Inflammatory Versus Proliferative Processes in Epidermis

TUMOR NECROSIS FACTOR α INDUCES K6b KERATIN SYNTHESIS THROUGH A TRANSCRIPTIONAL COMPLEX CONTAINING NFκB AND C/EBPβ*

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Epidermal keratinocytes respond to injury by becoming activated, i.e. hyperproliferative, migratory, and proinflammatory. These processes are regulated by growth factors and cytokines. One of the markers of activated keratinocytes is keratin K6. We used a novel organ culture system to show that tumor necrosis factor α (TNFα) induces the expression of K6 protein and mRNA in human skin. Multiple isoforms of K6 are encoded by distinct genes and have distinct patterns of expression. By having shown previously that proliferative signals, such as epidermal growth factor (EGF), induce expression of the cytoskeletal protein keratin K6b, we here demonstrate that the same isoform, K6b, is also induced by TNFα, a proinflammatory cytokine. Specifically, TNFα induces the transcription of the K6b gene promoter. By using co-transfection, specific inhibitors, and antisense oligonucleotides, we have identified NFκB and C/EBPβ as the transcription factors that convey the TNFα signal. Both transcription factors are necessary for the induction of K6b by TNFα and act as a complex, although only C/EBPβ binds the K6b promoter DNA. By using transfection, site-directed mutagenesis, and footprinting, we have mapped the site that responds to TNFα, NFκB, and C/EBPβ. This site is separate from the one responsive to EGF and AP1. Our results show that the proinflammatory (TNFα) and the proliferative (EGF) signals in epidermis separately and independently regulate the expression of the same K6b keratin isoform. Thus, the cytoskeletal responses in epidermal cells can be precisely tuned by separate proliferative and inflammatory signals to fit the nature of the injuries that caused them.

Epidermis is our first line of defense from the environment and must often respond to various types of injury. Keratinocyte is the predominant cell type in the epidermis. When an injury occurs keratinocytes can become hyperproliferative, e.g. in wound healing, or they can become inflammatory, e.g. in contact dermatitis, or both, e.g. in psoriasis (1, 2). These responses are coordinated and orchestrated by growth factors and cyto-
restricted to the suprabasal layers of stratified epithelia (11, 14–16).

This poses an interesting conundrum: if both proliferative and proinflammatory signals induce K6 expression, is the same K6 protein induced by both, or does one of the K6 genes respond to the proliferative and the other to the proinflammatory stimuli? Our previous results have demonstrated that EGF or TGFα, the proliferative signals, induce the expression of keratins K6b and K16 (17, 18). Therefore, focusing on the K6b keratin, we decided to determine whether TNFα, which is a strong proinflammatory but not a proliferative signal in keratinocytes (19), affects its expression.

TNFα is produced by a wide variety of cells in response to infection or injury, primarily macrophages and monocytes but also by epithelial cells including epidermal keratinocytes. A low level of TNFα is present in the upper layers of the healthy epidermis, but its synthesis and release from keratinocytes is greatly augmented in allergic and irritant contact dermatitis, infection, UV irradiation, etc. (20, 21). In these pathological conditions, TNFα activates immune responses through inducing production of proteins such as amphiregulin, TGFα, IL-1α, IL-1 receptor antagonist, EGF receptor, and ICAM1 (22–26). Mice lacking TNFα develop normally but have delayed and prolonged inflammatory responses, confirming the role of TNFα in inflammation (27).

The signaling cascades mediating cellular responses to TNFα have been partly elucidated (28–30). There are two TNFα receptors, but keratinocytes express mainly the 55-kDa receptor, type I (31–33). The most direct TNFα effect involves proteins TRADD and TRAF2 and activates transcription factors NFκB and C/EBPβ. The NFκB family includes the proteins p65, p50, and c-Rel, which both homo- and heterodimerize among themselves (34). These proteins are stored latent in the cytoplasm, bound to the inhibitory protein, IκB. TNFα causes activation of IKKs, kinases that phosphorylate IκB and induce its degradation, which results in activation and nuclear translocation of the NFκB protein (28, 35–37). Knockout of IKKα has severe epidermal phenotype causing incomplete epidermal differentiation (38, 39). On the other hand a knockout of IKKβ is defective in signaling from TNFα to NFκB (40, 41). NFκB proteins can interact with C/EBPβ, AP1, and other transcription factors to regulate gene expression (42, 43). In keratinocytes in vitro overexpression of NFκB inhibits proliferation. In epidermis in vivo NFκB is present in all layers but is nuclear only in the suprabasal ones; this suggests a role for NFκB in epidermal differentiation (44). On the other hand, constitutive activation of NFκB in IκB knockout mice results in normal epidermal development and differentiation but a widespread, lethal dermatitis in the first few days of life (45).

