Activator Protein 1 Activity Is Involved in the Regulation of the Cell Type-specific Expression from the Proximal Promoter of the Human Profilaggrin Gene*

(Received for publication, November 17, 1995, and in revised form, June 21, 1996)

Shyh-Ing Jang, Peter M. Steinert, and Nedialka G. Markova‡

From the Laboratory of Skin Biology, NIAMS, National Institutes of Health, Bethesda, Maryland 20892-2755

The human profilaggrin gene is expressed in the granular layer during the late stages of terminal differentiation of the epidermis. In vitro transcription experiments show that the abundance of the mRNA and the specificity of the expression are regulated primarily at the level of transcription. We found that the 5′-flanking sequences control the transcription in a keratinocyte-specific mode and that as little as 116 base pairs preceding the mRNA initiation site is sufficient to restrict the transcription to epidermal cells in vitro. This specificity depends critically on the presence of an activator protein 1 (AP1) motif at position −77. Binding of c-Jun/c-Fos heterodimers to this sequence confers high levels of expression to the reporter constructs in cultured epidermal keratinocytes, while having little effect in HeLa cells. The transactivating properties of c-Jun are essential in this process. On the other hand, JunB and JunD, which are involved in transactivating the transcription of earlier epidermal differentiation markers, control profilaggrin expression through a pathway which does not depend on a direct binding at the AP1 site and is not cell-type specific. These data indicate that AP1 factors are involved in a complex, multipathway regulation of the profilaggrin gene expression.

The terminal differentiation of epidermal keratinocytes is a result of a tightly regulated program. Triggered by an unknown mechanism, keratinocytes become committed to terminal differentiation, cease to proliferate, leave the basal layer and progressively differentiate as they migrate through the spinous, granular, and the cornified layers of the epidermis (for reviews, see Refs. 1 and 2). The morphological and biochemical changes, characteristic for each stage of differentiation, reflect sequential expression of specific structural proteins that are unique to the epidermis (3–5). These include the keratin proteins of the intermediate filaments (6), the intermediate filament-associated protein profilaggrin (see Resing and Dale (4) and references therein) and cell envelope proteins such as involucrin (7) and loricrin (8, 9). Ultimately, activation of transglutaminase(s) leads to a covalent cross-linking of the envelope proteins and the formation of the protective stratum corneum (reviewed in Ref. 5).

Although in some cases post-transcriptional control exists (10, 11), the regulation of keratin and intermediate filament-associated protein gene expression appears to be achieved primarily at the level of transcription (12–17). The abundance of the transcripts for the basal cell keratins K5 and K14 is markedly reduced in the first spinous layer at the time when the induction of keratins K1 and K10 mRNA takes place (18, 19). Similarly, the induction of mRNA for the late differentiation markers profilaggrin and loricrin is coupled to reduced expression of transcripts for the early markers K1 and K10 (16, 17). The proper sequence of these events is ensured by several distinct mechanisms: indirect effects of cellular-specific differentiation programs that probably affect transcription factor levels (20); the chromatin structure of the genes at different stages of differentiation (for a review, see Oshima (21)) and their state of methylation (e.g. see Thorey et al. (22)); and direct modulation of transcription in specific cell types in response to specific extracellular signals such as calcium, retinoids, and growth factors (for reviews, see Blumenberg (2) and Fuchs (20) and the references therein).

In recent years a continued identification of regulatory sequences and transregulating proteins has helped to begin defining the individual components involved in the expression of endogenous and viral genes in keratinocytes (see Refs. 2, 20, and 21 and the references therein). Variations in the arrangement of binding sites for ubiquitously expressed transcription factors, such as Sp1, AP2, NF-κB, AP1, Oct1 create the potential for formation of unique nucleoprotein complexes that may contribute to the specificity and/or the level of expression (2, 23–32). In some cases the DNA recognition motifs do not correspond to any described target sequences for known transcription factors and may prove to be unique to the epidermal genes (e.g. see Refs. 25, 33, and 34). In several instances the factors are known and include keratinocyte-specific proteins such as KTF1/AP2 (35–37) and KTP1 (38), which are similar to the well characterized transcription factor AP2, the POU-domain proteins skn-1a/i and epoc-1 (39, 40), or as yet uncharacterized keratinocyte-specific transcription factors such as proteins 1 and 2 of Byrne and Fuchs (24) and KRF-1 (41).

The aim of this work is to explore the mechanisms involved in controlling the expression of the human profilaggrin gene. Human profilaggrin is synthesized in the late stages of epidermal differentiation from a large mRNA (42). Recently the organization of the profilaggrin primary transcript was characterized and the position of the transcription start site was determined (11, 43). In this study we show that the expression of the profilaggrin gene is regulated primarily at the level of transcription. The sequences 5′ to the transcription initiation site play an essential role in this regulation. Increasing por-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence should be addressed. Present address: The Living Skin Bank, Dept. of Oral Biology and Pathology, School of Dental Medicine, SUNY at Stony Brook, Westchester Hall, Rm. 105, Stony Brook, NY 11794-8702. Tel.: 516-632-2947; Fax: 516-632-9707.

