriate bands were quantified by densitometry.

INV and TGase protein levels

Levels of INV and TGase protein were assessed by protein electrophoresis and Western blotting, as described previously (16–18). INV protein was detected by incubation overnight at 4°C with a polyclonal rabbit anti-human INV antibody (a gift from R. Rice, University of California, Davis). TGase protein expression was measured as for INV expression, with modifications as described previously (16), allowing TGase to be detected by BC.1 primary antibody. Specific bands on autoradiograms were quantified by densitometry.

DNA constructs and PCR mutagenesis

The 3.7-kb INV promoter was a gift from J. Carroll (State University of New York, Stony Brook, NY). Deletional INV constructs were generated as described previously (28). The INV construct containing a mutant AP-1 site was prepared by site directed mutagenesis. The AP-1 site is underlined and the 5-base pair (bp) mutation is in upper case (see Fig. 5B).

Transfections

Keratinocytes were transfected as described previously (16–18). Briefly, keratinocytes were transiently transfected 1 day after plating (at a confluence of approximately 20–30%). A $10\text{-}\mu\text{g}/\text{ml}$ final concentration of Polybrene (dihexabromide; Aldrich, Milwaukee, WI), RSV- β -galactosidase ($0.2\text{ }\mu\text{g}$), and either INV promoter-luciferase construct ($2\text{ }\mu\text{g}$) or $0.5\text{ }\mu\text{g}$ of LXRE (TK-LXRE₃-luciferase), and $2\text{ }\mu\text{g}$ of DR0, DR1, DR2, DR3, DR4, or DR5 (all in TK-luciferase) (gifts from D. Mangelsdorf, Howard Hughes University, Dallas, TX) or $1\text{ }\mu\text{g}$ of PPRE-luciferase (kindly provided by A. Bass, University of California, San Francisco) were added to medium (KGM containing 0.03 mM calcium) in a final volume of 0.65 ml . Keratinocytes were incubated at 37°C for 5 h with gentle shaking each hour. Cells were rinsed with calcium- and magnesium-free phosphate-buffered saline solution (CMF-PBS), incubated at room temperature for 3 min with 10% glycerol in medium, and again rinsed with CMF-PBS. Cells were then incubated overnight with fresh medium and treated the next day as indicated for each experiment. Cells were rinsed and harvested in $250\text{ }\mu\text{l}$ of reporter lysis buffer (Promega, Madison, WI). The lysate was centrifuged at $10,000\text{ g}$ (4°C) for 2 min, and $10\text{--}20\text{ }\mu\text{l}$ of supernatant was assayed with luciferase substrate (Promega) and the β -galactosidase substrate Galacto-Light (Tropix, Bedford, MA) according to the manufacturer instructions. β -Galactosidase activity was used to normalize data and correct for variations in transfection efficiencies.

Isolation of nuclear proteins, gel shift assay

Nuclear proteins were prepared from preconfluent keratinocytes incubated in 0.03 mM calcium in the presence of CS or DMSO as vehicle for 24 h, using the method described by Dignam, Lebowitz, and Roeder (29). The AP-1 oligonucleotides used were 5'-TCGA-TATGCCG-TGAGTCA-GAGGGC-3' and 5'-TCGA-GCCCTC-TGACTCA-CGGGCATA-3'; AP-1 mutants were 5'-TCGA-TATGCCG-aactgCA-GAGGGC-3' and 5'-TCGA-GCCCCTC-TGcagtt-CGGGCATA-3' (AP-1 sites are underlined; lower-case letters denote mutations). Double-stranded oligonucleotides were end labeled with $50\text{ }\mu\text{Ci}$ of [^{32}P]ATP ($3,000\text{ Ci}/\text{mmol}$) in the presence of T4 polynucleotide kinase (Amersham, Arlington Heights, IL) for 60 min at 37°C and were purified with G-50 microcolumns (Amersham). Binding reactions were performed for 30 min at 4°C , using $5\text{ }\mu\text{g}$ of nuclear extract, buffer [10 mM Tris (pH 7.5), 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% glycerol], $2\text{ }\mu\text{g}$ of poly(dI-dC), and $60,000\text{ cpm}$ of ^{32}P -labeled oligonucleotide in a final volume of

15 μ l. Radioinert competitor DNA was added at a 100-fold molar excess. The samples were electrophoresed for 1.5 h on 5% acrylamide gels, using a 0.5 \times Tris-borate-EDTA running buffer, dried, and autoradiographed.

Supershift assay

Five micrograms of nuclear extract was preincubated for 2 h at 4°C in the presence of 20 μ g of antibody (Fra-1, Fra-2, Fos B, c-Fos, Jun D, c-Jun, or Jun B; all from Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated in the presence of 32 P-labeled AP-1 oligonucleotides as described above.

AP-1 protein levels

Protein levels of members of the AP-1 family were assessed by Western analysis. Briefly, 35 μ g of nuclear extract was fractionated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, which was then incubated in PBS with 5% nonfat dry milk and 0.05% Tween 20 for 1 h at 20°C. The membrane was then incubated for 16 h at 4°C in the presence of rabbit antibodies (diluted 1:5,000; Santa Cruz Biotechnology) specific for each AP-1 family protein. After four washes in 0.05% Tween 20 in PBS for 15 min each, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies (NA934; Amersham Life Science, Arlington Heights, IL) for 1 h at 20°C. The membranes were washed four times in 0.05% Tween 20 in PBS for 15 min each, incubated with SuperSignal Ultra chemiluminescent substrate (Pierce, Rockford, IL), and then exposed to film. Prestained standards (Bio-Rad, Hercules, CA) were used to determine molecular weight.

Statistics

Statistical analysis was performed with a Student's *t*-test.

RESULTS

Increased INV expression by CS

Prior studies by other investigators have shown that the activity and mRNA levels of TGase are elevated in NHK treated with CS (26). In the present study, we first examined the effects of CS on protein levels of both TGase and INV, an early differentiation marker, using Western analysis. As shown in **Fig. 1A**, protein levels of both TGase and INV were increased by CS compared with vehicle-treated controls. As shown in **Fig. 1B**, INV protein was increased in a dose-dependent manner, with 20 μ M producing near maximal effects. In contrast, no effect on TGase or INV protein levels was observed in NHK treated with cholesterol.

