Human involucrin whose gene transcription is directed by a 2456-nucleotide (nt) 5' non-coding region is a structural component of the epithelial cornified layer. Transient transfection assays demonstrated that this region is transcriptionally active in multiplying keratinocytes and is enhanced by 2 mM CaCl2 treatment. Calcium-independent transcriptional activity and the interaction with the AP-1 transcriptional factor was located on the proximal part (nt −159 to −1) of the 5' non-coding region. However, CaCl2 responsiveness was mapped to a distal 1185-nt fragment (nt −2456 to −1272). Moreover, this fragment potentiated the Herpes simplex thymidine kinase promoter in normal keratinocytes and is responsive to calcium treatment in a cell type-specific manner. Interestingly, the absence of a 491-nt fragment located between the two enhancer domains (nt −651 to −160) resulted in transcriptional activation in multiplying keratinocytes. This fragment interacts with AP-1 and the YY1 transcriptional silencer. It is concluded that human involucrin 5' non-coding region contains at least three regulatory domains, a distal CaCl2-responsive enhancer, a putative transcriptional silencer (that interacts with AP-1 and YY1), and a proximal enhancer/promoter (that interacts with AP-1). Thus, this study demonstrates the presence of particular transcriptional factors that can potentially regulate the human involucrin expression.

The differentiation of stratified epithelia requires the harmonious expression of several structural and regulatory proteins. The complex regulatory pathways that direct the transcription of epithelial differentiation-related genes are of particular importance in human disease. Involutcin, a precursor of the cornified envelope of terminally differentiated keratinocytes (1, 2), is apparently limited to primates (3). The involucrin protein has a molecular mass of 68 kDa and possesses a central glutamyl-rich domain formed with 39 repeats of a 10-amino acid cassette (4) which is required for the cross-linking activity of the calcium-dependent epithelial transglutaminase during cornified envelope formation (5–8).

The human involucrin gene is about 6000 nt in size composed of two exons of 43 and 2107 nt, respectively, separated by an intron of 1188 nt (4). A 2456-nt noncoding sequence located 5' of the first involucrin exon has transcriptional regulatory elements that control its transcriptional activity (9–11). Analysis of in vitro and in vivo results show that the involucrin gene activation depends on the interaction of transcriptional factors present in the keratinocyte nucleus (9–11).

In vitro and in vivo experiments correlate the presence of involucrin transcripts and protein following the progression of keratinocytes from the basal layer to terminal differentiation state (12–14). Thus, the transcriptional factors required for specific involucrin gene transcription may also be necessary for expression of other epithelial terminal differentiation-related genes (15–17). Interestingly, several genes related to terminal differentiation, such as involucrin, profilaggrin, and loricrin, are located on chromosome 1q21 (18).

The 5'-noncoding region controlled the expression of the involucrin gene in transient transfection of cultured human keratinocytes (9). This region was divided functionally into two portions: the proximal 900-nt promoter region with the putative TATA box and an upstream 1600-nt region with necessary elements for the proper expression of the involucrin gene (9). Interestingly, the entire 2456-nt 5'-noncoding segment activity is tissue-specific in transgenic mice, suggesting that the basic regulatory elements of the involucrin gene are widespread in mammals (10, 19).

The reported sequence of the 900-nt proximal promoter region active in keratinocytes contains putative target sites for the AP-1 family of transcriptional factors (9, 11). Moreover, the addition of TPA, an AP-1 activator, moderately activates this region in transient transfection assays using cultured rat cells. The latter suggests that AP-1 could be necessary for involucrin expression. Furthermore, the proximal 900-nt enhancer was activated by overexpression of c-fos and c-jun oncogenes, components of AP-1 (11). Treatment of normal keratinocytes with calcium, TPA, or vitamin A depletion (20–26) are able to increase involucrin mRNA levels. However, how these compounds directly regulate the involucrin promoter region is not clear.

To investigate the transcriptional regulation of the involucrin gene, several constructs of the 2456-nt 5'-noncoding region were transfected into cultured human keratinocytes during multiplication (0.1 mM CaCl2) or differentiation (2 mM CaCl2).