1 The abbreviations used are: AP1, activator protein 1; TAM, transactivating mutant; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde phosphate dehydrogenase; NHEK, normal human epidermal keratinocytes; PCR, polymerase chain reaction.
Regulation of Profilaggrin Expression

Amplified fragment of the ubiquitously expressed human transcripts were precipitated twice and equal amounts of radioactivity for 30 min at 37°C, and then with proteinase K for 30 min at 37°C. The reaction mixture was incubated in a buffer containing 10 mM HEPES (pH 7.9), 70 mM KCl, 7 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 200 units/ml RNasin (Promega, Madison, WI), 500 mM ATP, UTP, and CTP and 100 μCi [α-32P]GTP (3000 Ci/mmole, DuPont NEN) for 30 min at 30°C. Labeled transcripts were digested with 10 units of RNase-free DNase I (Boehringer Mannheim) for 30 min at 37°C, and then with protease K for 30 min at 37°C. The transcripts were precipitated twice and equal amounts of radioactivity were hybridized for 4 days at 50°C to Zeta-probe (Bio-Rad) immobilized plasmids as suggested by the manufacturer. The filters were exposed in a PhosphorImager (Molecular Dynamics) for 3 days and scanned.

Nuclear Run-on Analysis—Nuclei were isolated from cultured cells as described previously (17). The in vitro elongation of the nascent transcripts was performed in 25-μl reactions. Nuclei (15 μg) were incubated in a buffer containing 10 mM HEPES (pH 7.9), 70 mM KCl, 7 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 200 units/ml RNasin (Promega, Madison, WI), 500 mM ATP, UTP, and CTP and 100 μCi [α-32P]GTP (3000 Ci/mmole, DuPont NEN) for 30 min at 30°C. Labeled transcripts were digested with 10 units of RNase-free DNase I (Boehringer Mannheim) for 30 min at 37°C, and then with protease K for 30 min at 37°C. The transcripts were precipitated twice and equal amounts of radioactivity were hybridized for 4 days at 50°C to Zeta-probe (Bio-Rad) immobilized plasmids as suggested by the manufacturer. The filters were exposed in a PhosphorImager (Molecular Dynamics) for 3 days and scanned.

Nuclear Extracts and Mobility Shift Assays—Nuclear extracts were prepared according to Schreiber et al. (49) with slight modifications. The cell pellets were resuspended in ice-cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μl/ml bovine pancreatic DNase. The nuclear extracts were then incubated with 5 μg/ml of bovine pancreatic DNase. Third passage NHEK cells were used for transfection experiments, and preparation of nuclear extracts. Cos-7, HeLa, MCF-7, HepG2, F9, and NIH-3T3 cells were purchased from American Tissue Culture Collection (ATCC). NHEK were grown and maintained according to the manufacturer's recommended procedures. HaCaT cells were a gift from Dr. Norbert E. Fusenig and were grown in Dulbecco's Modified Eagle's medium supplemented with 4.5 g/liter glucose, 10% fetal bovine serum, and nonessential amino acids (Life Technologies, Inc.). Keratinocyte KC18-2-40 cells were a gift from Dr. Craig Woodward (NCI, NIH) and were grown in keratinocyte-SFM medium (Life Technologies, Inc.).

Transfection Experiments—Recombinant plasmid DNA was introduced into NHEK, HeLa, Hep G2, MCF-7, and F9 cells, or DOTAP (Boehringer Mannheim) for NIH-3T3 and Cos 7 cells following the manufacturer's recommendations. Typically, 2–3 × 10⁶ cells were plated in six-well culture plates 16–20 h before transfection. Transfections were done when cultures reached 90–70% confluency. Transfection efficiencies were always monitored by use of a thymidine kinase β-galactosidase construct (Clontech, Palo Alto, CA). In Lipofectin transfections, cell cultures were washed once at 37°C with phosphate-buffered saline, and then were preincubated for 30 min at 37°C with either keratinocyte-SFM (Life Technologies, Inc.) for NHEK, HaCaT, and KC18-2-40 cells, or Opti-MEM I medium (Life Technologies, Inc.) for HeLa, F9, MCF-7, and Hep G2 cells. For each well, 1 μg of reporter plasmids and 0.5 μg of thymidine kinase β-galactosidase were mixed with 6 μg of Lipofectin and incubated for 20 min at room temperature. The lipid/DNA mixture was then added into each well and incubated for 16–18 h in the case of the keratinocyte, MCF-7, and F9 cells, or 3–4 h in the case of HeLa and Hep G2 cells. At the end of the transfection period the medium was replaced with the medium in which the cells normally grow, and for NHEK cells the concentration of the Ca2⁺ in the keratinocyte growth medium was adjusted to 1.2 mM. Cells were harvested 50–60 h posttransfection. For DOTAP transfections, the plasmids (1.0 μg of reporter constructs and 0.5 μg of thymidine kinase β-galactosidase) were mixed with 14 μg of DOTAP in HEPES buffer solution (pH 7.3) and incubated for 20 min at room temperature. The transfection mixture was added to the medium of each cell type for 16–18 h. The media were then replaced and cells cultured for another 50–60 h. Cellular extracts were prepared as described by Poitier et al. (46). Aliquots were used for CAT assay, β-galactosidase assay (Promega), and total protein quantitation (47). CAT activity was determined by the continuous enzyme assay of Neumann et al. (48). Cellular extracts of untransfected cells and of cells transfected with the pCAT-Basic vector were used as negative controls, while the activity of CAT gene controlled by SV40 early promoter in the pCAT- Promoter vector and by the SV40 early promoter and enhancer in the pCAT-Control vector (Promega) served as positive controls. To monitor the transfection efficiency, the β-galactosidase expressing vector YTKs (Clontech) was co-transfected. The values for CAT were normalized by protein content and β-galactosidase activity. The relative CAT values are the averages of at least three independent experiments, each with duplicate samples.

