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Epidermal growth factor and transforming growth factor α specifically induce the activation- and hyperproliferation-associated keratins 6 and 16

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ABSTRACT **Epidermal injury results in activation of keratinocytes which produce and respond to growth factors and cytokines and become migratory. Activated keratinocytes express a specific pair of keratin proteins, K6 and K16, distinct from the keratins in the healthy epidermis. Keratinocytes can be activated, for example, by binding of the appropriate ligands to the epidermal growth factor receptor (EGFR). We have analyzed the effects of EGFR activation on keratin gene transcription by transfecting DNAs containing keratin promoters linked to a reporter gene into primary cultures of human epidermal keratinocytes in the presence or absence of EGF or transforming growth factor α (TGFα), two growth factors that activate EGFR. The activation of EGFR had no effect on the promoters of simple epithelial, basal-layer-specific, or differentiation-specific keratins. In contrast, the expression of K6 and K16 was strongly and specifically induced. A 20-bp DNA segment of the K16 gene promoter conveyed the EGF regulation, functioned in a heterologous construct, and therefore constituted an EGF-responsive element. A nuclear protein specifically bound to this element and to the analogous sequence of the K6 promoter. Thus, EGF specifically induces K6 and K16, markers of activated keratinocytes, via nuclear proteins that bind to EGF-responsive elements in the promoters of these keratin genes.**

The keratinocyte, the predominant cell type of the epidermis, provides mechanical protection, prevents water loss, and forms the first line of immunological defense as well. Upon releasing its contacts with the basement membrane, a keratinocyte leaves the basal layer, elaborates markers of differentiation, enucleates, and becomes a part of the insoluble crosslinked protein network, known as stratum corneum, that provides the mechanical and water barrier (1). The immunological function of the keratinocyte appears in pathological conditions—e.g., during wound healing and in allergic and inflammatory reactions. In response to epidermal injury, keratinocytes become "activated"; i.e., they produce and respond to growth factors and cytokines, become migratory, and can produce components of the basement membrane (2). The extracellular signals that induce keratinocytes to start differentiating or to become activated are not known.

The extracellular milieu can convey signals to cells via surface receptors. After ligand binding, signals are conveyed by interactions with secondary messengers that commonly involve protein phosphorylation and dephosphorylation (3). These signals are eventually conveyed to nuclear proteins that regulate gene expression (4). Among the regulated genes are those encoding additional regulators of gene expression. By this mechanism receptors, when activated, can initiate a cascade of events that leads to major morphological, developmental, and differentiative changes.

Among the most intensely studied cellular receptor signaling pathways are those involving epidermal growth factor (EGF) and its receptor (EGFR) (3, 5). Activation of EGFR results in major pleiotropic changes in many cell types, including proliferation, degradation of extracellular matrix, and increased motility (6, 7). Upon binding to EGF the EGFR dimerizes, activating its intracellular protein-tyrosine kinase, which initiates a cascade of phosphorylation events that include cytoplasmic and nuclear proteins, thus conveying signals from the cell membrane to the nucleus (8–10).

Epidermal keratinocytes both produce and respond to transforming growth factor α (TGFα) (11), a polypeptide that interacts with the EGFR with effects similar to those of EGF (12). Keratinocytes respond to the activation of the EGFR by proliferating, degrading components of the extracellular matrix, and becoming migratory (13–17). This response is induced not only under pathological conditions but also during normal morphogenesis of epithelial structures, such as mammary gland ducts (18). EGFR is present at a very early stage of embryonic skin development and whereas such epidermis contains EGFR in all cell layers, in adults EGFR is primarily expressed in the basal and, to a lesser degree, the deepest suprabasal layers (19). TGFα production is increased in cutaneous malignancies (20, 21), can be induced by tumor promoters (22), and is greatly elevated in psoriasis (23).

The commonly used phenotypic markers of epithelial development and differentiation are keratins, a large family of proteins that form the intermediate filament network in all epithelial cells. Keratins 5 and 14 (K5 and K14) are expressed in basal keratinocytes, K1 and K10 in differentiating keratinocytes, and K6 and K16 in activated keratinocytes (24–26). Because EGF and TGFα have such profound effects on keratinocyte physiology and because keratins are the markers for various pathways of keratinocyte differentiation, we have analyzed, on the molecular level, the effects of EGF and TGFα on keratin gene expression. We have engineered DNA constructs in which keratin gene promoters drive expression of the chloramphenicol acetyltransferase (CAT) reporter gene, and have transfected them into human epidermal keratinocytes in the presence or absence of EGF or TGFα. All transfected promoters are transcribed at somewhat higher levels in the presence of EGF or TGFα, but the effect is greatest on the promoters of the K6 and K16 genes, which are transcribed at 5 times higher levels relative to the other keratin promoters and control viral promoters. The increase

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; EGF-RE, EGF-responsive element; TGF, transforming growth factor; CAT, chloramphenicol acetyltransferase; HEK, human epidermal keratinocyte; K*n*, keratin *n*.

