Identification of an involucrin promoter transcriptional response element with activity restricted to keratinocytes

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The involucrin proximal promoter was examined for response elements that confer cell-type specificity. Using a segment spanning positions −157 to +41, three possible response elements were identified by their protein-binding activity using DNase I footprinting. From distal to proximal, they were: an activator protein-1 (AP-1) site (previously identified) overlapping an Ets-like site; a second Ets-like site located 13 bp more proximally; and an extended region designated footprinted site A (FPA). Mutation of the distal Ets-like site had essentially no effect on the transcriptional activity in transfections, while mutation of the proximal site reduced the activity by half. FPA was shown by electrophoretic mobility-shift assay (EMSA) to be comprised of two separable binding sites, FPA1 (distal) and FPA2 (proximal). While mutation of FPA2 had only a modest effect on transcriptional activity in transient transfections, mutation of FPA1 reduced transcriptional activity to approx. 20% of that obtained with the intact promoter. Additional mutations of FPA1 indicated that the active region comprises positions −85 to −73 (GTGGTGAAACCTGT). The molecular masses of the major proteins binding to this site were shown by UV cross-linking to be approx. 40 and 50 kDa, while minor bands were observed at 80 and 110 kDa. Since the involucrin promoter exhibits much higher transcriptional activity in keratinocytes than in other cell types in transfection assays (indicating that cell type specificity of expression is retained), the comparative influence of FPA1 was examined. While mutation of the AP-1 site affected transcriptional activity similarly in all cell lines tested, mutation of FPA1 decreased activity substantially in keratinocytes, but not in NIH-3T3 and HeLa cells, evidence for a contribution to cell-type specificity of expression. Furthermore, a correlation between the sensitivity to FPA1 mutation and amount of involucrin expression in different keratinocyte cell lines was evident. EMSA showed that NIH-3T3 and HeLa cells lacked the same FPA1 DNA–protein complex as keratinocytes. However, the amount of complex formed with nuclear extracts from several keratinocyte lines did not correlate well with the level of involucrin expression. Other factors, such as differences in post-translational modification or co-activators, must account for varied transcriptional response mediated by this site among keratinocyte lines.

Key words: activator protein-1, cell-type specificity, Ets, footprinted site A, transfection specificity.

INTRODUCTION

The study of gene expression in human epidermis is anticipated to help understand, and ultimately to alleviate, many medical problems of genetic and environmental origin that affect skin. Knowledge of the normal function is a prerequisite to determining the basis for abnormalities that cause disease. Healthy epidermis is a constantly renewing epithelium, consisting of a basal layer of proliferating cells and more superficial layers of cells advancing through a program of terminal differentiation as they move outward [1]. Eventually, the cells flatten and terminally differentiate, forming the stratum corneum, the outermost layer responsible for the barrier function of skin. These corneocytes are ultimately lost when they are sloughed from the surface.

As the cells progress through their differentiation programme, they transcribe new sets of genes [2,3]. The identities of many of these genes have been determined, cDNA and genomic clones have been obtained, and the stage of differentiation at which they are expressed has been elucidated. Efforts continue to determine how this precisely timed transcription is coordinated. The existence of a cell culture system that recapitulates many stages of differentiation of keratinocytes, the major cell type in the epidermis, has made it possible to study the promoters of epidermally expressed genes *in vitro* and to determine some of the requirements for transcription. One of these genes encodes involucrin [4], a substrate for keratinocyte transglutaminase [5], a calcium-activated enzyme localized at the cell periphery where it crosslinks involucrin and several other proteins to form the cornified envelope [6,7]. Involucrin is synthesized after cells cease proliferating and move out of the basal layer. Some of the regions of the involucrin gene promoter that confer proper tissue-specific expression in transgenic animals [8–10], and are required for transcription in cell culture [11–16], have been determined. The interplay between different transcription factors that bind to these identified regions and are responsible for correct gene regulation is still being investigated. Thus far, a distal region containing an activator protein-1 (AP-1) site [12,15] flanked by an SP1 site [16], a proximal region with a second AP-1 site [12,15], and a CCAAT/enhancer binding protein (C/EBP) site [17] have been described. Other studies have pointed to the existence of a unique factor implicated in calcium responsiveness [14] and a transcriptional silencer [13]. The present study con-

Abbreviations used: AP-1, activator protein-1; BrdU, 5-bromo-2′-deoxyuridine; C/EBP, CCAAT/enhancer binding protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FPA, footprinted site A; rB, rat bladder; SCC, squamous cell carcinoma; SIK, spontaneously immortalized keratinocyte; SV40, simian virus 40.

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centrates on the identification of transcription factors that contribute to the activity of the proximal promoter and identifies a new protein-binding site that has restricted transcriptional activity in transfection assays, thus contributing to cell-specific expression of the involucrin gene.

EXPERIMENTAL

Cell culture

Keratinocytes employed were the spontaneously immortalized SIK line derived from human foreskin epidermis [18]; the human squamous cell carcinoma lines SCC4 and SCC9 [19]; and rB, a continuous cell line derived from rat bladder epithelium [20]. All cells were cultured in a 3:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, supplemented with 5% fetal bovine serum, 0.4 μg/ml of cortisol, 5 μg/ml of insulin, 5 μg/ml of transferrin, 20 pM triiodothyronine and 0.18 mM adenine. Medium for SIK cultures was also supplemented with epidermal growth factor and cholera toxin, both at 10 ng/ml. For SCC and SIK cultures, a feeder layer of lethally irradiated 3T3 cells was employed [21]. HeLa and MCF7 cell lines were cultured in the DMEM/Ham's F-12 media mixture used for keratinocytes, but without cortisol. NIH-3T3 cells were grown in DMEM containing 10% bovine serum.