In the co-transfection experiments 1.0 μg of the profilaggrin-CAT constructs were co-transfected with 0.5 μg of expression vectors containing c-jun (Rous sarcoma virus-cDNA, obtained from M. Karin) and c-fos (pRF fos M3, obtained from E. Adamson) cDNAs under the control of the Rous sarcoma virus long terminal repeat region, JunB and c-Jun TAM cDNAs (a gift from M. Bissel) under the control of the cytomegalovirus promoter, JunD cDNA (pSG5-JunD, a gift from Dr. L. Lauer) under the control of SV40 early promoter, or with the same amount of the corresponding expression vectors alone.

Nuclear Extracts and Mobility Shift Assays—Nuclear extracts were prepared according to Schreiber et al. (49) with slight modifications. The cell pellets were resuspended in ice-cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml bovine pancreatic DNase. The nuclear extracts were then incubated with 5 μg/ml of bovine pancreatic DNase, and left to swell on ice for 15 min. Nonidet P-40 was then added to 0.6% and the suspension...
was homogenized with 20 strokes in a tightly fitting glass homogenizer. After centrifugation, the nuclear pellets were resuspended in ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol, and the mixture of protease inhibitors, and vigorously shaken at 4 °C for 15 min. The nuclear debris was discarded by centrifugation for 15 min at 14,000 rpm, and the extracts were aliquoted and stored at −70 °C until used.

Mobility shift experiments were performed with 5–20 μg of nuclear extracts and 4–8 × 10^4 cpm (about 1 ng) of gel-purified, end-labeled, double-stranded DNA fragments. Typically, the binding reactions were carried out in 20 μl containing 10 mM Tris-HCl (pH 7.5), 65 mM NaCl, 5 mM dithiothreitol, 5 mM MgCl2, 0.05% Nonidet P-40, 10% glycerol, 1 μg/ml bovine serum albumin, and 25 μg/ml poly(dI-dC) as a carrier for 30 min at 4 °C. In competition experiments a 100-fold molar excess of the cold competitor was preincubated with the extracts for 30 min at 4 °C before the labeled DNA fragment was added. Antibody interference experiments were carried out by adding 2 μg of the corresponding commercially available antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to the binding reactions and preincubating in the absence of specific probe for 2 h at 4 °C, followed by incubation in the presence of the probe for additional 30 min at 4 °C. The antibodies were: anti-c-Jun/AP1 (N), Jun B (N-17), Jun D (329); anti-c-Fos (4). The complexes were resolved on nondenaturing 6% polyacrylamide gels in 0.5 × TBE buffer for 1 h at 14 V/cm. The gels were autoradiographed at room temperature.

RESULTS

**Profilaggrin Gene Expression Is Controlled Primarily at the Level of Transcription—**In *vivo*, human profilaggrin mRNA first appears in the granular cells of the differentiating epidermis (50). As with many late differentiation markers, studies of profilaggrin expression have been significantly limited by the availability of *in vitro* systems in which the gene is properly transcribed. Primary cultures of normal human keratinocytes have been shown to contain profilaggrin mRNA, although the abundance of the message is lower than in the epidermis (16). In established human epidermal cell lines (*e.g.* HaCaT, KC18-2-40) the abundance is even lower, and in some of them (*e.g.*, RHEK cells) the profilaggrin message cannot be detected at all (11). We compared the relative amounts of profilaggrin transcripts made by different cell types of epidermal and nonepidermal origin. These included NHEK cells, grown under differentiating conditions in 1.2 mM Ca2+, the simple epithelial HeLa cells, and NIH-3T3 mouse fibroblasts. The results from the reverse transcriptase-PCR amplification of the corresponding total RNAs with profilaggrin specific primers are shown in Fig. 1a. The relative amount of profilaggrin message in the foreskin RNA (lane 4) was defined as 100%. Neither NIH-3T3 (lane 1), nor HeLa cells (lane 2) contained detectable profilaggrin mRNA. The abundance of profilaggrin mRNA in NHEK cells was estimated to be 50–75% of that in the foreskin (lane 3). These results reflected the steady-state level of profilaggrin mRNA, but did not clarify the question of whether profilaggrin transcripts were initiated in the nonepidermal cells and were subsequently rapidly degraded. To address this, the amount of the nascent transcripts was estimated by nuclear run-on experiments (Fig. 1b). No profilaggrin RNA was detected in either fibroblast (lane 1) or HeLa (lane 2) nuclei, whereas preinitiated profilaggrin transcripts were elongated in NHEK nuclei (lane 3). Thus it appears that the key step in the regulation of profilaggrin gene expression is at the level of transcription.

**Profilaggrin 5′-Flanking Sequences Regulate the Expression of the Gene—**To explore the role of the 5′-flanking sequences in controlling profilaggrin transcription, a series of fragments encompassing different lengths of this region were cloned into the pCAT-Basic vector (Fig. 2a) and their regulatory potential was compared to pCAT-Promoter and to pCAT-Control. In NHEK cells (Fig. 2b) the longest fragment tested (~1532/9) showed an activity that was 10 times higher than the activity conferred by the SV40 early promoter in the pCAT-Promoter vector. Gradual deletions of the sequences between ~1532 and ~78 (construct ~78/9) had little effect on the level of expression. Further deletion to ~59 (construct ~59/9), however, reduced the activity 2-fold, to about four times that of pCAT-promoter. Elimination of 19 more base pairs completely abolished transcription. Construct ~41/9 was not capable of conferring detectable levels of CAT in the pCAT-Basic construct. Similarly, construct ~81—~31, in which the sequences from ~30 to ~9 were deleted, did not show any CAT activity. Taken together, these data indicated that the region between ~59 and ~9 is essential for transcription. Thus it encompasses the core promoter of the profilaggrin gene. Sequences involved in positive regulation and accounting for the high activity of the 5′-flanking region reside between positions ~59 and ~78.