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; nt, nucleotides; CAT, chloramphenicol acetyltransferase; HP1, HP2, HP3 and HP4, proximal footprints; H1, H2, H3, and H4, distal footprints; SV40, simian virus 40; TK, thymidine kinase.
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conditions. Involucrin transcription is shown to be regulated by several functional elements: a distal cell type-specific 1100-nt upstream enhancer (nt -2456 to -1272) responsive to calcium stimulation and a possible transcriptional silencer (nt -159 to -1/1) unaffected by calcium concentration. Further DNA-protein characterization of the silencer and proximal enhancer/promoter regions established that AP-1 and YY1 are the main transcriptional factors interacting with these elements.

MATERIALS AND METHODS

Plasmids and Oligonucleotides—The p2.6CAT plasmid contains the entire 2456 nt from the human involucrin 5’-noncoding region of p1.3H6B plasmid (4) cloned in the pCAT-basic vector (Promega Corp., Madison, WI) using synthetic HindIII and XbaI linkers (Fig. 1A). p827CAT plasmid contains the polymerase chain reaction-amplified fragment from nt -784 to 43 (with addition of HindIII site) from the involucrin 5’-nontranscribed region of p1.3H6B plasmid in pCAT-basic. A series of nested deletions was constructed from p827CAT, the p97CAT and p220CAT reporter plasmids contain the PstI-XbaI and Apal-XbaI fragments, respectively, and p610CP with the 610-nt HindIII fragment cloned in the pCAT-promoter vector (Promega Corp.), which possesses the SV40 early promoter (Fig. 1A). The p1.1TKM construct contains the 1185-nt HindIII-Rsal fragment from p2.6CAT (Fig. 1A) cloned upstream the herpes simplex type 1 thymidine kinase promoter from pT7Km vector (27). plN220 and plN630 were obtained inserting the Apal-Xbal or HindIII-Apal fragments from p827CAT in pUC19 (Fig. 3B).

The complete nucleotide sequence from p2.6CAT insert is recorded in GeneBank™ (accession number U23404). All constructs were sequenced using Sequenase (Amersham Corp.) or the chemical degradation method (28). All oligonucleotides (Table I) were synthesized in an Applied Biosystems 391 DNA synthesizer.

Cell Culture—HeLa cervical carcinoma cells and MRC-5 human fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cervical carcinoma cell line C-33A was grown in Dulbecco’s modified Eagle’s medium/F-12 (1:1) medium supplemented with 7% fetal bovine serum. Secondary cultures of neonatal human foreskin keratinocytes were obtained essentially as described previously (33). All buffers were freshly prepared and contained the protease inhibitors aprotinin, leupeptin, antipain, chymostatin, pepstatin (5 μg/ml each), and benzamidine (2 mM) to prevent nuclear factor proteolysis (Sigma). Protein concentration was measured with 2 mM CaCl2 12 h post-transfection. Cell harvests were performed on confluent cultures by addition of 2 mM CaCl2

Transient Transfections and CAT Assays—Normal human keratinocyte cultures, 60-70% confluent in 100-mm tissue culture dishes, were transfected with 10 μg of total plasmid DNA from different deletion constructs as described under “Materials and Methods.” The average CAT activities relative to the promoterless vector pCAT-basic were obtained from at least three independent experiments 48 h post-transfection. The transcriptional start site is represented by an arrow. The various deleted constructs, the putative TATA box, the restriction sites for Apal, HindIII, PstI, RsaI, and XbaI, as well as the SV40 minimal promoter (5V) and the herpes simplex type 1 thymidine kinase promoter (TK) are indicated.

Cell-type-specific enhancer activity of the human involucrin distal enhancer region, pTKM and p1.1TKM plasmids (10 μg) were transfected into multiplying human keratinocytes, MRC-5 fibroblasts, and C-33A cell line. Cells were harvested 48 h post-transfection. Because of the different transfection efficiencies, the activities are plotted relative to the SV40 enhancer/promoter.

as indicated before (32).