Preparation of nuclear extracts

Nuclear extracts were prepared by a modification of the method of Dignam et al. [22,23]. Briefly, cultures were scraped from the dishes into isotonic PBS, collected by centrifugation at 1850 ***g*** for 5 min, then incubated in hypotonic buffer [10 mM Hepes (pH 7.9 at 4 °C), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM dithiothreitol (DTT)], and lysed by homogenization. Nuclei were pelleted by centrifugation at 3300 ***g*** for 15 min and extracted by addition of one volume of high-salt buffer [20 mM Hepes (pH 7.9) at 4 °C, 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.6 M KCl, 0.2 mM EDTA and 0.5 mM DTT] equal to the volume of nuclei. Both the hypotonic and high-salt buffers contained PMSF (0.2 mM) as a protease inhibitor, and sodium pyrophosphate (30 mM) plus sodium vanadate (50 μM) as phosphatase inhibitors. Extracts were cleared by centrifugation in a microfuge at 16000 ***g*** for 15 min at 4 °C, then stored at −80 °C.

DNase I footprinting

A proximal promoter footprinting probe covering positions −10 to −307 was prepared by PCR, subcloned and verified by sequencing. For labelling, the insert was excised with an enzyme pair leaving one 3′ and one 5′ overhang, treated with calf alkaline phosphatase, purified, then labelled with T4 polynucleotide kinase. The probe was added to a 50 μl mixture containing 100 μg of nuclear extract from post-confluent SCC9 cells, 25 mM Tris/HCl (pH 8), 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT and 1 μg of poly[d(I-C)]·poly[d(I-C)]. After a 10 min incubation at room temperature to permit binding of protein to the DNA probe, 50 μl of a 5 mM $CaCl_2$/10 mM $MgCl_2$ solution was added, followed by increasing amounts of DNase I (Promega) for 3 min. The reaction was stopped by addition of 90 μl of stop solution (0.2 M NaCl, 30 mM EDTA, 1% SDS, 100 μg/ml of yeast tRNA), then incubated for 30 min with proteinase K (100 μg/ml final concentration), extracted with phenol/chloroform (1:1, v/v) and precipitated with ethanol. Samples without protein were prepared in parallel. The products were analysed by electrophoresis on a 6% sequencing gel. Sequencing reactions were run in parallel, using a primer with the same 5′ end as the labelled end of the footprinting probe.

Electrophoretic mobility-shift assay (EMSA)

Complementary oligonucleotides spanning the regions of interest (usually 20–30 bases) were synthesized by the Protein Structure Laboratory (University of California, Davis, CA, U.S.A.), desalted and annealed. Sequences of one strand only are given in Table 1. A 1–3 base 5′ overhang was designed at each end to allow labelling by fill-in with Klenow polymerase (3′ to 5′ exo-; New England Biolabs, Beverly, MA, U. S.A). After labelling, probes were purified by centrifugation in spin columns (Chromaspin 10; ClonTech, Palo Alto, CA, U.S.A.). Nuclear extract aliquots, containing 5 μg of protein, were incubated with 200 ng of poly[d(I-C)]·poly[d(I-C)] in a buffer containing 25 mM Tris/HCl (pH 8), 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT and 0.05% Nonidet P40. Unlabelled competitors, if any, were added at this time. After 10 min at room temperature, the probe was added (10–200 fmol), and incubation was continued for an additional 10 min. The binding reaction was then loaded on to a non-denaturing 4% polyacrylamide gel. Results were obtained by autoradiography or phosphorimaging (SI model; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

When competitions with different unlabelled oligonucleotides were being quantified and compared, care was taken to ensure that the probe was present at saturating concentration. This was achieved by addition of unlabelled oligonucleotide to the radioactive probe.

The buffer composition was optimized for protein binding to DNA. Binding of protein to the footprinted site A1 (FPA1) was reduced severalfold by an increase in the concentration of KCl from 50 to 150 mM during incubation. In contrast, FPA2 binding was affected little by this variable. Addition of $CaCl_2$ (1 mM), $MnCl_2$ (1 mM) or $ZnCl_2$ (0.5 nM) had no effect on binding activity, while inclusion of Nonidet P40 at 0.05% increased binding nearly twofold.

Transient transfections

Cells were plated 1–2 days before transfection at $0.5–2 \times 10^5$ cells per 3.5 cm culture well or $2–3 \times 10^5$ cells per 6 cm dish. Transfections in 6-well culture plates were done using calcium phosphate/DNA co-precipitation with 5 μg of reporter DNA and 25 ng of pRL-cytomegalovirus per 3.5 cm well. The total amount of DNA was brought to 10 μg per 3.5 cm culture well with the promoterless pGL3-vector. Promoterless pGL3 alone had little detectable luciferase activity above machine background. For transfection in 6 cm dishes, twice the amount of each plasmid was used. The precipitate was added to the cells and left overnight, except for transfection of NIH-3T3 cells, which were incubated for 4–6 h only, followed by a 3 min shock with 15% glycerol in isotonic PBS. After removal of the precipitate, fresh culture medium was added and the cultures were incubated for 2–3 additional days before harvesting. Firefly and renilla luciferase activities were measured in the cell extracts using the dual luciferase kit from Promega. The renilla luciferase values were used to normalize for dish to dish variation in transfection efficiency.

The involucrin promoter used for transfections was obtained from genomic subclone pλI-3H6B [4] by PCR. Restriction sites were included at the 5′ ends of the primers for convenient subcloning into the pGL3-basic luciferase reporter vector (Promega). The largest promoter tested extended to position −2466, terminating just 3′ to the *Hin*dIII site. The 3′ end of all promoter constructs was at position +41, which is just 5′ of the

Table 1 Sequences of oligonucleotides used

For oligonucleotides used in mobility shift, the sequence of the two strands is given and, for mutants, only the changed bases are shown. Most mutagenesis reactions require two oligonucleotides. Both strands are given only when they are not completely overlapping. S indicates sense strand (same strand as the RNA transcript) and AS indicates the antisense strand. Two mutants (FPA1 M1 and FPA2) were produced by a method that required only one nucleotide. The mutated bases are underlined.