We next sought to determine by Northern analysis whether increased INV mRNA levels might underlie the induction of INV protein by CS. A significant increase in INV mRNA levels was observed in CS-treated NHK, and again no significant effect was observed after cholesterol treatment (**Fig. 2**). The effects of CS on INV mRNA levels were dose dependent, with maximal effects at 20 μ M CS. These data indicate that CS, but not cholesterol, increases INV protein and mRNA levels in NHK.

Inhibition of cholesterol synthesis does not underlie the effects of CS on differentiation

CS inhibits one of the key enzymes in cholesterol synthesis, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)

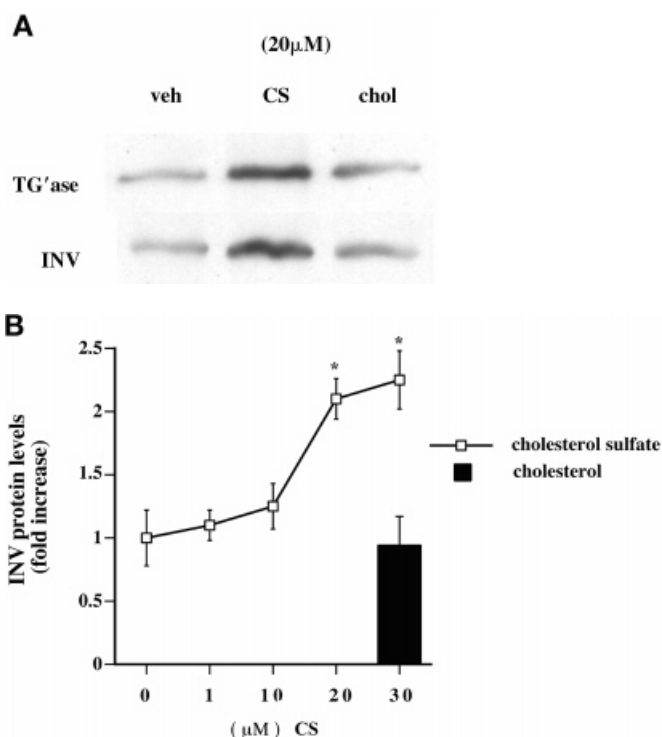


Fig. 1. Protein levels of TGase and INV are increased by CS. **A:** NHK were grown in 0.03 mM calcium and incubated in the presence of 0.05% DMSO vehicle (veh), 20 μ M CS, or 20 μ M cholesterol (chol). Western analysis was performed as described in Materials and Methods. A representative blot is shown. **B:** Dose-dependent effects of CS on INV protein levels. NHK were grown in 0.03 mM calcium and incubated together with increasing concentrations of CS (0–30 μ M) or with 30 μ M cholesterol (chol). Graph represents the averages (means \pm SEM) of data from two separate experiments (two different donors). It should be noted that CS but not cholesterol increases INV protein levels. * $P < 0.01$, $n = 6$.

reductase, and inhibits cholesterol synthesis in NHK (30). To determine whether a decrease in cholesterol or cholesterol metabolites, such as isoprenoids, might underlie the induction of INV mRNA levels by CS, we next measured mRNA levels in NHK treated with CS in the presence or absence of mevalonate, the product of HMG-CoA reductase. Mevalonate alone (200 μ M) had no effect on INV mRNA levels, and CS alone increased mRNA levels approximately 2.2-fold. Moreover, this increase was not affected by the addition of mevalonate together with CS. These data suggest that a reduction in intermediates in the cholesterol synthetic pathway is not the basis for the increase in INV mRNA levels by CS.

CS regulates transcription of the INV gene

Studies by others have shown that CS regulates the transcription of the TGase gene (26). To determine whether CS is also a transcriptional regulator of INV, NHK were transfected with a 3.3-kb fragment of the 5' promoter region of the INV gene coupled to a luciferase reporter. As shown in **Fig. 3**, INV promoter activity was significantly increased by 20 μ M CS, to levels similar to mRNA levels, indicating regulation of INV transcription by CS.

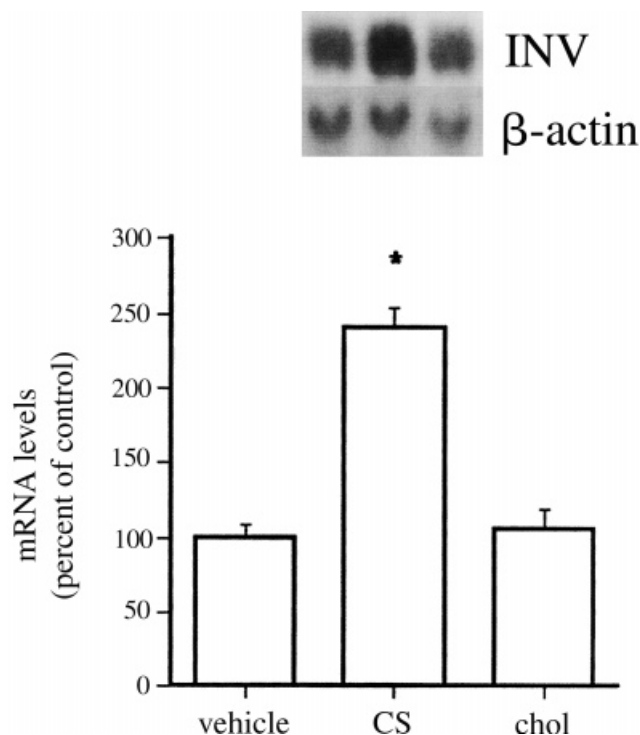


Fig. 2. Induction of INV mRNA by CS. NHK were incubated in media containing 0.03 mM calcium in the presence of 0.05% DMSO (vehicle), 20 μ M CS, or 20 μ M cholesterol (chol). Fifteen micrograms of total RNA was isolated and subjected to Northern analysis as described in Materials and Methods. Data presented here (means \pm SEM) represent the average of three separate experiments; * $P < 0.01$. Inset: Representative Northern blot.