TNFα as well as other extracellular stimuli activate C/EBPβ (46–48). The mechanisms that activate C/EBPβ have not been fully characterized. C/EBPβ, also known as NF-IL6 or LAP, interacts with many other transcription factors, such as the RB protein, the glucocorticoid receptor, Myc and, importantly for our studies, with AP1 and NFκB (42, 49–54). In epidermis the C/EBP proteins are differentially expressed during differentiation (55, 56). Whereas knockout mice lacking C/EBPβ have no cutaneous phenotype (57), overexpression of C/EBPβ in keratinocytes causes growth arrest and induction of early differentiation markers (58).

To determine the roles of TNFα-activated transcription factors in regulating K6b keratin gene expression, we have used the clone containing the promoter of the human K6b keratin gene (59, 60). The promoter contains several sites that bind transcription factors responsive to extracellular stimuli (5, 59, 60). By using transfection experiments, gel shifts, and footprinting, we have mapped the TNFα-responsive element. We determined that both NFκB and C/EBPβ act through the same DNA sequence. Only C/EBPβ binds this DNA directly and NFκB does not. By using specific inhibitors and antisense oligonucleotides, we have shown that both NFκB and C/EBPβ are essential for the regulation by TNFα, and we propose that a complex containing NFκB and C/EBPβ binds the K6b promoter through the C/EBPβ DNA binding domain to convey the TNFα signal. Finally, we physically separated the DNA element responsive to TNFα, NFκB, and C/EBPβ from the element responsive to EGF and AP1, thus showing that the inflammation and hyperproliferation in keratinocytes are distinct and independent processes.

**EXPERIMENTAL PROCEDURES**

**Organ Culture Explants of Normal Human Skin—**Pieces of normal human skin were obtained immediately after surgery. They were cut into pieces approximately 5 mm³ and incubated in keratinocyte basal medium, KBM (keratinocyte-SFM, Life Technologies, Inc.) with or without TNFα (50 ng/ml, Intergen), in a humidified incubator at 37 °C for 24 h. Generally, we use 24-well culture dishes with up to 5 pieces in the same well and enough medium to just cover the explants. The explants were counted in a Coulter Counter (Sartorius) and frozen. Sections, 4–6 μm thick, were obtained with a cryostat (Miles Laboratories), fixed with methanol/acetic for 10 min, incubated with anti-keratin K6 antibody (Progen Biotechnik GMBH) at 4 °C overnight, treated with peroxidase-conjugated anti-mouse IgG secondary antibody (Vecstain ABC-mouse IgG kit from Vector Laboratories), at room temperature for 1 h, incubated with ABC complex (Vector Laboratories) at room temperature for 1 h, and treated with 3,3′-diaminobenzidine-tetrahydrochloride (Dojindo Corp.) and 0.01% H₂O₂ in Tris, pH 7.6, for 2 min. The samples were observed and photographed under the light microscope (Microphot-FXA, Nikon). Additional antibodies used were used from Monosan, Uden Holland; antibodies specific for keratins K5, K8, K10, and K18 were from Progen, Heidelberg, Germany; antibodies specific for keratins K19 and K17 were from Neomarkers, Freemont, CA; and antibodies specific for K14 and for NFκB and C/EBPβ were from Santa Cruz Biotechnology.