**Profilaggrin 5′-Flanking Sequences Exhibit Cell Type Specificity—**To characterize the cell type specificity of the 5′-flanking region, several epidermal, simple epithelial and nonepithelial cells were transfected with six different constructs (Table I). Although the activity of pCAT-Promoter construct, used as a reference, was similar among the different cell lines, the activities from the profilaggrin constructs varied widely.

As expected from above, construct ~41/9 was not active in any of the cells. The other three profilaggrin constructs showed a constitutive promoter activity that was about 0.5 times that of pCAT-Promoter in the nonepithelial cells and 1.5–3.5 times that in simple epithelial cells. The highest expression was observed in the NHEK cells where the profilaggrin gene is normally abundantly transcribed and also in the two other keratinocyte cell lines, *e.g.* HaCaT and KC18-2-40, which express less profilaggrin. The levels of expression were several-fold higher than the pCAT-Promoter and only 30–50% of this high activity was generated by the sequences (~59 to +9) of the core promoter. The keratinocyte-specific enhancement was exerted primarily by positive elements residing in the region between ~59 and ~78 (Table I and Fig. 2).

**Specific Protein Binding to the Profilaggrin Proximal Pro-
Regulation of Profilaggrin Expression

**Fig. 2.** Transient expression of the CAT reporter gene under the control of profilaggrin 5′-upstream sequences. a, fragments spanning the designated lengths of the profilaggrin 5′-flanking region were subcloned into reporter vectors carrying the chloramphenicol acetyltransferase gene, CAT. The pCAT-Basic, pCAT-Promoter, and pCAT-Control vectors are described under “Materials and Methods.” b, NHEK cells were transfected and the activity of CAT was determined. The values for CAT were normalized by protein content and β-galactosidase activity of the cotransfected vector pTKβ. They represent the average of at least three independent experiments, each with duplicate samples. The relative CAT values represent the fold increase in expression of CAT over the background expression of the pCAT-Basic vector.

**moter Region**—Examination of the region −59 to −78 revealed a perfect consensus AP1 regulatory motif at position −60 to −77 (Fig. 3a), which bound strongly to one footprinting unit of recombinant human c-Jun protein (data not shown). Gel mobility shift experiments were performed to determine whether it represents a site for protein interactions that might lead to the observed increase in the CAT activity in the NHEK cells. When a 5′-end labeled double-stranded oligonucleotide probe (Fig. 3b, lane 1), spanning the sequences between −82 and −57 and encompassing the AP1 site (Fig. 3a), was combined with NHEK nuclear extracts, a major complex (A) was formed (lane 2). The specificity of the binding was established by competing with a 100-nucleotide excess of the unlabeled probe (lane 3) or with poly(dI-dC), as an unspecific competitor (lane 4). Oligonucleotides carrying consensus recognition sequences for a number of transcription factors were also assessed. Of these, a consensus AP1 oligonucleotide was the only one to compete for complex A binding as effectively as the probe itself (compare lanes 3 and 5). Oct, AP2, and Sp1 consensus sequences did not interfere with the formation of complex A (data not shown). Competition were also performed with a series of oligonucleotides (Fig. 3a) bearing point mutations (M1 and M2) and deletions (D1-D3) of the AP1 motif or of the sequences immediately adjacent to it. Of these, all fragments that contained the wild-type AP1 motif competed (Fig. 3b, lanes 6 and 7), whereas oligonucleotides in which the AP1 sequence was mutated (lane 8) or deleted (lanes 9 and 10) did not. These data show that the formation of complex A depends critically on the intact AP1 recognition motif.

To identify the proteins in complex A, we used antibodies against several transcription factors known to be associated with AP1. First we explored the sensitivity of the binding to antibodies specific for each of the Jun proteins. Under the experimental conditions, preincubation with the corresponding antibodies resulted both in decreased intensities of complex A and in the appearance of a supershifted band (Fig. 3c, compare lane 1 with lanes 2–4), thus indicating that all three members of the Jun family contributed to the AP1-mediated complex formation. In similar experiments antibodies against the ATF/CREB transcription factors known to form heterodimers with the AP1 proteins did not interfere with complex A formation (data not shown). Of all the antibodies recognizing the Fos transcription factors, only the antibody c-Fos (4), specific for c-Fos p62 protein and non-cross-reactive with other members of the Fos family, prevented the formation of complex B (Fig. 3c, lane 5). In control experiments complex A was found to be insensitive to anti-human IgG (lane 1) and to antibodies against AP2, Sp1, or Oct transcription factors (data not shown). Thus, it appears that the AP1 activity involved in complex A formation in vitro in cultured NHEK cells includes c-Fos and all the three members of the Jun family, namely c-Jun, JunB, and JunD.

**Mutations in the AP1 Sequence Alter the Activity of the Proximal Promoter**—To examine the role of the AP1 motif in the activity of the proximal promoter region, we cloned several deletion variants (Fig. 4a) of this sequence into the pCAT-Basic vector and assessed the ability of each to drive the expression of the CAT gene in NHEK cells (Fig. 4b). Invariably, elimination of the AP1 motif from the context of the promoter region caused 60–70% drop in the activity compared to the wild type sequence. The expression levels of all the AP1-deletion constructs were similar to that of the core promoter (construct −59/+9), thus suggesting that the AP1 site is essential for the enhancement exerted by the sequences −116 to −59 in keratinocytes (Table I and Fig. 2).