CS does not *trans*-activate LXR, PPAR, or DR1 through DR5 elements in NHK

We have shown that fatty acids and clofibrate stimulate INV transcription in NHK, and increase epidermal differentiation via activation of PPAR α (16), a nuclear receptor that is present in NHK (18, 31). Because CS increases

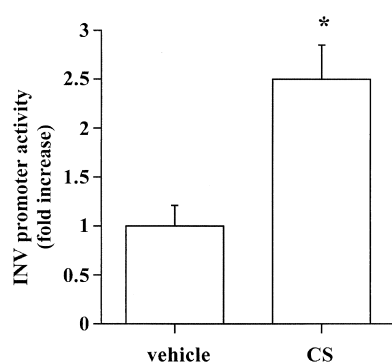


Fig. 3. INV transcription is increased by CS. NHK were transiently transfected with a 3.7-kb INV-luciferase construct as described in Materials and Methods, and treated the next day for 24 h with either 0.05% DMSO (vehicle) or 20 μ M CS. Data presented here (means \pm SEM) represent the average of three separate experiments; * $P < 0.01$, $n = 9$. Data were normalized to β -galactosidase activity to correct for variations in transfection efficiency.

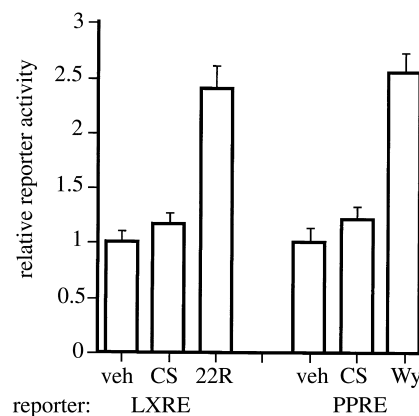


Fig. 4. CS does not activate an LXR response element (LXRE) or a PPAR response element (PPRE) in NHK. NHK were transfected with TK-LXRE₃-LUC (0.5 μ g) or with PPRE-LUC (1 μ g) together with RSV- β -galactosidase (0.2 μ g) to correct for variations in transfection efficiency as described in Materials and Methods. Cells were then exposed to vehicle (<0.05% DMSO) alone (veh), 20 μ M CS, 5 μ M (22 R)-hydroxycholesterol (22R), or 10 μ M Wy-14,643 (Wy). Data are expressed as means \pm SEM and represent means \pm SEM of three separate experiments.

fatty acid levels in preconfluent keratinocytes (30), we sought to determine whether CS might directly or indirectly activate PPAR α . As shown in **Fig. 4**, CS had no significant effect on NHK PPRE activity, while, in contrast, the PPAR α activator Wy-14,643 stimulated PPRE activity 2.6-fold.

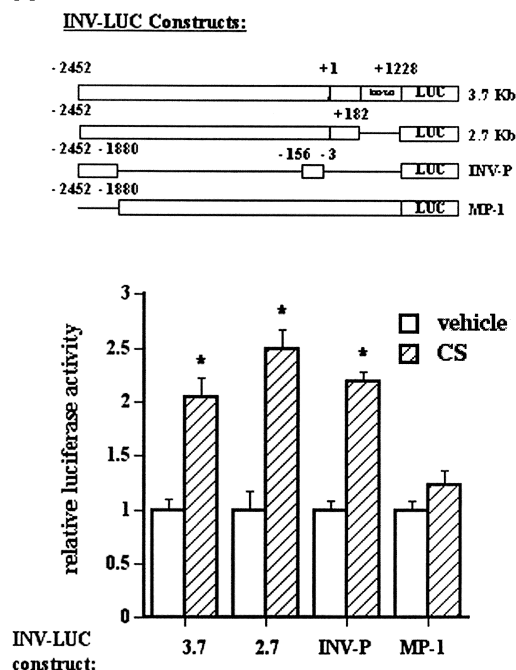
We have also shown that LXR α and LXR β , nuclear receptors that are activated by oxysterols, are present in NHK, and that oxysterols *trans*-activate an LXR response element transfected into NHK (17). To determine whether CS might exert its effects on differentiation via LXR, we transfected NHK with an (LXRE)₃-luciferase reporter and measured changes in reporter activity in response to CS. As shown in **Fig. 4**, LXRE activity in NHK was not significantly affected by CS treatment whereas LXRE activity is increased more than 2-fold by (22 R)-hydroxycholesterol treatment. These data suggest that the effects of CS on NHK differentiation are not mediated by either PPAR or LXR activation.

To determine whether CS may exert its effects by activation of an as-yet unidentified member of the nuclear hormone receptor family, we tested its ability to *trans*-activate via either the DR1, DR2, DR3, DR4, or DR5 response element. NHK were transfected with TK-luciferase constructs containing these consensus sequences and then treated with CS. DR1–DR5 reporter activities were not affected by CS treatment (not shown).

An AP-1 site in the CS-responsive region of the INV gene is important for inducibility

We next assessed the effects of CS on the activity of a variety of deletional INV promoter constructs so as to localize a region in the INV gene required for transcriptional activation by CS (**Fig. 5A**). Internal deletion of the intron at bp +182 to +1228 or of the regions containing bp –1880 to –156 and bp –3 to +1228 did not affect CS re-

A



B

INV construct:

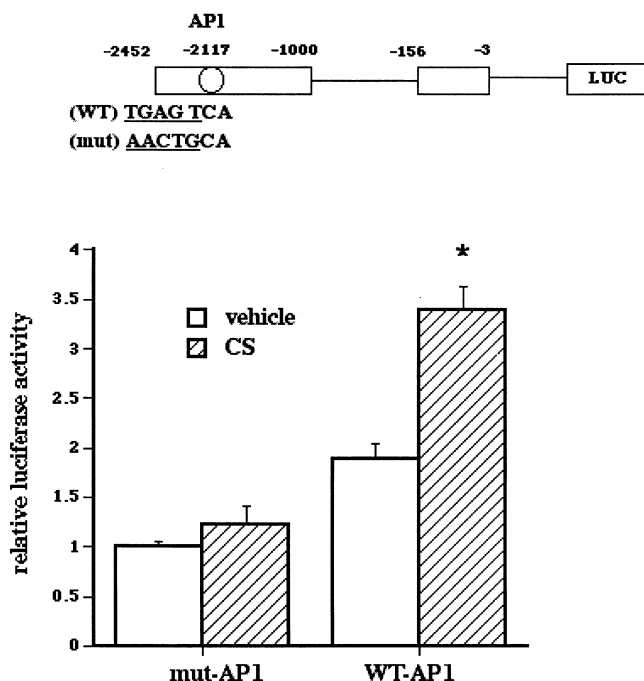


Fig. 5. Effects of CS on reporter activity in NHK transfected with INV promoter-luciferase constructs. A: NHK were transiently transfected with 2.0 μ g of the corresponding INV-LUC construct, which does not include the ATG nucleotide sequence, together with 0.2 μ g of RSV- β -galactosidase as described in Materials and Methods, and were then incubated for 24 h in the presence of vehicle (open columns) or 20 μ M CS (hatched columns). Previous studies (28) have examined the effect of these constructs on basal expression. The 2.7-kb construct expression is 3-fold, the INV-P expression is 30-fold, and the MP-1 expression is 0.25-fold compared with the 3.7-kb construct. It should be noted that luciferase activity was easily measured with all constructs. B: NHK were transfected with an INV-luciferase construct containing either a wild-type (WT-AP1) or a mutated (mut-AP1) AP-1 site with 0.2 μ g of RSV- β -galactosidase as described in Materials and Methods and treated as described in (A). Data presented here (means \pm SEM) represent the average of two separate experiments (three controls and three CS per experiment); * $P < 0.005$.