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in transcription is dependent on a short DNA sequence that is similar in K6 and K16 gene promoters and that specifically binds a nuclear transcription factor. These results indicate that EGF and TGFα activate keratinocytes and specifically induce the transcription of the activated-keratinocyte-specific keratins K6 and K16.

MATERIALS AND METHODS

Plasmids pSV2CAT, pRSVZ, K5CAT, K6CAT, K8CAT, K10CAT, K14CAT, and K18CAT have been described (27–29). We have cloned K19CAT from polymerase chain reaction (PCR)-amplified human DNA, using the K19F and K19R oligonucleotides (Table 1), whose sequences were based upon the published sequence of the human K19 gene (30). The sequence of cloned DNA was found to be identical to the reported sequence of K19 (M.B., unpublished). K17CAT was engineered using K17F and K17R oligonucleotides in PCR with 2F12 λ phage as template (31). Note that K14 and K17 genes derive from the human keratin pseudogene locus. Their sequences are 93% identical to the functional genes and retain the regulatory elements of the functional genes (27). K3CAT contains the promoter of the human K3 gene (32); its construction will be described elsewhere.

Deletion mutants of K6 and K16 DNAs were created by using the same reverse-oriented primers, K6R and K16R, and two series of forward primers described in Table 1 in PCR with K6CAT and K16CAT as templates. The PCR products were digested with *Pst* I and *Xba* I and cloned into a similarly digested pGCAT vector. Synthetic oligonucleotides that contained one or three copies of the putative EGF-RE were ligated into the *Bam*HI site of p-CAT-promoter vector (Promega). The orientation of the insert was ascertained by digestion with *Sst* I or *Sst* II. All DNAs used in transfections were purified through two successive CsCl/ethidium bromide equilibrium gradient centrifugations.

Human epidermal keratinocytes (HEKs) were purchased from Clonetics and grown in 60-mm dishes in serum-free keratinocyte medium (GIBCO), except as indicated. Bovine pituitary extract was treated by the growth-factor-inactivation method (33). The cells were expanded through two 1:3 passages before transfection, so that they were transfected in their third or fourth passage, before they showed any sign of senescence or differentiation. HEKs were incubated in EGF-free, serum-free medium for 1 hr before transfection using Polybrene followed by dimethyl sulfoxide shock (28). EGF and TGFα (Boehringer Mannheim and Promega) were added (20 and 40 ng/ml, respectively) to the transfected cultures immediately after the dimethyl sulfoxide shocks. After 48 hr the transfected cells were harvested by scraping into phosphate-buffered saline and washed twice. The cell disruption and enzyme assay procedures have been described (28).

Nuclear extracts of HEKs were prepared according to Dignam *et al.* (34), with the following modifications. Pelleted cells (5 min, 800 × *g*, 4°C) were resuspended in 2 volumes of buffer A (1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol/10 mM Hepes, pH 7.9) and then broken by 20–30 strokes of a glass Dounce homogenizer (B pestle). The resulting nuclei were pelleted by centrifugation (17,000 rpm in a Beckman SW41 rotor at 4°C), suspended in 3 ml of buffer C [420 mM NaCl/225 mM EDTA/1.5 mM $MgCl_2$/25% (vol/vol) glycerol/20 mM Hepes, pH 7.9/0.5 mM phenylmethanesulfonyl fluoride/0.5 mM dithiothreitol/1 mM benzamidine with leupeptin (0.5 μg/ml) and pepstatin A (1 μg/ml)]. Nuclei were broken by 15–20 strokes of a Dounce homogenizer (B pestle) and gently stirred at 4°C for 30 min. Cell debris was removed by centrifugation (16,500 rpm in an SW41 rotor at 4°C). The clear supernatant was dialyzed for 5 hr at 4°C against 1 liter of buffer D (200 mM EDTA/100 mM KCl/20% glycerol/0.5 mM dithiothreitol/20 mM Hepes, pH 7.9, with protease inhibitors as in buffer C above).