Gel-shift Assays—Nuclear extracts from keratinocytes or HeLa cells were incubated on ice with 0.5-1 μg of poly(dI-dC)-(Pharmacia Biotech Inc., Alameda, CA) and 1 ng of [32P]-end-labeled DNA in 2 × BD buffer (24 mM HEPES, pH 7, 8, 1.0 mM EDTA, 15 mM NaCl) and lysed with three freeze-thaw cycles in 0.25% Tris-HCl, pH 8.0; protein was quantified employing the Bradford method (32). Standardized amounts of lysate protein were incubated with 0.25 μCi of [32P]-choloroformphenol (50 mCi/mmol, Amersham Corp.) and 0.66 mM acetyl-CoA (Sigma) in a final volume of 115 μl. The acetylation reactions were carried out at 37 °C for up to 4 h. The samples were extracted with ethyl acetate (J.T. Baker Inc.) and loaded in TLC plates (Sigma). Chromatography was developed with chloroform:methanol (19:1) and exposed to Hyperfilm radiographic films. For competitive studies, the reaction mixtures were preincubated with different amounts of unlabeled competitor oligonucleotide before the addition of labeled DNA. Gel supershift experiments, reactions with the DNA-protein complexes were incubated at 4 °C with anti-c-Jun/AP-1 (sc-44; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-HPV16 E7 rabbit polyclonal antibodies for 6 h prior electrophoresis.

DNase I Footprinting—EcoRI-HindIII fragments from plN220 and plN630 plasmids (Fig. 3B) were asymmetrically end-labeled with either [γ-32P]-ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) or [α-32P]-dATP and DNA polymerase I Klono fragment (Boehringer Mannheim) and isolated through preparative 6% acrylamide gel electrophoresis. A standard DNA binding reaction was performed using 20-40 μg of total nuclear extract in 20 μl final volume. DNase I (Boehringer Mannheim) digestion was performed at 20 °C with
empirically determined concentrations and stopped using 600 mg/ml proteinase K (Boheringer Mannheim).

The nucleic acids were phenol-extracted and precipitated with ethanol. The pellets were washed with 70% ethanol and dissolved in gel loading buffer (80% formamide, 0.1% bromphenol blue, 0.1% xylene-cyanol) and denatured 5 min at 95°C prior electrophoresis through 6% polyacrylamide, 7M urea sequencing gels.

RESULTS

The Human Involucrin Gene 5'-Noncoding Region Contains Several Transcriptional Regulatory Domains—

The transcriptional regulation of the human involucrin gene was analyzed employing deletion constructs derived from p2.6CAT plasmid which express the CAT reporter gene under the control of the intact 5'-noncoding proximal 784-nt fragment. Normal keratinocytes were transfected with 10 μg of total plasmid DNAs from p2.6CAT, p827CAT, p220CAT, and p97CAT constructs. Transfected cells were grown in multiplying (0.1 mM CaCl2) or after differentiation induction (2 mM CaCl2) conditions as described under "Materials and Methods." Cell extracts were obtained 48 h post-transfection. Representative CAT chromatograms from three independent experiments are shown. pCAT-basic and pCAT-control vectors were used as negative and positive controls, respectively.

B, calcium responsiveness resides in the distal enhancer. pTKM and p1.1TKM plasmids (10 μg) were transfected into keratinocytes and processed as described above. The average CAT activities relative were obtained from three independent experiments.

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Fig. 2. Activity of the involucrin gene 5'-noncoding region in differentiation-induced keratinocytes. A, differentiation induction does not stimulate human involucrin 5'-noncoding proximal 784-nt fragment. Normal keratinocytes were transfected with 10 μg of total plasmid DNAs from p2.6CAT, p827CAT, p220CAT, and p97CAT constructs. Transfected cells were grown in multiplying (0.1 mM CaCl2) or after differentiation induction (2 mM CaCl2) conditions as described under "Materials and Methods." Cell extracts were obtained 48 h post-transfection. Representative CAT chromatograms from three independent experiments are shown. pCAT-basic and pCAT-control vectors were used as negative and positive controls, respectively. B, calcium responsiveness resides in the distal enhancer. pTKM and p1.1TKM plasmids (10 μg) were transfected into keratinocytes and processed as described above. The average CAT activities relative were obtained from three independent experiments.