**RT-PCR from Explant Tissue—**Explanted skin samples were incubated with or without TNFα for 16 h and harvested, and total RNAs were isolated utilizing RNasey RNA extraction kit from Qiagen (Santa Clarita, CA). Between 1 and 15 μg of RNA were subjected to RT-PCR, using an RT-PCR system from Promega (Madison, W1). By optimizing the number of cycles (30 cycles) and application amount of total RNA (1–9 μg), we achieved linear correlation between the amount of RNA added and the density of bands. We used commercial primers for glyceraldehyde-3-phosphate dehydrogenase (CLONTECH, Palo Alto, CA), and the K6 keratin primers are given in Table I. The PCR products were subjected to agarose-gel electrophoresis, visualized with ethidium bromide (Sigma) with a transilluminator from Ultraviolet Products (Upland, CA), and photographed with a photographing unit from Polaroid (Germany). The densities of bands were quantified by utilizing an image scanner (GT-9000 from Epson, Tokyo, Japan).

**Immunofluorescence of Cultured Keratinocytes—**Human epidermal keratinocytes in the fourth passage were plated on glass coverslips and grown for 24 h in KBM. The cells were then treated with TNFα, washed twice with phosphate-buffered saline, and then fixed and methanol/acetone (1:1) for 5 min. The coverslips were stained with NFκB and C/EBPβ-specific antibodies. As the secondary antibodies we used anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate absorbed with human serum proteins or anti-rabbit immunoglobulin G-fluorescence isothiocyanate conjugate absorbed with human serum proteins (both from Sigma). DNA Constructs—The plasmids containing keratin promoters and the control plasmids pRSVZ have been described previously (17, 18, 60). The plasmids containing the IL-8 promoter and (NFκB)3-CAT were gifts from J. Vilcek (52); and those expressing NFκB proteins were from A. Beg and D. Baltimore (35); those expressing C/EBPβ were from S. Clurman-Kiang (61); IκB and His-NFκB were from S. Ghosh (62, 63), and CHOP was from D. Ron (64).

Additional K6b promoter constructs were prepared by PCR with *Thermus aquaticus* DNA polymerase under conditions suggested by the manufacturer (Perkin-Elmer). All DNA primers, including the phosphorothiate-modified ones used in antisense experiments, were either synthesized on a Amersham Pharmacia Biotech Gene-Plus Synthesizer or provided by the Kaplan Comprehensive Cancer Center Core Facility.
They are listed in Table I. To create the deletions of the K6 promoter we used PCR with K6CAT as a template, a common proximal primer starting just upstream of the ATG translation initiation codon and a series of nested distal oligonucleotides (Table I). To create point mutations in the responsive element, we performed a two-round PCR mutagenesis procedure (65).

To clone the responsive element into a heterologous vector, we amplified the DNA using PCR and cloned it into the enhancer trap TK-CAT vector (Promega). The CAT activity of the resulting plasmid was compared with that of control plasmid TK-CAT (Promega). The CAT activity of the resulting plasmid was normalized for another 24 h or stimulated with TNF $\alpha$.

| K6 footprint short | ATTTCGCCGACTAAAGGAAGCGAAAATGCAATCTCGGTATTTCATAACTTTGTAATAATGC |
| K6 footprint long | ATTTCGCCGACTAAAGGAAGCGAAAATGCAATCTCGGTATTTCATAACTTTGTAATAATGC |

## Table I

### Oligonucleotides used in PCR, electrophoretic mobility shift assays, footprinting, RT-PCR, and antisense experiments

| Oligonucleotide | Sequence |
|-----------------|----------|
| K6Forward       | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| K6Reverse       | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| D268            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| D193            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| D172            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| D138            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| D111            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| I216            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| I193            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| I131            | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| I96             | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| AP1-forward     | CGCTGATGATGCTGACCGGAA |
| AP1-reverse     | TTTGGATCCACCTCGAGGSCATGTGCGATA |
| C/EBP           | TGGAGATTGAGGGGACTTTCCCAGGC |
| NF-κB-forward   | CGCTGATGATGCTGACCGGAA |
| NF-κB-reverse   | CGCTGATGATGCTGACCGGAA |
| K6 footprint short | ATTTCGCCGACTAAAGGAAGCGAAAATGCAATCTCGGTATTTCATAACTTTGTAATAATGC |
| K6 footprint long | ATTTCGCCGACTAAAGGAAGCGAAAATGCAATCTCGGTATTTCATAACTTTGTAATAATGC |