MOBILITY SHIFT

AP1-124	GCCTGTGGTGAGTCAGGAAGGGG
FPA	TAGAGTGGTGAAACCTGTCCATCACCT
FPA1	TAGAGTGGTGAAACCTGTCCA
M1	GTCC
M(GA)	TC
M(GT-5′)	TG
M(GG)	TT
M(TG)	GT
M(AA)	CC
M(AC)	CA
M(CT)	AG
M(GT-3′)	TG
M(CC)	AA
FPA2	AACCTGTCCATCACCTTC
M1	TGGT
AP2	AACTGCAGCCTCGGGCATAGAGGCT

MUTAGENESIS

FPA1 M1		5′	CCAACTAGAGTGG<u>GTCC</u>ACCTGTCCATCACC 3′
(GT)	S	5′	GTTGACCAACTAGA<u>TG</u>GGTGAAACCTGTCC 3′
(GG)	S	5′	GACCAACTAGAGT<u>TT</u>TGAAACCTGTCCA 3′
(TG)	S	5′	AGAGTGG<u>GT</u>AAACCTGT 3′
	AS	5′	TGGACAGGTTT<u>AC</u>CCACT 3′
(AA)	S	5′	AGAGTGGTG<u>CC</u>ACCTGT 3′
	AS	5′	TGGACAGGT<u>GG</u>CACCACT 3′
(AC)	S	5′	AGAGTGGTGAA<u>CA</u>CTGT 3′
	AS	5′	TGGACAG<u>TG</u>TTCACCACT 3′
(CT)	S	5′	TAGAGTGGTGAAAC<u>AG</u>GTCCA 3′
(GT)	S	5′	GAGTGGTGAAACCT<u>TG</u>CCATCACCTTCAAC 3′
(CC)	S	5′	GTGGTGAAACCTGT<u>AA</u>ATCACCTTCAACC 3′
FPA2		5′	GGTGAAACCTGTCC<u>TGGT</u>CCTTCAACCTGG 3′
ETS-106	S	5′	CAGGAAGGGGTTAGA<u>TTC</u>AGTTGACCAACTAGAGTGG 3′
	AS	5′	TCAACT<u>GAA</u>TCTAACCCCTTCCTG 3′
ETS-119	S	5′	TGAGTCAG<u>TTTT</u>GGGTTAGAGGAAGTTGACCA 3′
	AS	5′	CTCTAACCC<u>AAAA</u>CTGACTCACCACAGGCATCT 3′

intron. The proximal promoter region of all clones used in transfections was sequenced. Except for Ets and AP-1 site mutants, two independent clones were tested with similar results.

Mutagenesis

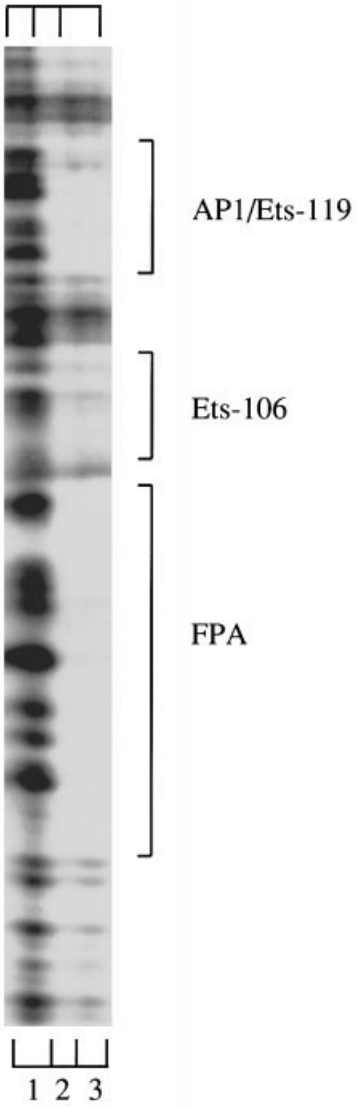
In most cases, mutagenesis of sites in the involucrin promoter was done using the Stratagene QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). This PCR-based method utilizes two mutant primers (Table 1) and the high fidelity *Pfu* polymerase, with selection against the original template by cutting with *Dpn*I. Mutagenesis was done in the pGL3-vector. After sequencing to verify the mutations, the promoter was excised and subcloned back into the pGL3-vector to avoid any possible mutation in the luciferase gene and 3′ processing signals.

Alternatively, FPA1(M1) and FPA2(M2) mutants were made by the phosphorothioate technique [24,25], using the Amersham Sculptor *in vitro* mutagenesis kit. The involucrin promoter was subcloned into M13mp18. Single-stranded mutant primer (Table 1) was annealed and extended by T7 polymerase in the presence of deoxycytidine 5′-[α-thio]triphosphate. The template strand was removed by nicking with *Nci*I, followed by digestion with exonuclease III. After re-polymerization with DNA polymerase I, which introduced the mutation into the second strand, the mutant DNA was transformed into XLI Blue-competent bacteria (Stratagene). Plaques encoding mutant DNA were identified by DNA sequencing and the involucrin promoter was subcloned back into pGL3.