**jun and c-fos Transcription Factors Regulate the Activity of the Profilaggrin Proximal Promoter in Cultured Keratinocytes**—When we explored which AP1 proteins modulate the activity of the core promoter in keratinocytes. For this we used the wild-type construct −116/+9, its deletion counterpart −116/+9 deletion AP1, and the core promoter construct −59/+9. These were co-transfected into NHEK cells with vectors expressing c-fos, c-jun, TAM, a dominant-negative mutant of c-jun with a truncated transactivating domain; junB or junD. As Fig. 5 shows, co-expression of c-fos cDNA had little effect on the activity. This is consistent with the known high levels of c-Fos protein in differentiating keratinocytes (51–53) and with the inability of c-fos alone to affect transcription. However, co-transfection with c-jun up-regulated the expression from construct −116/+9 and the simultaneous co-transfection of c-fos and c-jun had an even more pronounced effect, increasing the activity by 50%. In contrast, co-transfection of TAM dramatically reduced the activity to less than 10%. Significantly, the levels of expression from constructs −116/+9 deletion AP1 and −59/+9, both of which do not contain the AP1 binding site, were not affected by co-transfection with c-jun alone or with c-jun and c-fos together. Similar results (not shown) were observed when the effect of c-fos, c-jun, and TAM was assayed within the context of the entire 5′-flanking region by using the wild type and the deletion constructs shown in Fig. 4. These data suggested that c-jun and c-fos transactivate profilaggrin expression through binding at the AP1 site at position −77. Similar to c-jun, co-transfection with junD increased the
expression from construct −116/+9 by 2-fold (Fig. 5). However, the positive effect of junD was not exerted through interactions with c-fos, since no cooperativity was observed when junD and c-fos were co-transfected together. Moreover, this effect did not depend on binding at the AP1 site at position −77, since the expression was also elevated from both constructs −116/+9 deletion AP1 and −59/+9 in which the AP1 motif was deleted.

In contrast to c-jun and junD, junB down-regulated the expression to less than 10%. This reduction in CAT activity was proportional to the amount of the co-transfected junB expression vector (from 1 to 0.02 μg, data not shown). Again, the effect of junB was not dependent on binding at the AP1 site, since it was observed not only with construct −116/+9, which contained the AP1 motif, but also with the constructs in which the AP1 motif was deleted (Fig. 5 and see Figs. 6 and 7 below).

c-jun and c-fos Effect on the Profilaggrin Gene Expression Is Cell Type-specific—Since the sequences between −59 and −116 enhanced transcription only in epidermal cells (Table I), we were interested to know whether interactions involving the AP1 motif played a role in the cell-type specificity of the expression. To this aim, the expression of the constructs carrying the wild type and the AP1 deletion variants was analyzed in mouse embryonal carcinoma F9 cells and in HeLa cells.

Interactions in F9 Cells—F9 cells were chosen, since, first, they do not express the profilaggrin gene3 and second, in an undifferentiated state they do not contain c-Fos, have a low constitutive amount of JunD and barely detectable c-Jun and JunB transcription factors (54). As shown in Table I, none of the profilaggrin constructs expressed CAT even to the level of the SV40 promoter in the pCAT-Promoter construct. In agreement with its inability to bind directly to specific DNA sequences, co-transfection with c-fos had a very modest effect on the CAT activity of profilaggrin constructs, probably conferred by the complexes formed between the transfected c-Fos and the endogenous JunD (Fig. 6a). However, co-transfection of the c-jun expression vector resulted in a 14-fold increase in the level of CAT in construct −116/+9 and simultaneous co-transfection of c-fos and c-jun synergistically increased the expression up to 40-fold. In contrast, co-expression of TAM alone (Fig. 6a) or together with c-oes (data not shown), had no effect on the transcription. Thus, the cooperative effect of c-Fos and c-Jun on construct −116/+9 in F9 cells was similar to their effect in NHEK cells, but much more pronounced, due to the negligible endogenous levels of these factors in the undifferen-

TABLE I

| Cell type | pCAT-Promoter | pCAT-Control |
|-----------|---------------|--------------|
|           | dpm/A_{0.05} |              |
| Primary keratinocyte: | | |
| NHEK | 22,390 ± 1,411 | 3.71 ± 0.57 |
| Epidermal cell lines: | | |
| HaCaT | 21,514 ± 2,056 | 11.15 ± 0.61 |
| KC18-2-40 | 1,4520 | 7.68 |
| Epithelial cell lines: | | |
| HeLa | 26,292 ± 1,186 | 5.25 ± 0.57 |
| MCF7 | 16,805 ± 4,600 | 7.40 ± 0.61 |
| HepG2 | 39,930 ± 7,563 | 5.54 ± 0.15 |
| Nonepithelial cell lines: | | |
| Cos7 | 32,806 ± 766 | 2.42 ± 0.08 |
| NIH-3T3 | 43,120 ± 8,260 | 3.25 ± 0.46 |
| F9 | 12,340 ± 1,390 | 4.18 ± 0.56 |

* BKG, the CAT activity was equivalent to the activity of pCAT-Basic construct.