sponsiveness. In contrast, the region between bp -2452 and -1880 was required for activation by CS.

An AP-1-binding sequence is contained within the CS-responsive region of the INV gene (13, 32). Because CS is known to activate at least one PKC isoform that can activate AP-1 complexes (25), we next assessed the involvement of this AP-1 site in CS-induced INV transcription. As shown in Fig. 5B, mutation of the AP-1 site at bp -2117 to -2111 (TGAGTCA mutated to AACTGCA) resulted in loss of INV transcriptional inducibility by CS. Thus, CS increases INV transcription via an AP-1-dependent pathway.

CS increases AP-1 DNA binding

Differentiation-promoting agents, such as calcium and phorbol esters, induce AP-1 DNA binding activity (3, 28, 32). Here we examined the effects of CS on the binding of nuclear proteins to AP-1 DNA by gel shift analysis. As shown in Fig. 6, addition of nuclear extract to AP-1 oligonucleotide resulted in a band shift, and the intensity of the band was increased in extracts from NHK treated with CS. Addition of homologous oligonucleotide (AP) dramatically reduced binding to the probe, while addition of mutant AP-1 (mAP) resulted in no competition, suggesting that the binding is specific for AP-1. These data indicate that AP-1 DNA binding is increased during CS-stimulated differentiation.

AP-1 DNA complex composition in CS-treated NHK

Supershift analysis was next used to determine which AP-1 protein members are present in the CS-induced AP-1 complex. As reported by others, Fra-1, Jun B, and Jun D are the main AP-1 proteins in the complex in control NHK (33). Figure 7 shows the appearance of a super-shifted band in nuclear extracts from cells treated with CS. Fra-1 (F1) was the predominant protein in the complex, but Fra-2 (F2) and Jun D (JD) were also observed.

CS increases the protein and mRNA abundance of Fra-1, Fra-2, and Jun D

Western blots were used to assess alterations in the components of AP-1 from CS-treated keratinocytes (Fig. 8). PMA-treated cells were used as a positive control. PMA increased protein levels of Fra-1, c-Fos, and Jun D, but had little or no effect on Fra-2, Fos B, or Jun B (Fig. 8). With regard to c-Jun, PMA increased high molecular weight bands while decreasing low molecular weight bands. Although the significance of these multiple bands is unclear they are specific to the c-Jun antibody in that their detection is lost by preincubation of the antibody with c-Jun. CS treatment also resulted in increased levels of Fra-1 and Jun D protein to an extent similar to or greater than PMA (Fig. 8). In contrast to PMA, CS had no effect on either

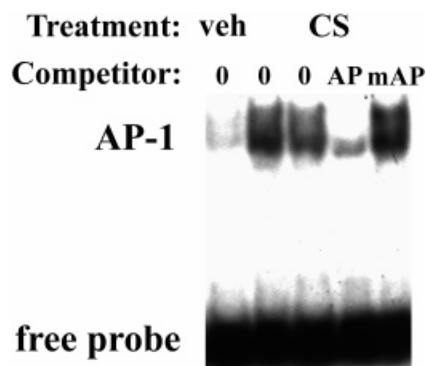


Fig. 6. CS treatment results in increased binding of nuclear extracts to AP-1 DNA. NHK were treated with vehicle ($<0.05\%$ DMSO; veh, lane 1) or $20\ \mu\text{M}$ CS (lanes 2–5) for 24 h and nuclear extracts were isolated as described in Materials and Methods. ^{32}P -labeled AP-1 was incubated with $5\ \mu\text{g}$ of nuclear extract in the absence or presence of a 100-fold molar excess of AP-1 (AP) or mutated AP-1 (mAP) competitor. The incubations were electrophoresed on nondenaturing gels, dried, and autoradiographed as described in Materials and Methods. The autoradiograph shown is representative of results obtained in three separate experiments.

c-Fos or c-Jun, but rather it increased Fra-2 protein expression (Fig. 8). These data show that CS increases the protein abundance of Fra-1, Fra-2, and Jun D, and suggest that CS and PMA act via overlapping but different pathways.

We next determined the effect of CS on mRNA levels of the AP-1 factors. As shown in Fig. 9, CS increased mRNA levels of Fra-1, Fra-2, and Jun D. These results show that the increase in Fra-1, Fra-2, and Jun D proteins is likely due to increased mRNA levels for these factors after CS treatment.

Additive effects of CS and PMA

We next sought to determine whether CS and PMA might induce differentiation via different pathways. As

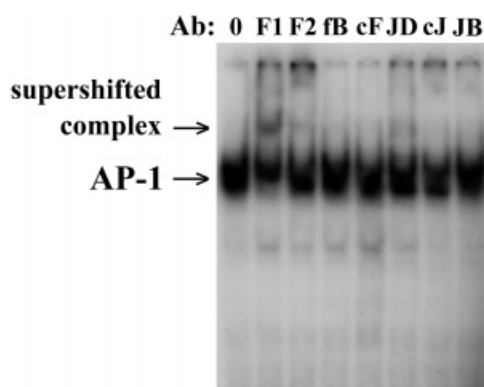


Fig. 7. Composition of the AP-1-DNA complex in CS-treated NHK. NHK were treated with $20\ \mu\text{M}$ CS for 24 h and nuclear extracts were isolated as described in Materials and Methods. Five micrograms of nuclear extract was preincubated for 2 h at 4°C in the presence of $20\ \mu\text{g}$ of antibody and then incubated in the presence of ^{32}P -labeled AP-1 oligonucleotides as described in Materials and Methods. F1, Fra-1; F2, Fra-2; fB, Fos B; cF, c-Fos; JD, Jun D; cJ, c-Jun; JB, Jun B. The autoradiograph shown is representative of results obtained in three separate experiments.