Table 1. Synthetic oligodeoxyribonucleotides used in PCR, cloning, or electrophoretic mobility-shift assays

Name*	Sequence (5′ to 3′)
K17F	TTTTCTAGAGCATGCCCCGGGCCAGCACCTT
K17R	TTTAAGCTTGCGGCCGCGGGAGGCAGGCACACA
K19F	TTTCTGCAGTTAAAGGGTGAGGCTC
K19R	TTTAAGCTTGGCGAGGCGGAGCACG
K6F	GATCTGCAGCACTCAGGGCATTGTCGAT
K6R	GATAAGCTTGAGATGAGAGGGCTTAGGA
K6F-292	TTTTCTAGAAGAATACTCTTATTGT
K6F-218	TTTTCTAGAGCAATCTCGGTATTTC
K6F-138	TTTTCTAGACAAGCTCACCTTCCAG
K6F-115	TTTTCTAGACCCAGCCCATGCTCTC
K6R-A	TTTCTGCAGGGTTCCAGAGATGAGA
K16F	TTTTCTAGAGGATCCCCACAACTGC
K16F-397	TTTTCTAGACCTGGGGACGCAGTTG
K16F-272	TTTTCTAGACTGGCCCCACACCCCC
K16F-212	TTTTCTAGAGGCTAATAATCCAGAG
K16F-192	TTTTCTAGAGAGTTGGACGGGACCG
K16F-132	TTTTCTAGAGAGGGCCCCGCCTTCC
K16R-A	TTTCTGCAGGGTGCCAAGGAGGGAG
K16EGF-RE-F	GGCTAATAATCCAGAGTGAG
K16EGF-RE-R	CTCACTCTGGATTATTAG
K6EGF-RE-F	CAAGCTCACCTTCCAGGACTA
K6EGF-RE-R	TAGTCCTGGAAGGTGAGCTTG
1× K16F	TTTGGATCCGAGCTCGGCTAATAATCCAGAGTGAGGAGTTGGACGGGACCGGGAGAGATCTAAA
1× K16R	TTTAGATCTCTCCCGGTCCCGTCCAACTCCTCACTCTGGATTATTAGCCGAGCTCGGATCCAAA
3× K16F	TTTGGATCCCCGCGGCTAATAATCCAGAGTGAGGGCTAATAATCCAGAGTGAGGCTAATAATCCAGAGTGAGATCTAAA
3× K16R	TTTAGATCTCACTCTGGATTATTAGCCTCACTCTGGATTATTAGCCCTCACTCTGGATTATTAGCCGCGGGGATCCAAA
RAREβ-F	AGCTTAAGGGTTCACCGAAAGTTCACTCGCAT
RAREβ-R	ATGCGAGTGAACTTTCGGTGAACCCTTAAGCT
AP-1-F	CTAGTGATGAGTCAGCCGGATC
AP-1-R	GAACCGGCTGACTCATCACTAG
AP-2-F	GATCGAACTGACCGCCCGCGGCCCT
AP-2-R	AGGGCCGCGGGCGGTCAGTTCGATC
SP1-F	GATCGATCGGGGCGGGGCGATC
SP1-R	GATCGCCCCGCCCCGATCGATC
NF1-CTF-F	ATTTTGGCTTGAAGCCAATATG
NF1-CTF-R	CATATTGGCTTCAAGCCAAAAT

*F, forward; R, reverse; RARE, retinoic acid-responsive element; AP-1, AP-2, SP1, and NF1-CTF indicate binding sites for transcription factors AP-1, AP-2, Sp1, and NF1/CTF.

Recessed 3′ ends of double-stranded annealed oligonucleotides were radiolabeled by filling in with Klenow DNA polymerase (Stratagene) in the presence of 40 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq).