3 N. G. Markova, unpublished results.

Interestingly, in F9 cells c-Jun enhanced transcription (up to 5-fold) from constructs −116/+9 deletion AP1 and −59/+9 in which the AP1 site was not present (Fig. 6a). Significantly, there was no synergistic interaction between c-Jun and c-Fos, suggesting that the transactivation by c-Jun from the AP1-deficient constructs did not depend on dimerization with c-Fos. Similarly, the positive effect of JunD (8–15-fold) did not require binding at the AP1 site at position −77 and was not affected by the presence of c-fos (Fig. 6a).

Co-transfection with junB failed to affect the low constitutive activity from all constructs. This could be due either to a lack of interactions with the co-transfected junB or to an active regulatory role for junB. To resolve these possibilities, mobility shift experiments were performed (Fig. 6b). Combining the AP1 containing oligonucleotide (Fig. 6b, lane 1) with F9 nuclear extract resulted in a barely detectable AP1-mediated band (Fig. 6b, lanes 2 and 3), consistent with the low levels of JunD activity in these cells. Nuclear extracts isolated from F9 cells transfected with c-jun or junB cDNA, produced strong retarded complexes A (Fig. 6b, lanes 4 and 7, respectively) which were affected by incubation with the corresponding c-Jun-specific (lane 5) and JunB-specific (lane 8) antibodies and by competition with a consensus AP1 oligomer (lanes 6 and 9). Together, these data indicated that the AP1 proteins have been successfully transfection into the F9 cells and were capable of interacting with the AP1 motif. However, c-Jun was the only one to up-regulate the expression through binding at this motif.

AP1 Interactions in HeLa Cells—Studying the HeLa cells could clarify whether the effect of AP1 was specific for epidermal keratinocytes or was common for other epithelial cells as well. Accordingly, we explored the DNA-protein interactions of the AP1 site with HeLa nuclear extracts (Fig. 7a). A retarded complex with a mobility similar to that of complexes A in NHEK and F9 cells was formed (lane 2) which was sensitive to competition with a consensus AP1 oligomer (lane 1). The bands marked with asterisks were competed by a number of oligomers, as well as by oligo(dIdC) and were therefore attributed to unspecific interactions. Consistent with the very low amount of jun B polyopeptides in HeLa cells that have not been stimulated with TPA (55), we could not detect binding to junB (Fig. 7a, lane 4). Preincubation of the binding reactions with the c-Jun-specific (lane 3), JunD-specific (lane 5), and the c-Fos-specific antibody (lane 6) caused supershifts of some of the complexes, indicating that c-Jun, JunD, and c-Fos-containing dimers are capable of binding at the profilaggrin AP1 site in the
Regulation of Profilaggrin Expression

HeLa nuclear extract.

We assessed the effect of these AP1 interactions on the expression of the CAT reporter constructs in HeLa cells. The activity of constructs $-59^2/9$ and $-116^2/9$ was very similar (Table I and Fig. 7b). A deletion of the AP1 motif within the fragment $-116^2/9$ reduced the activity by about 40%, while the difference between the activity of the wild type $-116^2/9$ and the core promoter $-59^2/9$ was less than 20%. Co-transfection with c-jun and junD expression vectors increased the activity from both AP1-containing and noncontaining constructs by 2–3-fold (Fig. 7b). The effect of junD was similar to that observed with the NHEK and F9 cells. However, the positive effect of c-Jun in HeLa cells, which have many characteristics of simple epithelia, differed markedly from that in the keratinocytes. Thus, co-transfection of TAM, the mutant c-jun in which the transactivating domain has been truncated, did not result in decreased CAT values from any of the constructs. Similarly, simultaneous co-transfection with c-jun and c-fos did not affect the levels of expression. Therefore, although c-jun- and c-Fos-containing complexes could be formed, the positive effect of c-Jun did not depend on the transactivating properties of c-Jun protein and, similarly to JunD, it did not involve dimerization with c-Fos. It must have been exerted through interactions with other transactivators through a pathway that does not depend on binding at the AP1 recognition site at position $-77$. It is possible that the effect was
caused by a cryptic AP1 binding site. However, an inspection of both profilaggrin and vector sequences in the vicinity of the CAT gene did not reveal a meaningful homology to consensus AP1 recognition motifs other than the AP1 site at position –77 of the profilaggrin gene. Second, we were not able to detect any additional interactions within the region –116/+9 that were sensitive to competition by consensus AP1 oligomers or by the profilaggrin AP1 site in any of the nuclear extracts (data not shown). Most likely the AP1 site-independent transactivation by c-Jun and JunD both in F9 and HeLa cells reflected the modulatory effect of the Jun proteins on the function of transcriptional regulators operating downstream from the AP1 motif (see below).

In HeLa cells the forced expression of JunB repressed transcription down to 10% from all constructs (Fig. 7b). Thus, while the mechanisms through which c-Jun modulated the activity of profilaggrin constructs were clearly distinct between keratinocytes and other epithelial cells, the effect of JunB was very similar in both cell types.

**DISCUSSION**

An AP1 Site Confers Cell Type-specific Expression to the Profilaggrin Gene in Cultured Keratinocytes—In this study we have shown that the primary step in the control of the epidermal-specific expression of the profilaggrin gene is at the level of transcription. The proximal promoter sequences play an important role in this control, at least in vitro. While competent in assembling the basal transcriptional machinery both in epithelial and nonepithelial cells, these sequences encompass elements that ensure high levels of expression only in epidermal cells (Table I).