Fig. 3. Human involucrin 5'-noncoding region. A, complete nucleotide sequence of the involucrin 2456-nt regulatory region. Nucleotide position number—2456 corresponds to the HindIII site of p2.6CAT plasmid. The sequence segment from nt—784 to 49 was reported previously (12). Predicted consensus sequences for several transcriptional factors within the proximal 784-nt (underlined) and restriction sites for Apal, HindIII, and PstI are shown. TATA box and transcription start site are double-underlined. B, plasmid constructs employed for DNase I footprint analysis. The Apal-XbaI and HindIII-Apal fragments from p827CAT plasmid are denoted in plN220 and plN630 plasmids, respectively. Black lines show the position of oligonucleotides employed in gel-shift assays (H1, H2, H3, H4, H4, 2072, and H4, 2126). The TATA box (vertical box) and Apal, HindIII, PstI, and XbaI restriction sites are shown.
Confirmation of the above observation was obtained with the p220CAT plasmid containing the \( \text{ApaI}-\text{XbaI} \) fragment from p827CAT, a 6-fold increase in CAT activity relative to p827CAT was observed (Fig. 1A). Therefore, the region upstream the \( \text{ApaI} \) site, probably has a negative regulatory element. To explore this possibility, the \( \text{PstI}-\text{PstI} \) fragment from p827CAT was cloned in the p610CP plasmid before the SV40 promoter and transfected to multiplying human keratinocytes. However, the observed CAT activity of p610CP was similar to that obtained with the SV40 promoter alone, denoting that other elements present in p827CAT construct are associated with the inhibitory function (Fig. 1A). None of the constructs presented activity when transfected into HeLa cells, which do not express involucrin (data not shown). The p1.1TKM construct was transfected in MRC-5 fibroblasts and C-33A cells to establish the cell type specificity of this enhancer. A mild relative activity of p1.1TKM was noticed in C-33A cells, whereas this same construct remained silent in MRC-5 fibroblasts (Fig. 1B). These results suggest that the elements regulating transcription from the distal enhancer are specific of epithelial-derived cells.

The p2.6CAT plasmid displayed 5-fold activation in calcium-induced differentiation conditions when transfected in keratinocytes stimulated to differentiate by increasing the \( \text{CaCl}_2 \) concentration to \( 2 \text{mM} \) in the absence of epidermal growth factor and bovine pituitary extract (Fig. 2A). These conditions stimulate 3–5-fold the transcription of the involucrin gene (34). However, activity of p97CAT, p220CAT, and p827CAT remained unchanged (Fig. 2A). Therefore, the calcium responsiveness should reside in the distal 1185-nt enhancer. To test this, the p1.1TKM construct was also transfected in normal keratinocytes under multiplying and differentiation conditions resulting in a significant increase on the p1.1TKM activity in calcium-induced differentiation conditions (Fig. 1A).

FIG. 4. DNase I footprint analysis of the human involucrin proximal enhancer/promoter region. A, nuclear extracts (35 \( \mu \text{g} \)) from multiplying (Ker) and 2 mM \( \text{CaCl}_2 \)-induced (Ki) human keratinocytes or HeLa cells were incubated with the end-labeled EcoRI-HindIII fragment from pN220 plasmid for DNase I footprinting as described under “Materials and Methods” and electrophoresed in 6% sequencing gels. Brackets show the regions covered by the HP-1, HP-2, HP-3, and HP-4 footprints in the upper and lower DNA strands. Numbers on the left side show the nucleotide position in the human involucrin 5′-noncoding region sequence. \( \text{Pu} \), purine chemical cleavage ladder. Triangles indicate DNase I hypersensitive sites. B, AP-1 competition footprint analysis. The labeled EcoRI-HindIII pN220 DNA fragment was incubated with 40 \( \mu \text{g} \) of human multiplying involucrin nuclear extract. Competition was performed by adding 0.5 and 1.0 \( \mu \text{g} \) of nonlabeled oligonucleotide containing a consensus AP-1 binding site (Table I) to the binding reaction for 10 min before incubation with DNase I. The relative location of the HP-2 and HP-3 footprints is indicated by brackets. Triangles show recovered sites. Pu, purine sequence ladder.