For electrophoretic mobility-shift assays, ≈7 μg of HEK nuclear protein was first incubated for 15 min on ice, with or without a 200-fold molar excess of double-stranded synthetic DNA used as nonradioactive competitor, in 25 μl of binding buffer [100 mM NaCl/20 mM Tris Cl, pH 8/10% glycerol/5 mM $MgCl_2$/1 mM dithiothreitol/2% polyvinyl alcohol/0.1 mM EDTA/4 mM spermidine with poly(dI-dC) at 80 μg/ml]. ^{32}P-labeled probe (80,000 cpm, ≈500 pg of DNA) was then

added and incubated for an additional 15 min on ice. The shifted band was detected in a 6% polyacrylamide gel (30:0.8 acrylamide/*N,N'*-methylenebisacrylamide weight ratio), after drying, by autoradiography at −70°C with intensifiers. DNAs were either synthesized (Pharmacia Gene Assembler) or purchased (Stratagene). As nonspecific competitor we used fragmented salmon sperm DNA (35).

RESULTS

EGF Specifically Induces K6 and K16 Genes. Transfection of K6 and K16 promoters in the presence of EGF resulted in a specific and significant, 4- to 5-fold increase in relative levels of CAT expression (Fig. 1). Treatment of HEKs with EGF (20 ng/ml) increased the levels of transcription of all transfected genes we have studied, including that of the pRSVZ control plasmid, but whereas the simian virus 40 promoter and all other keratin promoters were induced 20–100%, the K6 and K16 promoters were induced to significantly higher levels (Fig. 1).

The keratin promoters tested included those of the genes K8, K17, and K18, which are expressed in early embryo and simple epithelia; K5 and K14 found in basal layers of stratified epithelia; K3 and K10, differentiation-specific keratins expressed in cornea and epidermis, respectively; and K19, a keratin usually expressed in cells undergoing physiologic changes that involve disturbances in keratin synthesis. None of these keratin promoters responded to EGF to the same level as did the promoters of K6 and K16, which are associated with activated, hyperproliferative states.

TGFα binds to the EGFR and, in most systems studied, causes the same changes that EGF does. Indeed, at 40 ng/ml, TGFα had the same specific effect on K6 and K16 promoters as did EGF (Fig. 1*D*). These findings are in accord with those obtained with transgenic mice that express TGFα in their epidermis, where an increase in the expression of the endogenous K6 has been observed (36).

HEKs become quiescent when they reach confluence. EGF and TGFα induce proliferation of quiescent keratinocytes (11, 15), which causes the transcription of all transfected DNAs, not only K6 and K16, to be significantly increased (C.-K.J., unpublished).

Effects of EGF and TGFα Are Concentration-Dependent. The specific inductive effects on K6 and K16 genes have distinct optima for both EGF and TGFα, at 20 and 40 ng/ml, respectively. Other keratin promoters tested were not affected even at concentrations of EGF at which the induction of K6 and K16 waned. Further, EGF and TGFα appeared to compete for the same receptor in affecting K6 and K16 promoters, because when TGFα was added to cells grown in the presence of saturating levels of EGF, the TGFα effect was abrogated (data not shown).

The effects of EGF and TGFα on epidermal keratinocytes are quite rapid and increased migration can be detected within hours (13). In monolayers the changes are slower, but even within our experimental period there were visible phenotypic changes in keratinocytes (Fig. 2). EGF induced long processes and more distinct intercellular borders. This means that the induction of transcription of K6 and K16 genes is concurrent with development of long cellular processes and migration of keratinocytes in monolayers.

Localization of the EGF-Responsive Element (EGF-RE). To localize the sites of action of the EGF-dependent induction, we constructed several deletion mutants of the K6 and K16 DNAs. In the case of K16, the construct containing 212 bp was fully responsive to the EGF signal, but the 20-bp-smaller construct did not respond to EGF (Fig. 3). Thus, the EGF-RE of the K16 promoter is between −192 and −212.

In the case of the K6 promoter, three constructs, including the one that contained only 138 bp of K6 DNA, were fully responsive to induction by EGF (Fig. 3). The construct containing 115 bp was nonfunctional as a promoter and therefore its inducibility by EGF could not be directly