UV cross-linking

5-Bromo-2′-deoxyuridine (BrdU)-substituted probes were synthesized from an oligonucleotide containing the FPA1 (5′-TAGAGTGGTGAAACCTGTCCAGCTGGCCTCAGCTCC-3′) by extension of a primer (5′-GGAGCTGAGGCCAGC-3′) using Klenow polymerase (3′ to 5′ exo-) in the presence of [^{32}P]dCTP and BrdU triphosphate. Because the FPA1 is closely

flanked by other protein binding sites, the primer region is an artificial one that was shown by EMSA not to bind any proteins in keratinocyte nuclear extracts. Furthermore, the BrdU-substituted probe was shown to bind the same proteins in EMSA as the original FPA1 probe, based on identical shifted mobility and on competition with unlabelled FPA1 oligonucleotide. Binding reactions were performed as described for EMSA, the reaction was then loaded on to a 1% low-melt agarose gel. After electrophoresis, the gel was irradiated at 4 °C for 45 min at a 5 cm distance from a 302 nm transilluminator (Spectroline; Spectronics Corp., Westbury, NY, U.S.A.). The gel was sliced into 0.5 cm segments and the shifted band identified by Cerenkov counting. After addition of one-quarter volume of sample buffer [0.25 M Tris/HCl (pH 6.8), 4% SDS, 4% β-mercaptoethanol], the gel slice was melted and electrophoresed on a SDS/polyacrylamide gel (10%). Radioactive bands were located by autoradiography and relative molecular masses estimated by comparison with stained protein markers.



Sequences of Footprinted Regions

AP1/Ets-119	TGTGGTGAGTCAGGAAGGGG
Ets-106	GAGGAAGTTGA
FPA	TAGAGTGGTGAAACCTGTCCATCACCTTCA

Figure 1 DNase I footprinting of the involucrin proximal promoter

The region shown spans nucleotides −50 (bottom) to −140 (top). Lane 1 shows DNase I digestion of the probe (0.05 unit of DNase I) in the absence of protein. Lanes 2 and 3 show the digestion pattern in the presence of 100 μg of SCC9 nuclear extract with 0.15 and 0.45 unit of DNase I. Footprinted regions are indicated below the gel, for which sequences are given. Underlined bases conform to binding sites for known transcription factors. Sites have been named for these known factors. Putative Ets sites are distinguished by including the position of the most 5′ base of the binding site. The third site is named FPA, for footprinted site A, since the proteins binding to it are as yet unidentified.

RESULTS

Footprinting of the involucrin proximal promoter

Although by itself the proximal promoter (−157 to +41 bp) has low transcriptional activity compared with the 2.4 kb promoter, mutations in this region can substantially reduce the activity of larger promoter constructs. For example, mutation of an AP-1 site (AP-1-124, designated by the position of the 5′ base of the consensus sequence) reduces activity of the 2466 bp promoter contruct to 30–40% of that obtained with the native construct [12,15]. To determine whether other important transcription-factor-binding sites are found in this region, DNase I footprinting was undertaken using nuclear extracts from SCC9 cells. This keratinocyte line was chosen because it contains the highest

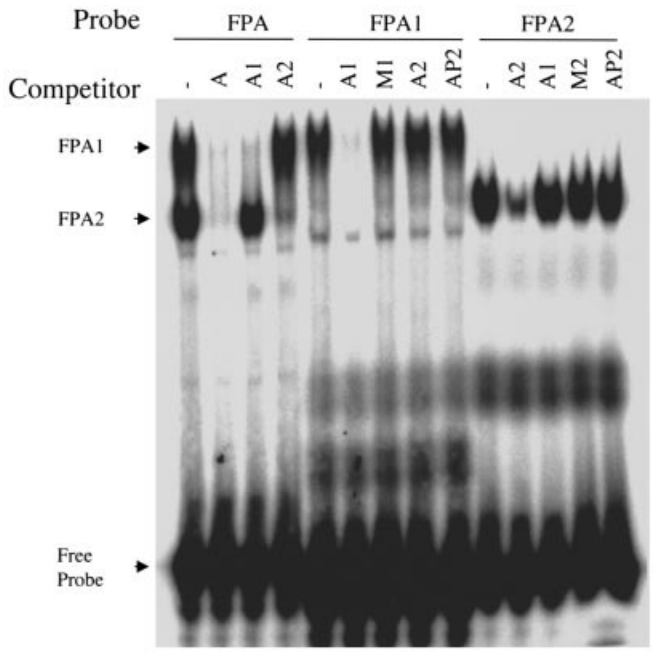


Figure 2 EMSA of proteins binding to FPA

SCC9 nuclear extracts (5 μg of protein) were incubated with the indicated probes (10 fmole). Competitors (100-fold molar excess) used were: -, none; A, entire footprinted region, FPA; A1, 5′ end of site FPA; A2, 3′ end of FPA; M1, 4 bp mutation of FPA1; M2, 4 bp mutation of FPA2; AP-2, derived from involucrin promoter, positions −629 to −653. Sequences of these oligonucleotides and the probes are listed in Table 1. Arrowheads indicate the complexes preferentially competed by the 5′ (FPA1) or 3′ (FPA2) ends of the FPA and, at the bottom of the gel, the unbound probe.

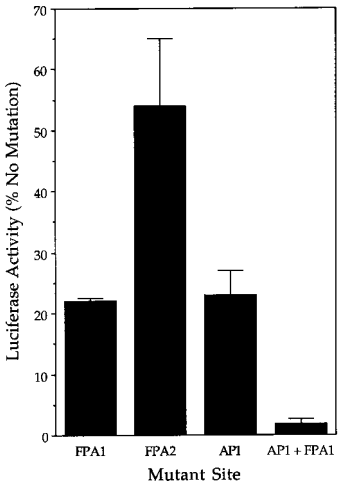


Figure 3 Transcriptional activity of proximal promoter mutants in transient transfections

The SIK line was transfected with plasmids containing the involucrin promoter (−2466 to +41) cloned upstream of the luciferase gene in plasmid pGL3-basic. The sites mutated, displayed along the horizontal axis, are FPA1 (mutant M1, Table 1), FPA2 (mutant M2, Table 1), AP-1 (mutant AP-1-1m, [15]) and AP-1 + FPA1 (contains both the FPA1 and AP-1 mutations). Activities of the mutant promoters, normalized to a promoter of the same length lacking mutations (100%), are displayed on the vertical axis ($n = 3$; means ± S.D.).