FIG. 5. The human involucrin promoter contains binding sites recognized by AP-1 factor. A, gel-shift assays were done incubating the \( ^3\text{P} \) end-labeled HindIII-EcoRI fragment from pN220 plasmid with 8 \( \mu \text{g} \) of total nuclear extracts from multiplying (Ker) or 2 mM \( \text{CaCl}_2 \)-induced (Ki) human keratinocytes on ice in the presence of 1 \( \mu \text{g} \) of poly[d(I-C)] as unspecific carrier. Competitions were performed by adding 100 and 200 molar excesses of the indicated nonlabeled competitor oligonucleotides before electrophoresis in 4% low ionic strength nondeaturing polyacrylamide gels. Arrows indicate the AP-1-specific retarded complexes. B, gel supershift experiments were done by incubating on ice the above described binding reaction mixture with 2 \( \mu \text{g} \) of anti-c-jun/AP-1 sc-44 or anti-HPV16 E7 polyclonal antibodies for 6 h prior electrophoresis. The positions of the AP-1 shifted and supershifted complexes are indicated by arrows.
calcium-treated keratinocytes (Fig. 2B).

Thus, three transcriptional regulatory domains are established: a distal enhancer with calcium responsiveness located between nt −2456 and −1272, a possible transcriptional silencer located between nt −651 and −160, and a proximal enhancer/promoter located between nt −160 and −1/+1.

Footprinting Analysis of the Human Involucrin Gene Proximal Promoter/Enhancer—The complete nucleotide sequence of the 2456-nt 5′-noncoding region revealed several putative transcriptional factor binding sites (Fig. 3A). AP-1, YY1, and TBP binding sites were located within the proximal enhancer/promoter and the putative transcriptional silencer domains (Fig. 3A). As a part of the analysis of the involucrin transcriptional regulation, DNase I footprinting assays were performed to detect nuclear factors capable of specifically interacting with these domains. Nuclear extracts from multiplying or 2 mM CaCl2-treated human keratinocytes and HeLa cells were incubated with end-labeled EcoRI-HindIII fragments from plN220 plasmid, which spans the proximal enhancer/promoter domain (Fig. 3B). The DNase I digestion patterns had several protected regions designated HP-1/HP-2 (nt −116 to −26), HP-3 (nt −139 to −119), and HP-4 (−156 to −143) separated by sites of enhanced DNase I sensitivity readily observable in both strands (Fig. 4A, black arrows). No noticeable difference was seen in the footprint patterns produced by nuclear extracts from multiplying or 2 mM CaCl2-treated keratinocytes or from HeLa cells.

HP-1 footprint includes the putative TATA box and a consensus sequence for the Sp-1 transcriptional factor 5′-GGAGGG-3′ (35). HP-2 overlaps HP-1 and is located on two putative AP-1 binding sites. The HP-3 footprint is localized over a third AP-1 binding site meanwhile HP-4 is associated to a putative Myb protein binding sequence 5′-CTGAA-3′ (6). Footprint assays of plN220 employing different amounts of a competitor oligonucleotide containing a bona fide AP-1 site from the human papillomavirus type 18 (HPV-18; Ref. 31), and nuclear extract from multiplying keratinocytes resulted in a dose-dependent competition of HP-2 and HP-3 footprints, suggesting that the nuclear factor involved is AP-1 (Fig. 4B).

AP-1 Binds to the Human Involucrin Proximal Enhancer/Promoter—Sequence analysis of the footprints produced by keratinocyte nuclear proteins indicates the presence of three potential 5′-TGAC/GTCA-3′ AP-1 binding sites coincident with HP-2 and HP-3 footprints (11, 36, 37). To examine whether the nuclear factors associated with HP-2 and HP-3 footprints indeed correspond to AP-1-related proteins, gel-shift competition assays were done with nuclear extracts from human keratinocytes cultures and with and without 2 mM CaCl2 and the EcoRI-HindIII fragment from plN220 as a probe. The complexes were efficiently competed by a 100-fold molar excess of nonlabeled wild-type AP-1 competitor oligonucleotide (AP-1) but not by a 200-fold molar excess of a mutated AP-1 (AP-1M) or adenovirus NF-1 (NF-1) binding sites (Fig. 5A and Table I). Similar results were obtained using nuclear extracts from HeLa cells (data not shown).