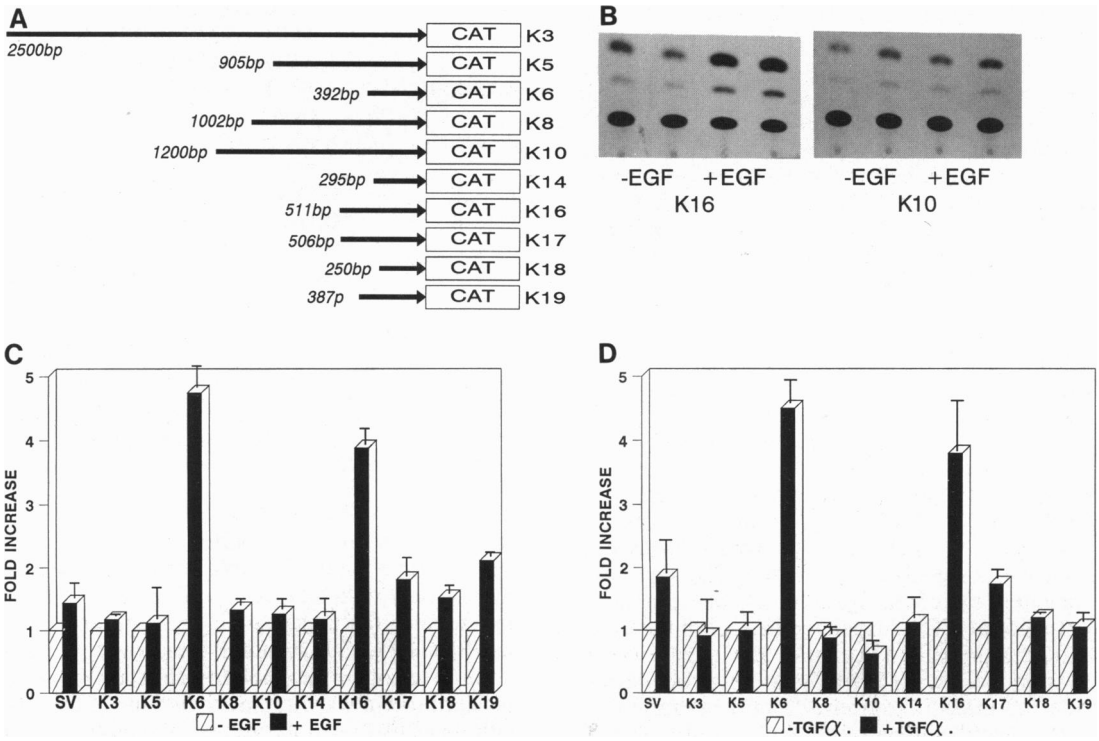

FIG. 1. EGF specifically induces K6 and K16 promoters. (*A*) Sizes of the constructs containing keratin promoters. (*B*) Effect of EGF on CAT levels after transfection. (*C*) The effect is specific for the K6 and K16 gene promoters. The increased CAT levels were normalized to the equivalent increases in β-galactosidase from the cotransfected pRSVZ vector (SV). Activities of the K6 and K16 promoters are induced approximately 4-fold relative to the controls. Each DNA construct was transfected in duplicate between 3 and 12 times. Error bars show the differences between duplicate transfections. (*D*) TGFα has the same effect on keratin promoters as EGF.

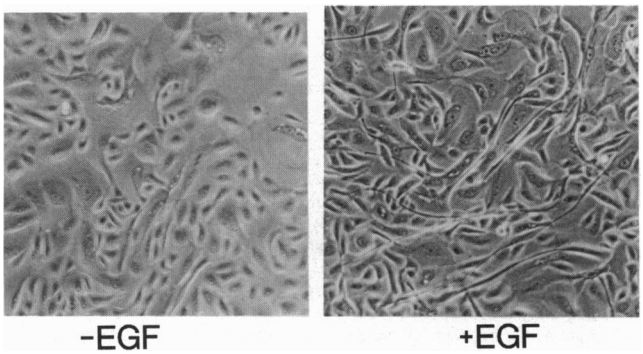

FIG. 2. Phenotypic changes seen during the course of the experiment. (*Left*) Cells grown without EGF. (*Right*) Cells that received EGF (200 ng/ml) for 48 hr after transfection.

determined. This means that the EGF-RE is within the 138 bp immediately upstream from the translation start.

The 20-bp sequence of the K16 promoter may contain the entire EGF-RE or just be a component of it. In the first case the sequence could confer EGF response on a heterologous DNA construct, and therefore we have cloned it into an enhancer trap vector. A single copy of the sequence was nonfunctional (data not shown); however, three copies in tandem constituted a functional enhancer that conferred EGF responsiveness (Fig. 3*B*). Curiously, only the construct containing the elements in the proper orientation, ENH2, was EGF-responsive; the reverse orientation, ENH1, did not work. We conclude that the 20-bp sequence is a functional