Deletion analysis defined the core promoter of the profilaggrin gene to be between positions –59 and +9. Sequences,
including the TATA box of the profilaggrin gene and a functional Sp1 site at position −55 reside in this region and are indispensable for the constitutive promoter activity. An immediately adjacent region (−78 to −59) works in conjunction with this core promoter to confer high levels of transcription in keratinocytes (Fig. 2, Table I). It accounts for more than 50% of the activity of the 5′-flanking region up to position −1532. Deletion of the AP1 recognition site in the context of this region or point mutations in it abolish the binding to proteins in the nuclear extracts and reduce the transcription in keratinocytes below the level of the core promoter (Figs. 3b and 4b).

The AP1-mediated Keratinocyte Specificity of Profilaggrin Transcription Depends on the Interactions between c-jun and c-fos—AP1 was first identified in HeLa nuclear extracts (56). It consists of heterodimers between individual members of the Jun and Fos families of transactivators or of homodimers between different Jun proteins. A notable feature of the AP1 transregulation is the diversity of the Jun and Fos proteins that can form the active complexes in the different cell types (55). In some cases junB has been shown to down-regulate genes which are normally transactivated by c-jun either directly, by forming heterodimers with c-jun, or indirectly, by down-regulating the transcription of c-jun gene (55, 57, 58). The level and the specificity of gene regulation achieved through interactions between different Jun and Fos family members could be expanded by structural and functional interactions with members of other families of transcription factors, including ATF/CREB (59), Oct (60), NF-κB (61), ets (62) (for a review, see Angel and Karin (55)).

There is increasing evidence implicating AP1 in regulating the expression of several epidermal genes. Among them are genes that are expressed at different stages of epidermal differentiation, the basal cell-specific keratins bovine K5 (29) and human K19 (30); the inducible bovine K6β (32); the transglutaminase TGM 1 (63, 64), which is expressed throughout the epidermis; the suprabasal cell specific K1 (65) and involucrin (31, 66, 67); and the marker of terminally differentiating keratinocytes, loricrin (27). Our finding that, at least in vitro, the activity of profilaggrin proximal promoter depends on the AP1 site at position −77 was particularly interesting in view of the recent data indicating that AP1 interactions at relatively similar positions are involved in regulating the activity of involucrin (31) and loricrin (27). Deletion of the AP1 motifs in the context of the entire regions resulted in 60% (31) and 85% (27) decrease in the activity, respectively.

Although the accumulating evidence points to the importance of AP1 activity, the expression pattern and the role of the individual members of the jun and fos families in controlling transcription in keratinocytes is still unclear. A study by Thierry et al. (68) suggested that the predominant jun activity in the epidermis is junB with some junD, but little or no c-jun, and that its expression is confined to the suprabasal layers (69). More recent reports, however, suggest that junB and junD are detectable in all epidermal layers (70), whereas significant amounts of c-jun are limited to the granular layer (70, 71). Differentiating epidermis contains high levels of c-fos with the expression being increased in the upper spinous and granular layers (51, 52).

In co-transfection experiments K19 (30) and TGM1 (64) have been shown to be up-regulated by c-jun. No data have been reported on the effect of junB. While in co-transfection experiments in fetal rat keratinizing epidermal cells, involucrin has been found synergistically up-regulated by c-jun/c-fos dimers (66), in bandshift assays using human foreskin keratinocytes Welter et al. (31) detected strong binding of the AP1 motifs to fra 1, junB, and junD, but not to c-jun or c-fos. To a certain extent these differences may reflect the different complement of AP1 factors involved in the regulation of the gene in rodent and in human cells.

In the profilaggrin-positive human foreskin epidermal cells the activity of the core promoter is enhanced exclusively by c-fos/jun heterodimers. The up-regulation requires a functional AP1 site at position −77 and depends critically on the transactivating ability of c-jun (Fig. 5). Notably, providing c-jun and c-fos activity to the AP1-deficient F9 cells triggers a transcription from all AP1 site-containing profilaggrin constructs in a manner similar to that observed in keratinocytes (see Fig. 6).

c-jun and junD Are Involved in the Positive Control of the Profilaggrin Core Promoter—In addition to the keratinocyte-specific enhancement which c-jun exerts through interactions at the AP1 motif at position −77, both c-jun and junD positively regulate profilaggrin core promoter activity independently on the binding at the AP1 site. The effect of junD can be observed in all three cell types. The effect of c-Jun is probably masked by the relatively high amounts of this protein in the differentiated keratinocytes but is clearly discernible in the F9 and in the nonepidermal epithelial HeLa cells. The transactivation of the core promoter exerted by both c-jun and junD does not depend on dimerization with c-fos. Interestingly, in F9 cells a truncation of the transactivating domain of c-jun completely abolishes the positive effect, whereas it does not affect the transcription in HeLa cells (compare Figs. 6a and 7b). One possible explanation is that in addition to the interactions at the AP1 site at position −77 c-jun and junD are involved in controlling profilaggrin transcription by modulating the transactivating function of other regulatory proteins which operate downstream of the AP1 motif. This may include modulating the levels of expression of the corresponding genes, or direct binding of Jun proteins to sequences different from canonical AP1 motifs, or protein/protein interactions of c-jun and junD with other transcription factors and auxiliary proteins. The cell type specific differences in the transcriptional activity of the reporter constructs lacking a functional AP1 site in response to a forced c-jun expression may thus reflect the diversity of the protein complement with which c-jun associates. It appears that at least two of these possibilities may be contributing to the AP1 control of profilaggrin expression. We have detected a relatively weak binding of recombinant c-jun to an octamer recognition sequence at position −15. The same site seems to be involved in multiple interactions with other transcription factors and with members of the basal transcriptional machinery. Significantly, mutations in the binding motif interfered with the binding of both recombinant c-jun and Oct 2 transcription factors, dramatically reduced the CAT activity and completely obliterated the effect of c-jun from all constructs. In interactions between Jun and octamer proteins over a single binding site have been documented in the interleukin 2 promoter (64). It is possible that similar interactions at the octamer binding site at position −15 may be responsible for the effect of the AP1 proteins in the absence of the binding motif at position −77 of the profilaggrin gene.