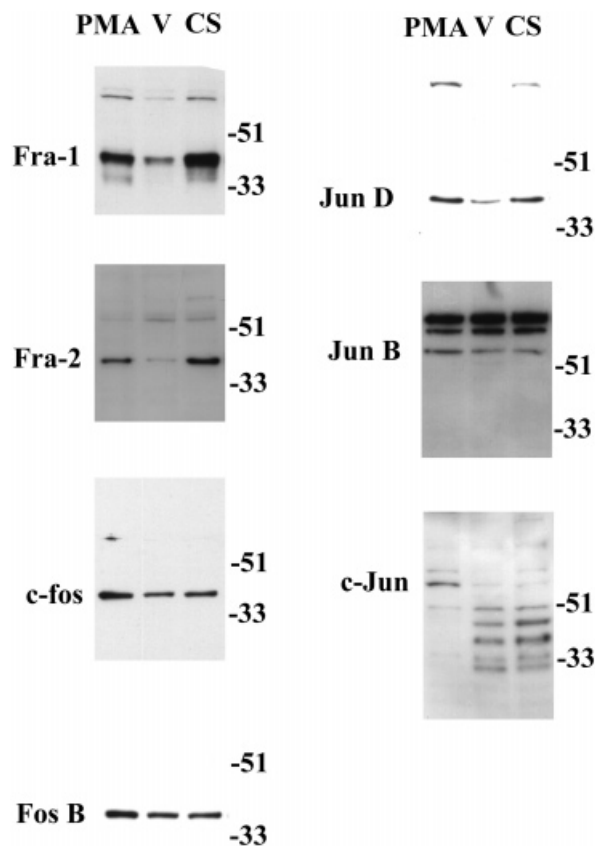


Fig. 8. AP-1 protein expression is differentially affected by CS and PMA. Nuclear extracts were isolated from NHK incubated with either vehicle (V; $<0.05\%$ DMSO), CS ($20\ \mu\text{M}$), or PMA ($10\ \text{nM}$) for 24 h as described in Materials and Methods. Forty micrograms of nuclear extract was fractionated and blotted, and blots were then incubated in the presence of each of the anti-AP-1 factors, and protein levels were detected by chemiluminescent substrate and exposure to film as described in Materials and Methods. The migration of molecular weight standards is marked. Representative blots are shown here; similar results were obtained in at least two separate experiments.

shown in Fig. 10, CS and PMA together at maximal doses further stimulate INV mRNA levels above levels stimulated by either compound alone. This suggests that the mechanisms by which CS and PMA stimulate INV expression differ, allowing for an additive/synergistic effect. Moreover, PMA causes a much greater increase in INV mRNA levels than CS, indicating that the pathway by which PMA activates differentiation is more potent than that by CS. However, CS is present normally in keratinocytes and the epidermis, whereas the physiologic relevance of PMA is open to question.

DISCUSSION

CS levels in the epidermis are regulated by its rate of synthesis, which is mediated by the enzyme cholesterol sulfotransferase, and its rate of degradation, which is catalyzed by the enzyme steroid sulfatase (19–22). Increased CS levels in the stratum corneum, resulting from decreased activity of the hydrolytic enzyme steroid sulfatase,

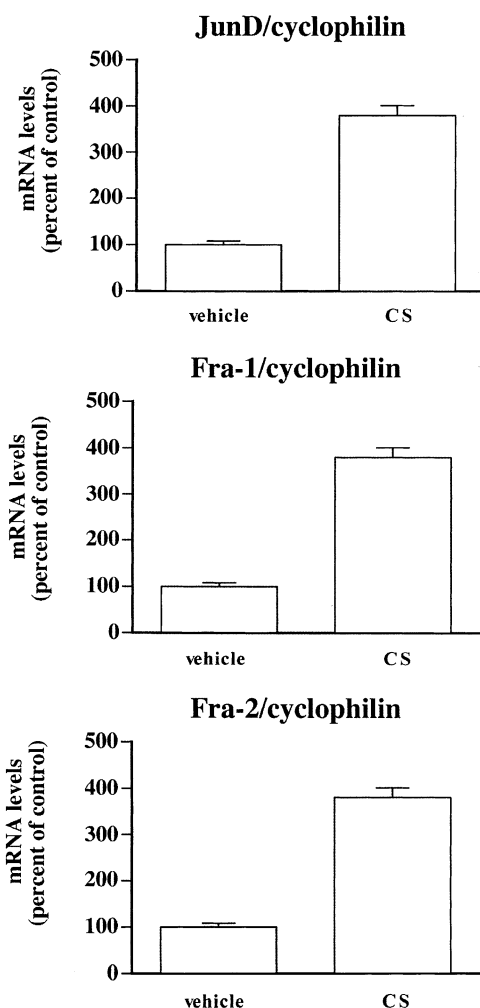


Fig. 9. CS increases mRNA levels of Fra-1, Fra-2, and Jun D in NHK. NHK were incubated in media containing 0.03 mM calcium and either 0.05% DMSO (vehicle) or 20 μM CS for 24 h. Poly(A)⁺ mRNA was isolated and Northern analysis was performed as described in Materials and Methods. Data normalized to cyclophilin mRNA levels are presented as means ± SEM, *n* = 3. * *P* < 0.001.

has long been recognized to cause abnormal corneocyte adhesion and desquamation (19, 23, 24). Recessive X-linked ichthyosis, a disorder of desquamation, is due to a defect in the steroid sulfatase gene. A regulatory role for CS in the nucleated layers of the epidermis has been described. Studies by other laboratories have shown that CS increases the expression of late markers of keratinocyte differentiation and the rates of cornified envelope formation (25, 26).