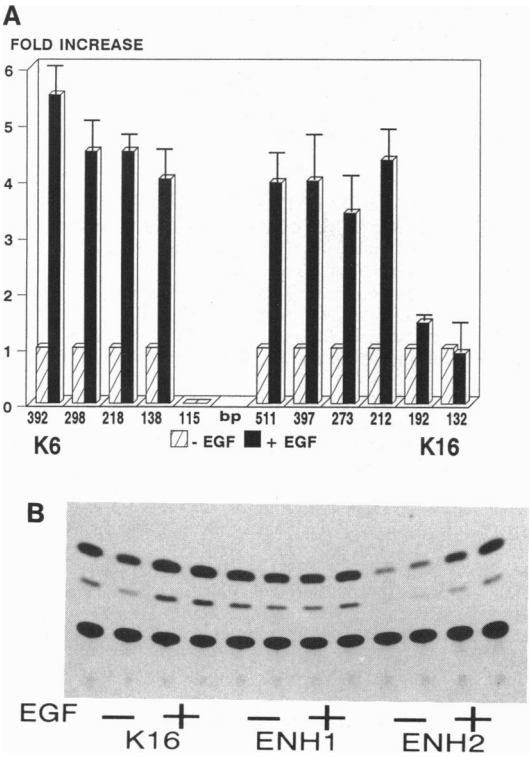

FIG. 3. Mapping the EGF-REs of K6 and K16 promoters. (*A*) Whereas the construct containing only 138 bp of K6 DNA was fully regulated by EGF, the 115-bp construct was inactive. In the K16 gene promoter the EGF-RE is between 212 and 192 bp upstream from the translation start. (*B*) The EGF-RE can be transferred to a heterologous promoter. All transfections into HeLa cells were performed in duplicate. CAT assays show that the K16 promoter and the construct containing three tandem copies of the site in positive orientation, ENH2, are inducible, but the construct with the insert in the reverse orientation, ENH1, is not.

EGF-RE but that it has stringent requirements for interaction with the transcription complex.

A Nuclear Protein Responsive to EGF Specifically Binds to EGF-RE. If the DNA sequence between −212 and −192 of the K16 promoter contains an EGF-RE, we would expect a nuclear protein to bind specifically to this sequence. Further, the sequence between −138 and −115 of the K6 promoter has similarities with the 20 bp of the K16 EGF-RE (Fig. 4*B*) and may contain the EGF-RE of the K6 promoter. If so, the K6 EGF-RE should compete for binding of the nuclear protein to the K16 EGF-RE. To test these possibilities we performed gel retardation analysis using synthetic oligonucleotides that contain the relevant sequences. Indeed, the putative K16 EGF-RE specifically bound a nuclear protein from HEKs (Fig. 4*A*). The binding was prevented by excess nonradioactive competitor of the same sequence. HeLa cells con-