In addition, gel supershift experiments were performed with nuclear extracts from multiplying keratinocytes to confirm the identity of the observed AP-1-specific complexes in the plN220 fragment. A decrease in the intensity of the specific DNA-protein retarded complex was observed in the presence of a specific rabbit polyclonal anti-c-jun/AP-1 antibody with the appearance of a clear supershifted band (Fig. 5B). In contrast, a heterologus rabbit polyclonal antibody directed against the human papillomavirus type 16 E7 protein did not affect the retarded complexes (Fig. 5B). Similar results were obtained with nuclear extracts from HeLa cells and CaCl2-treated keratinocytes (data not shown). Thus, it is concluded that AP-1 is the nuclear factor from normal keratinocytes associated with the proximal 159-nt enhancer/promoter.

Footprint Analysis of the Human Involucrin Transcriptional
Silencer—The nature of the nuclear factors associated with the transcriptional silencer region found in p827CAT construct was investigated using the cloned HindIII-ApaI 624-nt fragment in the pIN630 plasmid (Fig. 3B) and nuclear extracts from multiplying or CaCl2-treated keratinocytes and HeLa cells. Footprinting analysis revealed four protected regions, H1 (nt −222 to −166), H2 (nt −287 to −235), H3 (nt −313 to −292), and H4 (nt −387 to −317), respectively (Fig. 6). The overall footprinting pattern obtained with nuclear extracts from keratinocytes with and without CaCl2 treatment was similar, although a distinct reproducible difference occurred in the upper strand H4 footprint (Fig. 6). Interestingly, the sequence protected by H4 footprint contains a sequence track homologous to binding sites for the YY1 factor (38, 39). Distinct differences between keratinocytes and HeLa cells nuclear extracts were also noticed in H3 and H4 footprints (Fig. 6). H1 and H2 footprints displayed similar patterns for all nuclear extracts employed but with slight differences in the size of the protected zone for both strands (Fig. 6). The location of the H2 footprint in a DNA segment containing a putative AP-1 site suggests that AP-1 could also interact with this region.

Differential Nuclear Factor Binding in the Involucrin Gene Putative Transcriptional Silencer—Synthetic oligonucleotides containing the H1, H2, and H3 footprints from the pIN630 plasmid (Fig. 3B and Table I) were used to identify the associated nuclear factors as well as possible cell type- and stage-specific variations in standard gel-shift assays. To facilitate analysis, the H4 footprint was spliced into three different oligonucleotides H4 2072, H4, and H4 2126 (Table I). Several retarded complexes were noticed using both multiplying and calcium-induced keratinocytes nuclear extracts. The specificity of the DNA-protein interactions was tested by preincubating the nuclear extracts with a 100-fold molar excess of unlabeled homologous probe (Fig. 7). For H1 and H4 oligonucleotides, no specific competition was noticed when using the binding sites of AP-1, NF-1, and YY1 (data not shown). H1 and H2 oligonucleotides presented single band-specific retarded complexes, suggesting that only one factor may be implicated in these interactions (Fig. 7, panels H1 and H2). The specific retarded complex from H1 displayed a 2–3-fold increase in intensity with nuclear extracts derived from CaCl2-treated keratinocytes, but no noticeable difference was found for the H2 retarded complex (Fig. 7, panels H1 and H2).

H3 and H4 oligonucleotides produced a more elaborated gel-shift pattern. H3 presented at least two specific retarded complexes which were increased in nuclear extracts from CaCl2-treated keratinocytes (Fig. 7, panel H3). H4 oligonucleotide had two specific DNA-protein complexes with either nuclear extract, suggesting the interaction of multiple nuclear factors with this sequence (Fig. 7, panel H4).