amount of proteins binding to this region among the keratinocyte lines employed. This is particularly important for footprinting, since the footprint is evident only if all of the probe is bound. Extracts were prepared from post-confluent cells, a condition permitting expression of substantial amounts of involucrin mRNA and thus proteins required for its transcription. As shown in Figure 1, footprinting revealed several protein-binding regions: a previously identified AP-1 site; two Ets-like sites, one (Ets-119) overlapping the AP-1 site and the other 11 bp 3′ of the AP-1 site (footprinted site FPB, designated Ets-106); and an extended site (footprinted site FPA) covering approx. 30 bp and located approx. 30 bp 3′ from the AP-1 site. The roles of these binding sites in involucrin gene expression were explored further by EMSA and transcriptional assays of site-directed mutants.

FPA contains two separable protein binding sites

When an FPA probe was incubated with nuclear extract from keratinocytes and analysed by PAGE, two major complexes were observed (Figure 2). Using shorter probes, the lower mobility complex was assigned to the 5′ end of the footprinted region (designated FPA1), and the higher mobility complex to the 3′ end (designated FPA2). Binding of each of the shorter probes was competed with an excess of the same unlabelled oligonucleotide, but not with one specific for the other site or with a mutant or unrelated oligonucleotide. The results are illustrated in Figure 2 with SCC9 extracts because, among the various keratinocyte nuclear extracts tested, these exhibited the most binding activity to FPA1.

Transcriptional-activating activities of the FPA1 and FPA2 elements were assessed by site-directed mutagenesis and transfection. Since SCC9 cells transfect poorly under our standard conditions, another human keratinocyte line (the SIK line) was used for these experiments. SIK cells contain FPA-binding activity that is indistinguishable from that of SCC9 cells in

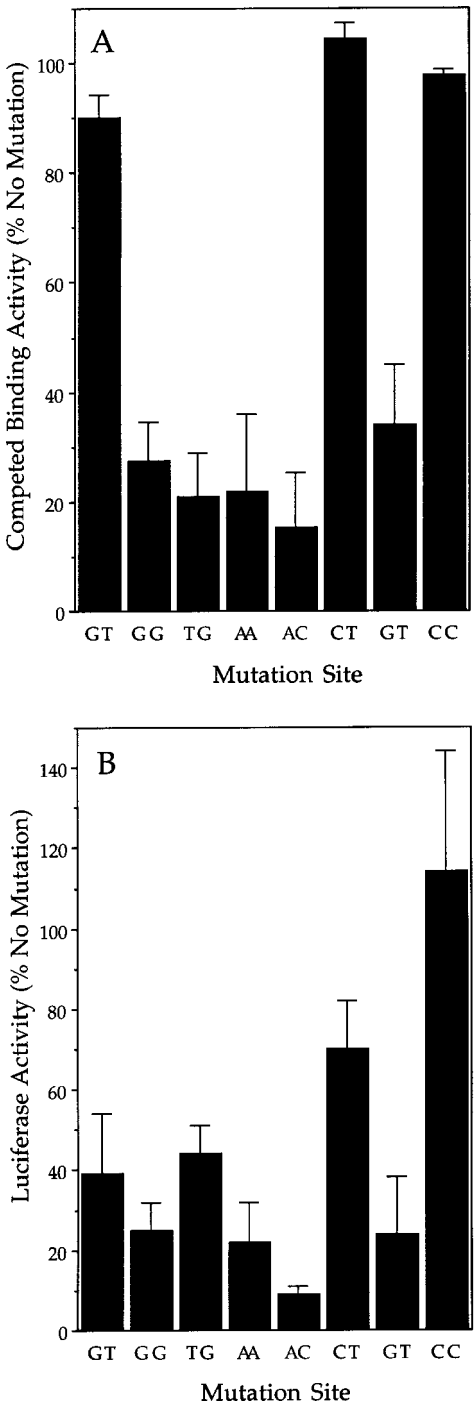


Figure 4 Effect of different FPA1 mutations on binding and transcriptional activities

Mutations of 2 bp were made across the FPA1 at the sites indicated along the horizontal axis. The sequence of the FPA1 without mutation is shown, and the transversion mutations introduced are listed in Table 1. (**A**) Effect of substitutions at each site on the abilities to bind protein in SCC9 nuclear extracts. Relative binding activities were estimated from the amount of specific binding of the FPA1 probe competed by a 10-fold excess of mutant oligonucleotide, expressed as a percentage of that competed by a similar amount of unlabelled native FPA1 oligonucleotide. Probes were used at a concentration saturating binding. (**B**) Effect of introducing the same mutations into the involucrin promoter (−2466 to +41) in the pGL3-vector and assaying transcriptional activity by transient transfection into SIK cells. Activities are expressed as a percentage of that obtained using the same construct without mutations ($n \geqslant 3$; means ± S.D.).

Table 2 Competition for FPA1 binding

An FPA1-site probe was incubated with SCC9 nuclear extracts with or without competitor double-stranded oligonucleotides and assayed by EMSA. One strand only of the oligonucleotides is shown. Values are residual FPA1 binding after competition expressed as % of binding obtained in the absence of competitor. 10× and 100× refer to fold excess of competitor over probe which was used at a concentration sufficient for saturation of binding.