In the present study we demonstrated that CS increases the levels of INV, a protein expressed during the early stages of keratinocyte differentiation. Treatment of pre-confluent NHK with CS increased protein and mRNA levels of INV approximately 2- to 3-fold. The increase in INV mRNA levels was primarily due to increased gene expression, as the reporter activity of a 3.7-kb INV promoter ligated into a luciferase vector was also increased 2- to 3-fold following CS treatment. Moreover, promoter deletion analyses demonstrate that a specific region of the INV

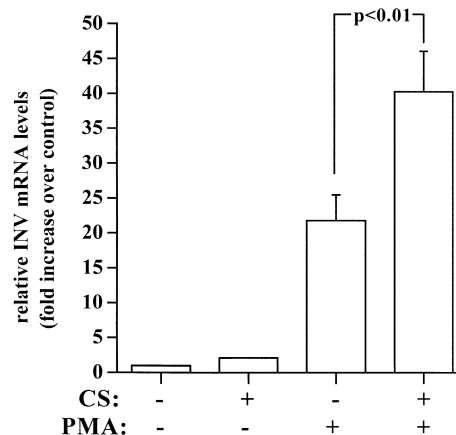


Fig. 10. CS further stimulates PMA-induced INV mRNA levels. NHK were treated for 24 h with either vehicle, CS (20 μM), PMA (10 nM), or CS and PMA together, and Northern analysis was performed as described for Fig. 2. Data normalized to actin mRNA levels are expressed as means ± SEM. Similar results were obtained in two separate experiments. * *P* < 0.01, *n* = 3.

promoter is important for mediating the CS effect. Specifically, deletion of the region spanning bp -2452 to -1880 results in loss of response to CS, indicating that an element within this portion of the promoter is essential for CS inducibility. An AP-1-binding site is present within this distal regulatory region of the INV promoter (at bp -2117 to -2111). In the present study we demonstrated that this AP-1 site is important for the transcriptional regulation of INV by CS, because the CS effect was lost on a 5-bp mutation of this AP-1 site. This AP-1 site has previously been shown to be important in the activation of INV transcription by calcium, phorbol esters, oxysterols, and PPAR activators (13, 17, 18, 28).

To elucidate further the mechanism by which CS stimulates the expression of INV we carried out gel-shift assays using an AP-1 oligonucleotide. Nuclear extracts from cells treated with CS contained increased quantities of a complex that bound AP-1 DNA, indicating that CS increases the AP-1 complex. The AP-1 complex consists of heterodimers of a Fos family member (Fra-1, Fra-2, c-Fos) and a Jun family member (c-Jun, Jun B, Jun D) or of a homodimer of two Jun family proteins (34). Supershift analyses by this and other laboratories (13, 28) indicate that Fra-1, which is localized in the suprabasal layers of the epidermis, is the main Fos family contributor to the formation of complexes that bind at the INV AP-1-5 site (bp -2117 to -2111), and that Jun B and Jun D are the main Jun family contributors. Here we showed that with CS treatment, Fra-1, Fra-2, and Jun D are the main AP-1 proteins that complex with the AP-1 regulatory site. Furthermore, we demonstrated, by Western and Northern analyses, that the protein and mRNA levels of Fra-1, Fra-2, and Jun D are increased by CS. It is likely, therefore, that CS treatment of NHK increases the levels of AP-1 proteins, which then stimulate INV gene expression by binding to the distal AP-1 site (bp -2117 to -2111). Other keratinocyte genes whose expression is stimulated during differentiation,


such as TGase, loricrin, and several of the keratin genes, also contain AP-1 elements, and therefore it is possible that CS coordinately regulates the expression of other genes important in keratinocyte differentiation by increasing Fra-1, Fra-2, and Jun D protein levels (35–40).

CS has been reported to activate PKC activity, and treatment of keratinocytes with the PKC inhibitor GF 109203X blocked the ability of CS to induce filaggrin and loricrin expression (25). PKC activation is well recognized to enhance AP-1 activity, and thus these data, coupled with our results, suggest that CS stimulates PKC activity that increases AP-1 protein levels and binding activity, thereby stimulating the expression of key genes required for keratinocyte differentiation. How CS stimulates PKC activity is unknown, but one could speculate, given its lipophilic nature and its structural similarities to a number of nuclear hormone ligands, such as oxysterols, vitamin D, and steroids, that it is recognized by a nuclear hormone receptor. However, our studies suggest that the effects of CS are not mediated by either PPAR or LXR. In addition, we were unable to demonstrate an effect of CS on *trans*-activating DR1, DR2, DR3, DR4, or DR5 consensus sequences. Whether CS is recognized by another member of the nuclear hormone receptor family or whether its effects are mediated by other pathways remains to be determined.

The epidermis is an active site of cholesterol and fatty acid synthesis, and our laboratory has shown that intermediates or products of these pathways are important regulators of keratinocyte differentiation (14–18). Fatty acids and the isoprenoid farnesol, which is derived from intermediates in the cholesterol biosynthetic pathway, activate PPAR α and thereby stimulate keratinocyte differentiation (16, 18). Oxysterols synthesized from cholesterol also induce keratinocyte differentiation, perhaps by activating LXR (see below) (17). Similar to what is observed with CS, the distal AP-1 site is required for the stimulation of keratinocyte differentiation by both PPAR α activators and oxysterols, suggesting that there are common pathways for inducing differentiation (16, 17). CS is therefore another lipid produced endogenously in the epidermis that can regulate keratinocyte differentiation (19, 25, 26).

Studies have recognized the important role of cholesterol and its metabolic products as regulators of gene expression (41). The activation of the sterol regulatory element-binding protein (SREBP) group of transcription factors, which regulate the expression of genes important in cholesterol and fatty acid metabolism, is dependent on proteolytic cleavage, which is regulated by cellular cholesterol levels (42). Sterols are postulated to interact with SREBP cleavage-activating protein in the endoplasmic reticulum, thereby regulating the activity of the proteases that release mature SREBP, which then migrates to the nucleus and alters gene expression (42). Cholesterol also has been shown to covalently bind to hedgehog proteins, which are key regulators of normal development (43). It is speculated that cholesterol binding to hedgehog enhances the association of hedgehog with the plasma membrane, thereby localizing its action (44). In addition to the direct effects of cholesterol, the oxidation of cholesterol

to specific oxysterols allows for the interaction of these cholesterol derivatives with LXR (45). In the liver the binding of oxysterols to LXR α has been shown to play an important role in the regulation of bile acid synthesis by stimulating cholesterol 7 α -hydroxylase expression (46, 47). We have shown that oxysterols stimulate keratinocyte differentiation in vitro (17) and epidermal differentiation in intact animals after topical treatment (48). In LXR α / β double knockout mice, oxysterols failed to induce differentiation, indicating that in the skin these receptors mediate the action of oxysterols (48). The present article demonstrates that another modification of cholesterol, sulfation, can also result in a compound that regulates gene expression and differentiation in keratinocytes. Whether CS regulates gene expression in other cells remains to be determined.