Cell type specificity was tested using nuclear extracts from HeLa cells. The similarity of the gel-shift pattern with H1 and
H2 suggests that nuclear factors are shared by HeLa cells and keratinocytes (Fig. 8, panels H1 and H2). In contrast, differences were observed between HeLa cells and keratinocytes with the H3 and H4 oligonucleotides. H3 had an extra upper DNA-Protein complex with HeLa nuclear extracts. The common complexes seem to be produced by a nuclear protein more abundant in HeLa cells than in keratinocytes (Fig. 8, panel H3). For H4 oligonucleotide, at least one DNA-Protein complex was absent from keratinocyte nuclear extracts, suggesting that in HeLa cells additional factors may interact with this region (Fig. 8, panel H4).

AP-1 and YY1 Transcriptional Factors Interact with the Human Involucrin Putative Transcriptional Silencer—As with plN220, several potential AP-1 binding sites were found within the plN630 fragment (Fig. 3A). Three of them coincide with the observed footprints H1, H2, and H3. Gel-shift competition experiments using nuclear extracts from multiplying keratinocytes demonstrated that only the H2 DNA-Protein complex is efficiently competed by a 30-fold molar excess of AP-1 homologous competitor (Fig. 9A). Similar results were obtained with HeLa and CaCl2-treated keratinocytes (data not shown). A 100-fold molar excess of either AP-1M or NF-1 competitor oligonucleotides had no effect on the H2 complex (Fig. 9A).

Gel supershift assays confirmed the AP-1 identity of the H2 complex using nuclear extracts from multiplying and CaCl2-treated keratinocytes and HeLa cells (Fig. 9A). For H4 oligonucleotide, at least one DNA-Protein complex was absent from keratinocyte nuclear extracts, suggesting that in HeLa cells additional factors may interact with this region (Fig. 8, panel H4).

Fig. 9. The H2 footprint corresponds to AP-1 transcriptional factor. A, gel-shift assays were performed using 1 ng of end-labeled H2 oligonucleotide as described in the legend to Fig. 5A (−) or with 30- and 100-fold molar excesses of the indicated competitor oligonucleotides and nuclear extracts from multiplying human keratinocytes. The arrow indicates the specific AP-1 retarded complex. B, gel supershift assays were performed as described in the legend to Fig. 8B using nuclear extracts from multiplying (Ker) and CaCl2-treated keratinocytes (Ki) and HeLa cells with the H2-end-labeled oligonucleotide in the presence of rabbit polyclonal sc-44 (anti-c-Jun/AP-1) or anti-HVPI-16E7 antibodies. The arrows show the position of the H2-AP-1 and supershifted complexes.

Fig. 10. YY1 transcriptional factor binds to the human involucrin silencer region. A, comparison of homologous YY1 sequences from different promoters. Boxes show conserved sequences. H4 2072 and H4 2126 are referred to oligonucleotides containing the 5' and 3' ends of the H4 footprint, respectively. B, YY1 interacts with the human involucrin putative transcriptional silencer. Gel-shift assays were done employing 1 ng of end-labeled H4 2072 or H4 2126 oligonucleotides (containing the putative YY1 sites from H4 footprint) and nuclear extracts from multiplying keratinocytes as described in the legend to Fig. 5A. For specific competition, 100- and 200-fold molar excess of the indicated competitor oligonucleotides was used prior electrophoresis through 4% nondenaturing low ionic strength polyacrylamide gels.
sponsible for specific involucrin transcriptional activity in normal keratinocytes. The similarity between the footprint and gel-shift patterns observed with both regions independent of mal keratinocytes. The similarity between the footprint and responsible for specific involucrin transcriptional activity in nor-
er/promoter region. Thus, the sum of the results suggests the Fra1, JunB, and JunD are the factors associated to the enhanc-
expression activates transcription from the proximal 784-nt enhancer or the transcriptional silencer regions, both being
consistent with this hypothesis, the intact 2456-nt noncoding region of the human involucrin 5'-noncoding region. Footprint sites in upper and lower DNA strands are indicated by cross-hatched boxes. The TATA box position is represented by a vertical box. Identified YY1 and AP-1 sites are shown as pentagons and hexagons, respectively. The arrow shows the direction and transcription start site. Restriction sites are provided as a reference.