JunB Down-regulates Profilaggrin Transcription in All Cell Types—In contrast to the positive effect of C-Jun and JunD, JunB down-regulates the expression both in epidermal and nonepidermal cells (Figs. 5–7). Since the activity is practically obliterated in all the constructs, irrespective of the presence of the AP1 motif, we cannot estimate to what extent the negative effect depends on interactions at the AP1 site. However, as the activity of construct −59/+9 is also affected, it appears that, at

---

4 S.-I. Jang, P. M. Steinert, and N. G. Markova, unpublished data.
least partially, JunB interferes with the transcription from the core promoter region, most probably through the same pathway through which c-Jun and JunD exert their AP1 site-independent effect. At present, it is not clear whether the strong down-regulation reflects an active suppressor role for junB or is due to competition with the positively acting c-jun in the in vitro experiments.

The Expression Pattern of the Jun Proteins May Be Involved in Determining the Specificity of Transcription of the Epidermal Differentiation Markers—Taking into account the restricted expression of c-jun in the granular layer of the epidermis, the keratinocyte-specific transactivation of profilaggrin promoter by c-Jun, and the strong negative effect of JunB, we may speculate that an interplay between the different Jun proteins may have an important role in determining the cell type and temporal specificity of the expression of the differentiation markers in the epidermis. Thus, while JunD-containing dimers may be responsible for maintaining the activity of the core promoter, JunB-containing dimers in the basal and suprabasal layers may be involved in the repression of the profilaggrin transcription at this earlier stage of differentiation. In concordance with this idea, JunB may be responsible for the high levels of expression of the earlier differentiation markers such as K1 (58) and involucrin (31). As the cells move upward to the granular layer, JunB is replaced by the now more abundant c-Jun, and this may be one of the key steps in the activation of the profilaggrin gene transcription in the late stages of epidermal differentiation. Conversely, the high levels of c-Jun may cause the down-regulation of the earlier expressed K1 and involucrin. In this respect it would be of considerable interest to explore the differential effect of the Jun proteins on the AP1-dependent transcription of other epidermal genes.

In conclusion, we have shown that, while operating as a constitutive promoter in a number of epithelial and nonepithelial cells, as little as 116 base pairs of profilaggrin 5′-sequences direct high levels of expression in cultured epidermal cells. The strong positive effect in keratinocytes is due primarily to interactions of c-Fos/c-Jun heterodimers at an AP1 site at position −77 and depends on the transactivating properties of c-Jun. This study thus constitutes the first detailed description of the mechanism by which AP1 activity affects the transcription of a late epidermal differentiation marker. The cell type specificity of the AP1 element is determined by the composition of the AP1 complexes in the different cells and possibly by interactions with additional transcriptional regulators. Elucidating the nature of these interactions is the goal of our future studies.

Acknowledgments—We thank George Poy for the synthesis of all oligonucleotides used in this study. We are grateful to Drs R. Oshima, M. Karin and A. Adamson (La Jolla) for the c-jun and c-fos expression vectors, to Dr. M. Birrer (Bethesda) for the junB and TAM expression vectors and to Dr. L. Lau (Chicago) for the junD expression vector. We thank Dr. N. Fusing for the HaCaT cell line and Dr. C. Woodward for the KC18-2-40 cells.

REFERENCES

1. Watt, F. M. (1989) Curr. Opin. Cell Biol. 1, 1107–1115
2. Blumenberg, M. (1993) Molecular Biology of the Skin, pp. 1–32, Academic Press, New York
3. Steinert, P. M., and Freedberg I. M. (1991) Curr. Opin. Cell Biol. 3, 1103–1112
Regulation of Profilaggrin Expression

63. Liew, F.-M., and Yamanishi, K. (1992) Exp. Cell Res. 202, 310–315
64. Yamada, K., Yamanishi, K., Kakegawa, A., Kibe, Y., Doi, H., and Yasuno, H. (1994) Biochem. Mol. Biol. Int. 34, 827–836
65. Lu, B., Rothnagel, J. A., Longley, M. A., Tsai, S. Y., and Roop, D. R. (1994) J. Biol. Chem. 269, 7443–7449
66. Takahashi, H., and Iizuka, H. (1992) J. Invest. Dermatol. 100, 10–15
67. Lopez-Bayghen, E., Vega, A., Cadena, A., Granados, S. E., Jave, L. F., Gariglio, P., and Alvarez-Salas, L. M. (1996) J. Biol. Chem. 271, 512–520
68. Thierry, F., Spyrou, G., Yaniv, M., and Howley, P. (1992) J. Virol. 66, 3740–3748
69. Wilkinson, D. G., Bhatt, S., Ryseck, R.-P., and Bravo, R. (1989) Development 106, 465–471
70. Welter, J. F., and Eckert, R. L. Oncogene 11, 2681–2687
71. Briata, P., D'Anna, F., Franz, A. T., and Gherzi, R. (1993) Exp. Cell Res. 204, 136–146