Antisense Strategies—Keratinocytes readily take up offered DNA. Specifically, short oligonucleotides can be introduced into these cells even in the absence of cationic lipid (69). We targeted the antisense oligonucleotides, in phosphorothioate form, to the sequences including and immediately upstream from the initiation codon. The oligonucleotides were stored at −70 °C in water until use. Their sequences are given in Table I. Our approach was first to transfect HeLa cells with a well characterized responding construct, (NFκB-1CAT, adding the antisense oligonucleotides both to the transfected DNA and to the culture medium of transfected cells. The oligonucleotides were added in combination with 5% polyacrylamide gels (29:1 = acrylamide-bisacrylamide). The gels were run for 30 min in 1× TBE buffer and then run for 2–2.5 h at 125 V. The gels were transferred onto filter paper, dried, and exposed to x-ray film (X-Omat, Eastman Kodak Co.) at −70 °C for 24–48 h with screen intensifiers.

### Purification of NFκB and C/EBPβ Proteins

The plasmid expressing GST-tagged C/EBPβ (61) was used to transform BL21(DE3) *E. coli* (U.S. Biochemical Corp.) which was grown in LB with ampicillin to $A_{600}$ of 0.9 and induced with 1 mM isopropyl-β-D-thio-β-galactopyranoside for 3 h. We used the GST-bulk purification kit that includes glutathione-Sepharose 4B and followed the procedures recommended by the manufacturer (Amersham Pharmacia Biotech). We prepared un-tagged protein using thrombin to remove the GST tag, but we found the tagged and the native proteins to have indistinguishable properties in gel shift and footprinting assays. The yield and purity of the proteins were assessed using standard SDS-polyacrylamide gels.
The plasmid expressing His6-NFkB was transfected into the same bacterial host, and its expression was induced the same way. The tagged protein was purified using the Xpress purification system (Invitrogen). We followed the manufacturer’s recommendations for isolation of both the native and the denatured-renatured protein, and we found that the native protein protocol gave significantly higher yields of active protein. The purified proteins were used in gel shift assays under same conditions used for the keratinocyte extracts, described above.

**DNase I Footprinting Method**—The oligonucleotide containing the C/EBPβ binding sequence, 150 ng, was labeled in a kinase reaction using [γ-32P]dATP. Then, 1.5 × 10^6 cpm of the oligonucleotide was used in the primer extension reaction with Klenow DNA polymerase (Roche Molecular Biochemicals) and purified by elution from a 2.5% agarose gel into TE buffer, pH 8, at 4 °C overnight. Two reactions were performed in parallel as follows: A + G Maxam-Gilbert sequencing (using the reagents and protocols from the NEN Life Science Products sequencing kit) (70) and DNase I footprinting. For the footprinting, 25 μl of the binding mix (see the gel shift protocol above), an amount of purified C/EBPβ protein, usually 50 ng, and 50,000 cpm of the probe were incubated at 4 °C. As a control, the C/EBPβ protein is omitted from one of the samples. Then, 50 μl of the solution containing 10 mM MgCl₂ and 5 mM CaCl₂ was added and incubated 1 min on ice. Next, 3 μl of the 1:25 dilution of the DNase I (5 units/ml, Roche Molecular Biochemicals), which we have found optimal for our conditions, was added, and the incubation continued exactly for 1 min on ice. The reaction was stopped by adding 90 μl of solution containing 20 mM EDTA, pH 8.0, 1% SDS, 0.2 mM CaCl₂ and 100 μg/ml of yeast RNA. Next was phenol extraction, followed by ethanol precipitation. The pellet was resuspended in 1.4 μl of 0.5 M urea, 1% Nonidet P-40 and after mixing, 4.6 μl of formamide loading buffer (U. S. Biochemical Corp.) was added. All samples were heated at 90 °C for 5 min, chilled on ice, and loaded onto a 12% sequencing polyacrylamide gel. Electrophoresis was run at 2,000 V for 2 h until the blue dye reached bottom of the gel. The gels were transferred onto filter paper, dried, and exposed to the x-ray film, as described above.