In summary, the present study demonstrated that CS stimulates keratinocyte differentiation and increases the expression of INV, an early differentiation marker. The increase in INV expression requires an intact AP-1 site (bp –2117 to –2111), and nuclear extracts from CS-treated keratinocytes demonstrate increased binding to this AP-1 site. This increased binding is due to increased levels of Fra-1, Fra-2, and Jun D in NHK treated with CS. Thus, we conclude that CS regulates keratinocyte differentiation by inducing AP-1 transcription factors, which in turn activate the genes required for the differentiation process. 

The authors appreciate the technical assistance provided by Sally Pennypacker (UC Cell Culture Facility, VA Medical Center). This study was supported by National Institutes of Health grants HD29706 and AR39639, AR19098, PO39448, and HL52839, and by the Medical Research Service, Department of Veterans Affairs Medical Center.

Manuscript received 8 September 2000 and in revised form 3 November 2000.

REFERENCES

1. Fuchs, E. 1990. Epidermal differentiation: the bare essentials. *J. Cell Biol.* **111**: 2807–2814.
2. Jackson, S. M., M. L. Williams, K. R. Feingold, and P. M. Elias. 1993. Pathobiology of the stratum corneum. *West. J. Med.* **158**: 279–285.
3. Eckert, R. L., J. R. Crish, and W. A. Robinson. 1997. The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol. Rev.* **77**: 397–424.
4. Robinson, N. A., P. T. LaCelle, and R. L. Eckert. 1996. Involucrin is a covalently cross-linked constituent of highly purified epidermal corneocytes: evidence for a common pattern of involucrin cross-linking in vivo and in vitro. *J. Invest. Dermatol.* **107**: 101–107.
5. Steinert, P. M., and L. N. Marekov. 1997. Direct evidence that involucrin is a major early isopeptide cross-linked component of the keratinocyte cornified cell envelope. *J. Biol. Chem.* **272**: 2021–2030.
6. Thacher, S. M., and R. H. Rice. 1985. Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. *Cell*. **40**: 685–695.
7. Steinert, P. M., and L. N. Marekov. 1995. The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isopeptide cross-linked components of the human epidermal cornified cell envelope. *J. Biol. Chem.* **270**: 17702–17711.
8. Hennings, H., F. H. Kruszezski, S. H. Yuspa, and R. W. Tucker. 1989. Intracellular calcium alterations in response to increased ex-