Site	Sequence	Residual binding	
		10×	100×
FPA1	TAGAGTGGTGAAACCTGTCCA	5	1
AP1	GCCTGTGGTGAGTCAGGAAGGGG	56	28
CRE	GTCATGGGATATGAGCTCATCCTTATTATG	65	52
VDRE (DR3)	TAGCTCCAGGTCACACAGGTCAAGGAATATTG	30	11
T3RE (DR4)	TAGCTCCAGGTCAACACAGGTCAAGGAATATTG	67	74
RARE (βRARE)	TATGCGAGTGAACTTTCGGTGAACCCTTCCTG	64	68
OCT	GTCTCATGAATATGCAAATCAGGGATCCCCTT	52	12
NFAT	AGCTCATGGGTTTCTCCACCAAGG	74	38
KRF-1*	ATTGCTTGCATAACTATATCCACTCCCTAAGTAAT	64	18
C/EBP	GTGCAGATTGCGCAATCTGCA	52	39

* HPV-18 binding site [34].

EMSA, making it an appropriate alternative. Figure 3 shows that FPA1 contributes substantially to the activity of the proximal promoter. In the context of the largest promoter available (2466 bp), mutating this site reduced transcriptional activity to $< 25\%$ of that given by the native sequence. By this test, the importance of the site was equivalent to that of the proximal AP-1 site, mutation of which is shown for comparison. Mutation of both sites virtually prevented transcription, giving approx. 2% of that by the native sequence. By contrast, FPA2 was less critical, judging from reduction in transcriptional activity to 50–60 % of the native level by its mutation. In smaller (157 bp) promoter constructs (results not shown), mutation of the FPA1 and AP-1 sites had nearly the same magnitude of effect as in the longer constructs. These elements thus account for the majority of the activity of this region.

Characterizing the FPA1

To determine which nucleotides are important for FPA1 binding and activity, a series of 2 bp transversion mutations were made across this region and tested for their relative abilities to compete in EMSA (Figure 4A) and for transactivating activity in transfections (Figure 4B). These results delineated the site as GTGGTGAAACCTGT. Except for the two 5′ bases, which appeared unimportant under the conditions of the binding assay but weakened transcriptional activity, mutations affected binding roughly in parallel with their effects on promoter activity.

Although the FPA1 has weak sequence similarity to several known transcription-factor-binding sites, oligonucleotides containing consensus sites for the latter competed only very weakly (Table 2). Of the eight sites chosen to test, the best competitor was a vitamin D receptor consensus-binding site, but 10-fold more of this response element was required to reduce binding to the level of a given amount of FPA1 oligonucleotide. These results suggest that the protein binding to this site has not yet been identified.

The apparent molecular mass of the protein(s) binding to the FPA1 was estimated by SDS/PAGE after UV cross-linking to a BrdU-substituted probe. This was done both by cross-linking of the protein–DNA complex in solution (results not shown), or by in-gel cross-linking after mobility-shift (Figure 5), with similar results. Two major bands of approx. 40 kDa and 50 kDa were observed, along with two minor bands of higher molecular mass (80 and 110 kDa). The latter may represent dimers of the two lower molecular mass proteins.

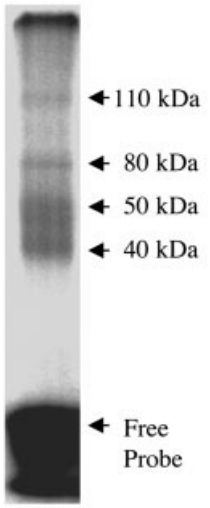


Figure 5 SDS/PAGE of protein cross-linked to a BrdU-substituted FPA1 probe

Estimated molecular masses of the four major complexes are indicated to the right of the lane.

Role of Ets-like sites in involucrin gene transcription

Two Ets-like sites located 3′ of the AP-1 site were detected by footprinting. One of these, Ets-119, partially overlaps the AP-1 site and the other is located at -99 to -106. That this is a common motif in several genes expressed in the outer layers of the epidermis [26–30] suggested they may be transcriptionally important. When these sites were mutated separately and tested within the context of the -2466 promoter, one of them, Ets-119, had little effect ($139 \pm 14\%$ of control), while the other, Ets-106,

Table 3 Comparison of involucrin and SV40 promoters in transient transfections

The involucrin promoter (−2466 to +41 in the pGL3-basic vector) and the pGL3-control vector, which contains the SV40 promoter and enhancer sequences, were transiently transfected into the indicated cell lines. Results show means ± S.D. of the ratio of activities obtained from 3 or more experiments. For ease of comparison, the last column shows the fold differences after normalizing to 1 for HeLa cells.

Cell line	Involucrin/SV40	Fold difference
rB	6.6 ± 2.1	1375
SIK	0.29 ± 0.008	59
SCC4	0.052 ± 0.012	11
MCF7	0.0125 ± 0.005	2.6
NIH 3T3	0.0062 ± 0.0019	1.3
HeLa	0.0048 ± 0.0035	1

reduced promoter activity by approximately half (56 ± 7% of control). Mutation of both Ets-106 and the AP-1 site decreased the activity more than from each individually, to 21 ± 1% of control. Insertion of five extra bases between Ets-106 and the AP-1 site had no effect on promoter activity (94 ± 15% of the activity of the promoter with no mutation).

Cell-type specificity of the involucrin promoter transcriptional activity in transient transfections

To determine how well the cell specificity of the involucrin promoter is maintained in transient transfection assays, involucrin promoter activity was compared with that of the simian virus 40 (SV40) promoter/enhancer in several cell lines (Table 3). Of all the cell lines tested, the involucrin promoter exhibited the highest relative activities in rB and SIK cells. Both of these cell lines express easily detectable amounts of involucrin mRNA. SCC4 cells, a cell line that expresses only trace amounts of involucrin, had the next highest relative activity, approx. 20% of that in SIK cells, while the involucrin promoter was approx. 100-fold less active than the SV40 promoter in the NIH-3T3, HeLa and MCF7 cell lines, which are also negative for involucrin expression. These results extend those of earlier reports using normal human epidermal cells in serum-free medium [12,13] and establish the rB and SIK lines as appropriate models for analysis of involucrin promoter activity.