Calcium-induced differentiation of transfected keratinocytes did not affect the activity of either the proximal promoter/enhancer or the transcriptional silencer regions, both being capable of interacting with AP-1. Additionally, no activity was registered with any reporter construct in fibroblasts, a cell type that contains AP-1. Furthermore, the p220CAT construct was inactive in transfected HeLa cells despite the interaction of AP-1 (data not shown), suggesting that a particular combina-
tion of AP-1 may be implicated in involucrin gene transcription. In agreement with this, Welter et al. 1995 (43) established that Fra1, JunB, and JunD are the factors associated to the enhancer/promoter region. Thus, the sum of the results suggests the existence of two different control mechanisms for involucrin gene transcription, one dependent on AP-1 activation and the other associated with calcium-dependent pathways.

Consistent with this hypothesis, the intact 2456-nt noncoding region is more efficient in the presence of 2 mM CaCl2. The enhanced activity requires the far upstream 1648 nt that includes the distal 1185-nt enhancer domain described in this work. Thus, the AP-1- and calcium-dependent involucrin regul-
atory pathways are apparently functionally and physically separable within the 5'-noncoding region.

Several putative binding sites for transcriptional factors are located within the 1185-nt distal enhancer. A detailed analysis is needed to establish the interaction and functional value of each of these factors in the context of calcium-induced differentiation.

Ying-Yang 1 or YY1 is a zinc finger protein related to the Krüppel family of transcriptional regulators of Drosophila melanogaster, with the unusual property of being able to acti-
rate or repress transcription initiation depending on the cellular context. Moreover, YY1 binding sites vary among cellular and viral promoters (44). On one hand, YY1 activates trans-
scription of c-myc (45), ribosomal proteins L30 and L32 (40), and cytochrome c oxidase genes (46) and the leaky late pro-
moter of herpes simplex (47) and the P6 promoter of B19 parvovirus (48). On the other hand, YY1 represses the regula-
tory regions from c-fos (49), the skeletal α-actin (50), human immunodeficiency virus type 1 (51), HPV-18 long control region (52), and the human cytomegalovirus major immediate early enhancer/promoter (53).

The ambivalent nature of YY1 as an activator or a silencer led to a hypothesis concerning the importance of this factor for human involucrin transcription. Although the abundance and function of YY1 in human keratinocytes are not known, the current results suggest that this factor may repress human involucrin transcription in multiply infected and calcium-treated ker-
atinocytes. Both, the 1185-nt distal enhancer and the 159-nt proximal enhancer/promoter are active, lacking the 624-nt fragment that contains the YY1 binding sites. Because YY1 physically interacts with other proteins (39, 54), it is possible that the mechanism of YY1 repression in the involucrin gene could be the association of YY1 with the Sp-1 basal transcription factor, whose putative binding site is present within the 159-nt enhancer/promoter. Accordingly, the substitution of the native involucrin TATA box with the SV40 promoter in the p610CP plasmid (that contains several Sp-1 sites in the 21 nt repeats) showed no increase in activity when compared with the control despite the presence of four AP-1 sites (Fig. 1A).

Site-directed mutagenesis experiments will be required to ver-
ify such interaction.

The association between two apparently antagonistic tran-
scriptional factors such as AP-1 and YY1 with the involucrin 5'-noncoding region resembles the epithelial-specific HPV-18 long control region, which also interacts with both factors in similar tissue-specific enhancer (AP-1) and transcriptional si-
lience (YY1) functions (31, 52). A particular combination of AP-1 containing JunB is responsible for the HPV-18 tissue-
rophism (31). Interestingly, oligonucleotides containing an AP-1 site from HPV-18 efficiently competed for the involucrin AP-1 complexes from the 159-nt promoter/enhancer. It has been shown that JunB is associated with involucrin transcrip-
tion (43). Therefore, functional association between YY1 and JunB can be proposed as a possible regulatory mechanism for epithelial expressed genes.

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