- ternal calcium in normal and neoplastic keratinocytes. *Carcinogenesis*. **4**: 777–780.
9. Yuspa, S. H., A. E. Kilkeny, P. M. Steinert, and D. R. Roop. 1989. Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J. Cell Biol.* **109**: 1207–1217.
10. Dlugosz, A. A., and S. H. Yuspa. 1994. Protein kinase C regulates keratinocyte transglutaminase (TGK) gene expression in cultured primary mouse epidermal keratinocytes induced to terminally differentiate by calcium. *J. Invest. Dermatol.* **102**: 409–414.
11. Denning, M. F., A. A. Dlugosz, E. K. Williams, Z. Szellasi, P. M. Blumberg, and S. H. Yuspa. 1995. Specific protein kinase C isozymes mediate the induction of keratinocyte differentiation markers by calcium. *Cell Growth Differ.* **6**: 149–151.
12. Younus, J., and B. A. Gilchrist. 1992. Modulation of mRNA levels during human keratinocyte differentiation. *J. Cell Physiol.* **152**: 232–239.
13. Welter, J. F., J. F. Crish, C. Agarwal, and R. L. Eckert. 1995. Fos-related antigen (Fra-1), junB, and junD activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects on promoter activity. *J. Biol. Chem.* **270**: 12614–12622.
14. Feingold, K. R. 1991. The regulation and role of epidermal lipid synthesis. *Adv. Lipid Res.* **24**: 57–82.
15. Hanley, K., Y. Jiang, D. Crumrine, N. M. Bass, R. Appel, P. M. Elias, M. L. Williams, and K. R. Feingold. 1997. Activators of the nuclear hormone receptors PPAR α and FXR accelerate the development of the fetal epidermal permeability barrier. *J. Clin. Invest.* **100**: 705–712.
16. Hanley, K., Y. Jiang, S. S. He, M. Friedman, P. M. Elias, D. D. Bikle, M. L. Williams, and K. R. Feingold. 1998. Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPAR α . *J. Invest. Dermatol.* **110**: 368–375.
17. Hanley, K., D. C. Ng, S. S. He, P. Lau, K. Min, P. M. Elias, D. D. Bikle, D. J. Mangelsdorf, M. L. Williams, and K. R. Feingold. 2000. Oxysterols induce differentiation in human keratinocytes and increase AP-1 dependent involucrin transcription. *J. Invest. Dermatol.* **114**: 545–553.
18. Hanley, K., L. G. Komuves, D. C. Ng, A. M. Lefebvre, S. S. He, P. Lau, D. D. Bikle, M. L. Williams, P. M. Elias, J. Auwerx, and K. R. Feingold. 2000. Farnesol stimulates differentiation in epidermal keratinocytes via PPAR α . *J. Biol. Chem.* **275**: 11484–11491.
19. Epstein, E. H., Jr., M. L. Williams, and P. M. Elias. 1984. The epidermal cholesterol sulfate cycle. *J. Am. Acad. Dermatol.* **10**: 866–868.
20. Epstein, E. H., Jr., J. M. Bonifas, T. C. Barber, and M. Haynes. 1984. Cholesterol sulfotransferase of newborn mouse epidermis. *J. Invest. Dermatol.* **83**: 332–335.
21. Elias, P. M., M. L. Williams, M. E. Maloney, J. A. Bonifas, B. E. Brown, S. Grayson, and E. H. Epstein, Jr. 1984. Stratum corneum lipids in disorders of cornification. *J. Clin. Invest.* **74**: 1414–1421.
22. Ranasinghe, A. W., P. W. Wertz, D. T. Downing, and I. C. Mackenzie. 1986. Lipid composition of cohesive and desquamated corneocytes from mouse ear skin. *J. Invest. Dermatol.* **86**: 187–190.
23. Koppe, J. G., A. Marinkovic-Ilsen, Y. Rijken, W. B. de Groot, and A. C. Jobsis. 1978. A sulfatase deficiency. *Arch. Dis. Child.* **53**: 803–806.
24. Shapiro, L. J., R. Weiss, M. M. Buxman, J. Vidjoff, and R. L. Diamond. 1978. Enzymatic basis of typical X-linked ichthyosis. *Lancet*. **2**: 756–757.
25. Denning, M. F., M. G. Kazanietz, P. M. Blumberg, and S. H. Yuspa. 1995. Cholesterol sulfate activates multiple protein kinase C isoenzymes and induces granular cell differentiation in cultured murine keratinocytes. *Cell Growth Differ.* **6**: 1619–1626.
26. Kawabe, S., T. Ikuta, M. Ohba, K. Chida, E. Ueda, K. Yamanishi, and T. Kuroki. 1998. Cholesterol sulfate activates transcription of transglutaminase 1 gene in normal human keratinocytes. *J. Invest. Dermatol.* **111**: 1098–1102.
27. Gibson, D., A. V. Ratnam, and D. D. Bikle. 1996. Evidence for separate control mechanisms at the message, protein, and enzyme activation levels for transglutaminase during calcium-induced differentiation of normal and transformed human keratinocytes. *J. Invest. Dermatol.* **106**: 154–161.
28. Ng, D. C., S. Shakaee, D. Lee, and D. D. Bikle. 2000. Requirement of an AP-1 site in the calcium response region of the involucrin promoter. *J. Biol. Chem.* **275**: 24080–24088.
29. Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**: 1475–1489.
30. Williams, M. L., S. A. Rutherford, and K. R. Feingold. 1987. Effects of cholesterol sulfate on lipid metabolism in cultured human keratinocytes and fibroblasts. *J. Lipid Res.* **28**: 955–967.
31. Rivier, M., I. Safonova, P. Lebrun, C. E. M. Griffiths, G. Ailhaud, and S. Michel. 1998. Differential expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes. *J. Invest. Dermatol.* **111**: 1116–1121.
32. Banks, E. B., J. F. Crish, J. F. Welter, and R. L. Eckert. 1998. Characterization of human involucrin promoter distal regulatory region transcriptional activator elements—a role for SP1 and AP1 binding sites. *Biochem. J.* **331**: 61–68.
33. Rutberg, S. E., E. Saez, S. Lo, S. I. Jang, N. Markova, B. M. Spiegelman, and S. H. Yuspa. 1997. Opposing activities of c-Fos and Fra-2 on AP-1 regulated transcriptional activity in mouse keratinocytes induced to differentiate by calcium and phorbol esters. *Oncogene*. **15**: 1337–1346.
34. Angel, P., and M. Karin. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta*. **1072**: 129–157.
35. Disepio, D., A. Jones, M. A. Longley, D. Bundman, J. A. Rothnagel, and D. A. Roop. 1995. The proximal promoter of the mouse loricrin gene contains a functional AP-1 element and directs keratinocyte-specific but not differentiation-specific expression. *J. Biol. Chem.* **270**: 10792–10799.
36. Liew, F. M., and K. Yamanishi. 1992. Regulation of transglutaminase 1 gene expression by 12-O-tetradecanoylphorbol-13-acetate, dexamethasone, and retinoic acid in cultured human keratinocytes. *Exp. Cell Res.* **202**: 310–315.
37. Yamada, K., K. Yamanishi, A. Kakizuka, Y. Kibe, H. Doi, and H. Yasuno. 1994. Transcriptional regulation of human transglutaminase 1 gene by signaling systems of protein kinase C, RAR/RXR and Jun/Fos in keratinocytes. *Biochem. Mol. Biol. Int.* **34**: 827–836.
38. Ma, S., L. Rao, I. M. Freedberg, and M. Blumenberg. 1997. Transcriptional control of K5, K6, K14 and K17 keratin genes by AP-1 and NF-kappaB family members. *Gene Expr.* **6**: 361–370.
39. Casatorres, J., J. M. Navarro, M. Blessing, and J. L. Jorcano. 1994. Analysis of the control of expression and tissue specificity of the keratin 5 gene, characteristic of basal keratinocytes. Fundamental role of an AP-1 element. *J. Biol. Chem.* **269**: 20489–20496.
40. Lu, B., J. A. Rothnagel, M. A. Longley, S. U. Tsai, and D. K. Roop. 1994. Differentiation-specific expression of human keratin 1 is mediated by a composite AP-1/steroid hormone element. *J. Biol. Chem.* **269**: 7443–7449.
41. Edwards, P. A., and J. Ericsson. 1999. Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. *Annu. Rev. Biochem.* **68**: 157–185.
42. Brown, M. S., and J. L. Goldstein. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane bound transcription factor. *Cell*. **89**: 331–340.
43. Porter, J. A., K. E. Young, and P. A. Beachy. 1996. Cholesterol modification of hedgehog signaling proteins in animal development. *Science*. **274**: 255–259.
44. Beachy, P. A., M. K. Cooper, K. E. Young, D. P. von Kessler, W. J. Park, T. M. Hall, D. J. Leahy, and J. A. Porter. 1997. Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. *Cold Spring Harbor Symp. Quant. Biol.* **62**: 191–209.
45. Janowski, B. A., P. J. Willy, T. R. Deni, J. K. Falek, and D. J. Mangelsdorf. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR α . *Nature*. **383**: 728–731.
46. Lehmann, J. M., S. A. Kliewer, L. B. Moore, T. A. Smith-Oliver, B. B. Oliver, J. L. Su, S. S. Sundseth, D. A. Winegar, D. E. Blanchard, T. A. Spencer, and T. M. Willson. 1997. Activation of the nuclear receptor LXR by oxysterol defines a new hormone response pathway. *J. Biol. Chem.* **272**: 3137–3190.
47. Peet, D. J., S. D. Turley, W. Ma, B. A. Janowski, J. M. Lobaccaro, R. E. Hammer, and D. J. Mangelsdorf. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell*. **93**: 693–704.
48. Komuves, L., K. Hanley, J. Lobaccaro, M. Williams, P. Elias, D. Mangelsdorf, and K. R. Feingold. 2000. *J. Invest. Dermatol.* **114**: 780 (Abstract 173).