The possibility that some promoter elements confer transcriptional activity only in keratinocytes was investigated by transfecting promoter deletions and mutations into different cell lines (Figure 6). In each case, the activity was expressed relative to the activity of the intact 2466 bp promoter. In all of the cell lines tested, mutation of the proximal AP-1 site decreased the activity of the involucrin promoter, and this was not correlated with endogenous involucrin mRNA levels. In contrast, mutation of the FPA1 affected transcriptional activity only in involucrin-expressing cells. The magnitude of the decrease was inversely correlated with involucrin expression levels; the rB and SIK cell lines were the most sensitive, NIH-3T3 and HeLa were unaffected, and SCC4 cells were intermediate. Finally, mutation of Ets-106 affected promoter activity in human, but not rodent cell lines and was not correlated with involucrin expression, while mutation of Ets-119 had little effect in any of the cell lines tested.

These transfection experiments revealed a correlation of sensitivity to mutation of the FPA1 with expression of the endogenous involucrin gene. To determine whether the correlation

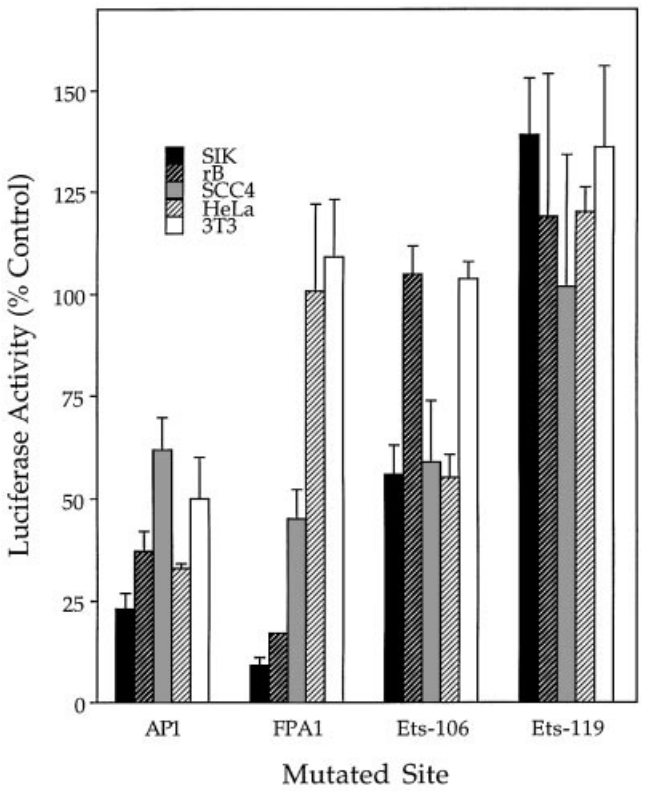


Figure 6 Effect of mutation of sites in the involucrin proximal promoter on transcriptional activities in different cell lines

The mutated sites, displayed along the horizontal axis, are AP-1 (AP-1-1m, [15]); FPA1 [M(AC)]; Ets-106 and Ets-119 (sequences of the latter three mutants shown in Table 1). Involucrin promoter constructs (−2466 to +41 in pGL3) containing the indicated mutations were transiently transfected into the various cell lines as shown. Luciferase activities are expressed as a percentage of that obtained by transfecting in parallel the same involucrin promoter construct with no mutation.

reflected the relative amounts of protein binding to the FPA1, EMSA were performed using nuclear extracts from the various lines (Figure 7). All the keratinocyte lines displayed binding activity with the FPA1 probe, but NIH-3T3 extracts lacked binding activity altogether (Figure 7A, lane 6), and HeLa extracts gave a complex with increased mobility (Figure 7A, lane 5). However, among the various keratinocytes, the amount of FPA1-binding activity did not correlate with involucrin expression. The SIK and rB cell lines contained higher amounts of involucrin mRNA than SCC9 and SCC4 cells, but showed less binding activity. Other factors, such as differences in post-translational modificaton or co-activators, could account for differences in transactivation. By contrast, all the nuclear extracts produced substantial binding to the FPA2 probe (Figure 7B), although the mobility of the complex was slightly increased when extracts from rodent cell lines were used.

DISCUSSION

In transfection experiments, sequential 5′ deletions of the involucrin promoter revealed easily detectable residual activity in the proximal promoter. Deletion of DNA between −160 and −40 resulted in a large drop in transcriptional activity, between 5- and 10-fold [12,15]. An AP-1 site, located at −124, contributes

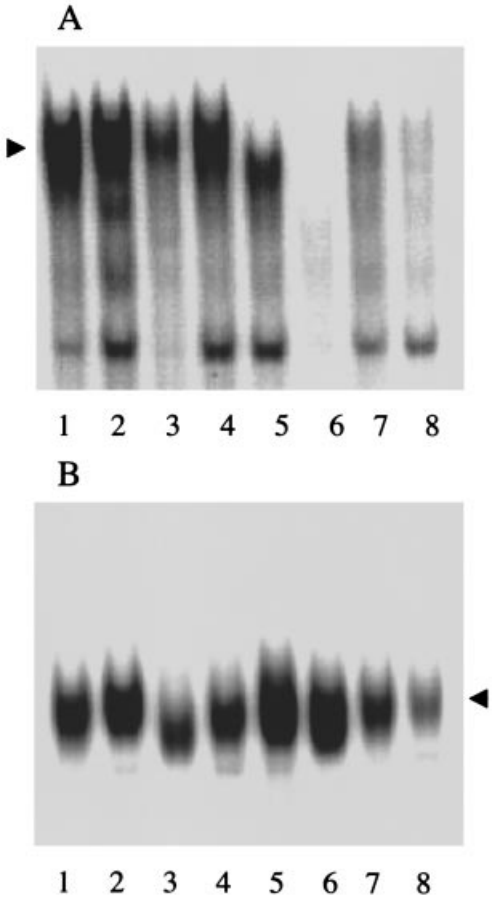


Figure 7 DNA–protein complexes formed with FPA probes in different cell lines

EMSA was performed using FPA1 (**A**) and FPA2 (**B**) probes. Nuclear protein (5 μg) extracted from various cell lines was employed as indicated: lane 1, preconfluent SCC9 (little involucrin mRNA); lane 2, two-week post-confluent SCC9 (greatly elevated levels of involucrin mRNA); lane 3, rB cells (high levels of involucrin mRNA); lane 4, SCC4 (very low levels of involucrin mRNA); lane 5, HeLa (no involucrin mRNA); lane 6, NIH-3T3 (no involucrin mRNA); lane 7, confluent SIK (low involucrin mRNA levels); lane 8, post-confluent SIK (high involucrin mRNA levels). The arrowhead indicates the mobility of the FPA1 (**A**) or FPA2 (**B**) complex.