**RESULTS**

**TNFα Activates NFκB and Induces Expression of Keratin K6 Protein in Normal Human Skin**—Whereas healthy interfollicular epidermis does not contain K6 keratin, this protein is present in many inflammatory and hyperproliferative diseases, where it is induced by the growth factors and cytokines that orchestrate the inflammatory responses (14, 59). EGF and TGFα cause keratinocytes to hyperproliferate and can induce the expression of K6b (18), but EGF and TGFα are not inherently proinflammatory. In contrast, TNFα does not cause keratinocytes to proliferate, although it is strongly proinflammatory (1). Therefore, we decided to determine whether TNFα could induce, in the absence of EGF/TGFα, the expression of K6b keratin in human epidermis. Because convenient systems for analysis of the effects of TNFα and other growth factors and cytokines in human skin in vivo have not been described, we developed a new and elegant nearly in vivo system that uses organ culture of human skin samples otherwise discarded during surgery (71–74). We obtained 5-mm diameter biopsies of human skin and placed them in culture medium to which we added TNFα. After 24 h the biopsies were frozen, sectioned, and the presence of K6 determined using specific antibodies. As a control we used a K17 keratin-specific antibody; the expression of K17 is induced by interferon γ but not by TNFα (17, 75). We found that TNFα strongly and specifically induced the expression of K6 in the 24-h period, whereas K17 was not induced (Fig. 1). Without TNFα, the presence of keratin K6 was detected in the perilocular and eccrine epithelia, where K6 is normally seen (Fig. 1). There was no difference in expression of K8, K18, K5, K14, or K10 between the specimens incubated with or without TNFα (not shown).

We note that at the edges of the biopsy after 24 h a weak presence of K6 can be detected even in the absence of TNFα (not shown). This is presumably due to the release of the endogenous IL-1 by the peripheral keratinocytes damaged dur-
TNFα Induces K6 Keratin Expression

Fig. 4. Gel shift assays of NFκB, C/EBPβ, and AP1 proteins using extracts from TNFα treated keratinocytes. Cultured cells were treated with TNFα for the number of minutes, indicated above the lanes, harvested, and protein extracts prepared. These extracts were allowed to bind to the NFκB consensus, a C/EBPβ-containing segment of K6b promoter or the AP1 consensus oligonucleotide (see Table I). The control lanes contained the 40-min time point with an excess of unlabeled oligonucleotide. The NFκB and C/EBPβ binding activities increase in the 1st h, whereas the AP1 binding activity does not.

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The expression of K6 at the edge of the biopsy is most prominent in the first suprabasal layer of keratinocytes. In contrast, K6 is present in all suprabasal layers of the TNFα-treated samples but most prominently in the granular layer, the layer of living cells most proximal to the medium that contains TNFα.

In TNFα-treated keratinocytes, subtle phenotypic changes are observed; cells treated for 24 h with TNFα are flatter, swirled, less tightly packed, and whereas their nuclei are more prominent, the cell-cell boundaries are less distinct (Fig. 2).

We performed semi-quantitative RT-PCR with primers specific for K6 mRNA. Incubation of ex vivo skin samples with TNFα resulted in an approximately 3-fold increase of keratin K6 messenger RNA level (Fig. 3). The linearity of the assay was confirmed by quantification of the RT-PCR bands obtained with different amounts of input RNA.

We next examined the effects of TNFα on the activation of transcription factors in skin. Several transcription factors respond to TNFα and transduce its signal to the nucleus; these include NFκB, AP1, and C/EBPβ (52, 53, 76, 77). Indeed, NFκB, which when activated by TNFα enters the nucleus, was predominantly cytoplasmic in untreated skin samples but exclusively nuclear in the treated ones. On the other hand, C/EBPβ was found in the nuclei of both treated and control skin explants. Both transcription factors behave similarly in culture and in vivo; C/EBPβ is always nuclear, whereas NFκB is cytoplasmic in unstimulated cells and enters the nucleus upon stimulation (not shown).