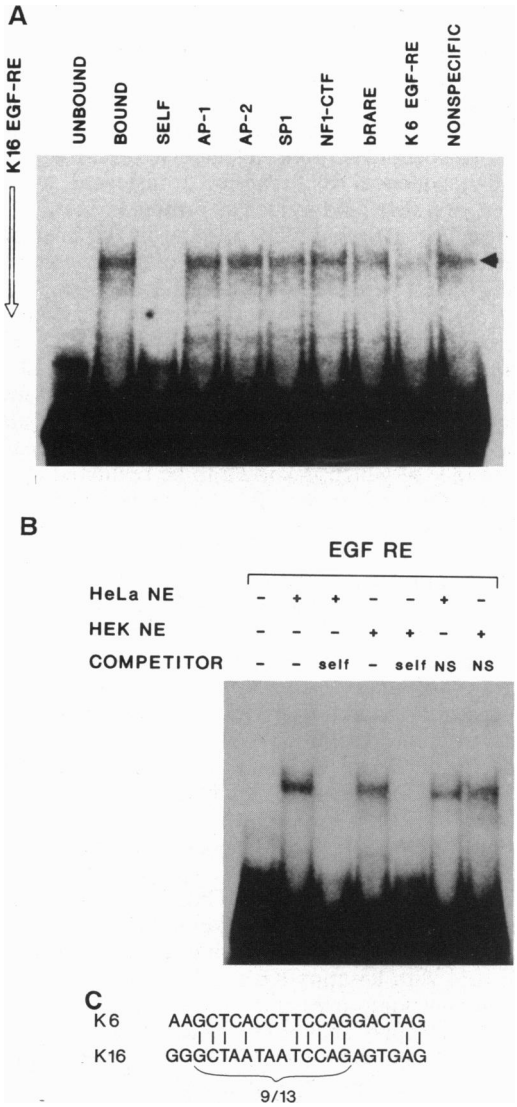

FIG. 4. Nuclear protein binds to EGF-RE. (*A*) Electrophoretic mobility-shift assay using HEK nuclear extracts and K16 EGF-RE oligonucleotide probe. Open arrow shows the direction of electrophoresis, and filled arrowhead marks the shifted band. Nonspecific DNA and consensus sequences recognized by various growth factors did not compete for binding, but an excess of unlabeled oligonucleotide did compete (SELF), as did the putative K6 EGF-RE. (*B*) Identical mobility shift produced by a protein from HeLa cell nuclei. NS is a nonspecific competitor, salmon sperm DNA. NE, nuclear extract. (*C*) Comparison of the relevant sequences of the K6 and K16 promoters. Vertical lines mark identical nucleotides.

tained an apparently identical binding activity in their nuclei (Fig. 4*B*). Consensus binding sequences for common transcription factors did not prevent binding, but the analogous sequence from the K6 promoter, the putative K6 EGF-RE, did. The two sequences share a 9/13-bp identity, which is the best candidate for the EGF-RE consensus site (Fig. 4*C*).

DISCUSSION

To analyze the molecular effects of EGF and TGFα on the expression of the keratin genes that are markers for the pathways of keratinocyte differentiation, we have transfected constructs containing promoters of human keratin genes into primary HEK cultures in the presence and absence of EGF or TGFα. The activation of EGFR causes in keratinocytes a general increase in mRNA and protein synthesis, and transcription of all transfected DNAs is enhanced. But our results indicate that EGF and TGFα specifically increase the transcription of K6 and K16 genes, the keratin genes expressed in hyperproliferating, activated keratinocytes.

The mechanism of induction is unknown, but it functions at the level of transcription initiation; because our constructs produce nearly identical mRNAs, we expect posttranscriptional events to be the same in all transfections. The induction depends on specific response elements, EGF-REs, that are similar in sequence, that can be transferred to another promoter, and that bind a nuclear protein.

Various EGF-REs have been found in the promoters of other regulated genes (37–40). Perusal of the sequences of the EGF-REs characterized in the K6 and K16 genes did not identify any similarity with those elements. Screening of DNA sequence data bases (GenBank and EMBL, February 1993) with 9-bp consensus sequence of the two EGF-REs identified homologs in the coding regions of many mammalian genes, including the EGFR gene, but in the 5′ flanking sequence of only two; the rabbit uteroglobin and rat calbindin genes, neither of which is known to be regulated by EGF.

Whereas K6 and K16 are often associated with hyperproliferation, there are circumstances under which expression of K6 and K16 is dissociated from cell proliferation. For instance, blocking DNA replication *in vitro* with mitomycin D permits continuous synthesis of K6 and K16 in corneal epithelial cells (41), and TGFβ, while blocking proliferation, does not prevent K6 and K16 synthesis in HEKs (42). *In vivo*, synthesis of K6 and K16 can occur without the concomitant expression of a proliferation-specific nuclear antigen recognized by the Ki-67 antibody (43). It has therefore been suggested that K6 and K16 mark an alternative pathway of keratinocyte differentiation. This alternative pathway results in keratinocytes that are migratory and "activated" in the sense that they produce and respond to immunological signals, such as interleukins 1 and 6, and growth factors, such as TGFα, TGFβ, and EGF (2). Thus, K6 and K16 may be considered markers of activated keratinocytes.

The specific replacement of the basal-cell-specific keratins K5 and K14 with keratins K6 and K16 during keratinocyte activation may point to the function of both pairs of keratins: K5 and K14 strongly anchor the basal cells to the underlying basal lamina, whereas K6 and K16 allow migration of keratinocytes in their activated phenotype.

That activation of EGFR specifically induces K6 and K16 offers potentially significant avenues for treatment of epidermolysis bullosa simplex, a severe congenital disorder caused by dominant mutations in the K5 or K14 gene (44, 45). In this disease, epidermal cells that survive into the suprabasal layers and substitute differentiation-specific K1 and K10 for the basal-cell-specific keratins, K5 and K14, can form a functional epidermal barrier. Perhaps specific induction of K6 and K16 may enhance survival of keratinocytes in the basal layer of epidermolysis bullosa patients, thus reducing the severity of this disease.

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