to the activity, but does not entirely account for it, since mutation of that site did not decrease activity to the level of that conferred by the promoter containing only the 40 bp upstream of the transcription initiation site. Earlier investigations have suggested the presence of other potentially important transcription factor binding sites [13]. These were attributed to additional AP-1 sites, a Myb site and a complex covering the TATA box, and an SP1-like site; however, the biological relevance of these sites has not been established and the proteins binding have not been well characterized. Although the present footprinting results are similar to that study, some of the footprinted regions are shifted from those reported and centre over an Ets-like site at -106 and an extended region from -90 to -60 attributable to two separable DNA–protein complexes. One of these, FPA1, corresponds to a putative AP-1-like site already described, but present EMSA show that this site was not competed preferentially with an oligonucleotide containing a consensus AP-1 site, suggesting a different protein composition. Cross-linking experiments revealed bound proteins of 40 and 50 kDa with minor components at 80 and 110 kDa. Although some of these bands are of the correct size to be of the fos or jun families, at least one component must be different to account for the altered site recognition.

Recently a C/EBP site located at -144 to -135 has been shown to be required for induction of involucrin gene transcription by phorbol esters [17]. This site was not footprinted with extracts in present experiments, possibly because they were not from phorbol ester-treated cultures. FPA1 bears a resemblance to some reported C/EBP sites, most notably nucleotides 7451–7459 in HPV 11 [31], but since a commercial C/EBP consensus oligonucleotide competed only poorly for FPA1 binding, another factor must bind to the latter site. The FPA2 sequence resembles CCAAT-binding elements in sequence and location (approx. -70 bp) and is abundant in nuclear extracts of every cell culture line tested (results not shown). While we have determined that an oligonucleotide containing a consensus nuclear factor 1 site does not compete (results not shown), other possible CCAAT-binding protein candidates have not yet been explored, since mutation of this site had only a small effect on promoter activity.

FPA1 was shown to be functionally important, since mutation decreased promoter activity to approx. 20% of that obtained with the intact promoter. Mutation of both the FPA1 and AP-1 sites reduced the activity considerably more than mutating either alone. Although mutation of the AP-1 site reduced transcription similarly in all cell lines tested, including those that do not express the involucrin gene, mutation of FPA1 was effective only in keratinocytes. These results suggest that FPA1 may contribute to cell-specific transcription *in vivo*. Since a transgene containing a distal-enhancer region, but lacking the FPA1, still exhibited proper tissue-specific expression [10], this proximal element is not entirely essential. One possibility is that binding of an as yet unidentified protein to FPA1 increases the overall level of transcription in keratinocytes, a feature that is difficult to assess in transgenic experiments due to differences in copy number and influences of the integration site on transcriptional activity. Alternatively, this site may accelerate the onset of involucrin gene expression, which was delayed in transgenic animals lacking this region. Conservation of the FPA1 between humans and mice [32] also supports its potential importance.

In addition to a plethora of AP-1 sites, Ets-like sites are a recurring motif in the proximal promoters of genes expressed in keratinocytes. In some of these genes, mutation of an Ets-like site resulted in a large decrease in activity [27] or loss of Ca^{2+} [28,30] or phorbol ester [30] responsiveness. Recent cloning of an epithelial-specific Ets protein [30,33] supports the idea that these sites play a special role in keratinocyte transcription. The footprinting of two Ets-like sites in the human involucrin proximal promoter, and conservation of one of these sites in the mouse promoter [32], suggested a role in transcription of this gene also. Contrary to expectation, mutation of one of these sites (Ets-119) had little effect on promoter activity, while mutation of the other (Ets-106) resulted in only a modest decrease, to approximately half that of the intact promoter, in human keratinocyte cell lines, and no effect in a rat keratinocyte cell line. Although one or both of these sites could contribute in some way to Ca^{2+} or phorbol ester responsiveness, the sites responsible for this regulation have been mapped elsewhere. Ca^{2+} responsiveness has been attributed to a distal element [13,14] and phorbol ester responsiveness relies primarily on a C/EBP site at -144 [17] and the AP-1 site at -124 [12]. Alternatively, either or both of the Ets sites could be involved in regulation by some as yet unidentified agent.

In summary, two new protein binding sites in the involucrin promoter have been identified, one (FPA1) contributing substantially to the transcriptional activity of the promoter. The base pairs comprising the site have been delineated by showing effects on both protein binding and transcriptional activity of mutations across the footprinted region, and molecular masses of proteins binding to the site have been determined by UV cross-linking. Possible binding by known transcription factors that have similar binding sequence preferences has ruled out any of these proteins as candidates and strongly suggests we have identified a binding site for a novel transcription factor. The FPA1-response element has activity limited to keratinocytes, in contrast with a nearby AP-1 site that has transcriptional activity in all cell lines tested. This, and the observation that EMSA showed that keratinocytes produce a unique protein–DNA complex using the FPA1 as probe, point to a role for this transcription factor in cell-specific transcription of the involucrin gene.

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