TNFα greatly increased the NFκB DNA binding activity (Fig. 4). The activity is detectable 20 min after addition of TNFα, peaks at 1 h, and then returns to the basal level in the next hour. This time course parallels closely the one of NFκB nuclearization. Within the K6b promoter sequence we found a cluster of C/EBP sites (see below). By using this cluster as a probe in gel shift assays, we found a rapid activation of a DNA binding activity (Fig. 4). Enhanced DNA binding is observed 5 min after addition of TNFα, peaks at 1 h, and then returns to basal level. These data suggest that addition of TNFα activates both NFκB and C/EBPβ transcription factors in human epidermal keratinocytes. The AP1 consensus binding activity was fairly high even in the absence of TNFα, most likely due to the EGF in the medium, and did not change under the influence of TNFα (Fig. 4).

TNFα Activates the K6b Promoter through NFκB and C/EBPβ Transcription Factors—Because the regulation of keratin gene expression occurs at least partly at the level of transcription, we transfected keratinocytes and HeLa cells with a DNA construct that contains the K6b gene promoter driving the CAT reporter and then incubated the cells in the presence or absence of TNFα. We found that in both cell types TNFα activates the K6b promoter dose-dependently (Fig. 5). The IL-8 gene promoter, used as a positive control, was similarly activated. The effect of TNFα is specific for K6b; promoters of several other keratin genes available were not activated by TNFα. Although we cannot exclude the possibility that TNFα-responsive elements in the other keratin genes lie outside of the available sequences, with the exception of K17, these keratins are not associated with inflamed and proliferative conditions in skin. K17 is induced by interferon γ and not by TNFα (Fig. 1 and Refs. 17 and 75). The results of the transfection experiments therefore confirm those obtained in vivo (Figs. 1 and 3); TNFα specifically and dose-dependently activates the promoter of the K6b keratin gene.

In the promoter of the K6b gene one finds consensus binding sites for NFκB, C/EBPβ, and AP1 transcription factors. We have shown previously that the co-transfection of vectors expressing NFκB and AP1 strongly induces the K6b promoter (60). Here we show that co-transfection of C/EBPβ also strongly induces K6b promoter (Fig. 6). Furthermore, NFκB, C/EBPβ, and AP1 synergize, providing several hundred-fold higher promoter activity when overexpressed together.

To confirm the involvement of NFκB in the induction of keratin K6b promoter activity, we performed transfection assays using a vector expressing IκB, an inhibitor of NFκB. The co-transfection of IκB suppressed the constitutive activity of the K6b promoter, the effect exactly opposite from that of NFκB (see below, Fig. 7).

We used antisense oligonucleotides targeting NFκB, IκB, or C/EBPβ as an alternative approach to examine the involvement of NFκB and C/EBPβ in the induction of K6b. The phos-

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phorothioate oligonucleotides specific for NFκB and IκB mRNAs were designed to bind the initiation codon and the sequences immediately upstream, sites that commonly confer efficient antisense blocking (78). The antisense oligonucleotides were added to the transfected DNA mixture and subsequently to the medium of the transfected cells. Including the antisense DNA into the transfection mixture has the advantage of ensuring that the cells that received the transfected DNA also received the antisense oligonucleotides. A major advantage of our choice of sequences is the fact that NFκB and IκB have opposing effects on the reporter. The suppression of NFκB synthesis, of course, should inhibit the NFκB function, but the suppression of IκB synthesis should enhance the NFκB function because IκB is an inhibitor of NFκB. Therefore, the system is internally controlled. Nonspecific effects of the oligonucleotides, e.g. suppression of transcription commonly observed in most systems, will be equivalent in both transfected cultures, so that the two sequences serve as a control for each other. This approach has been used before in another cell type (78).

When we tested the effects of the antisense oligonucleotides on the regulation of the K6b promoter we found that AS-NFκB not only reduced the constitutive activity of K6CAT but also greatly inhibited its induction by TNFα (Fig. 7). In contrast, AS-IκB allowed a substantial induction by TNFα and abolished the suppression by co-transfected IκB. Thus the AS-IκB oligonucleotide effects are antagonistic to those of AS-NFκB, as expected.

Next we examined the effects of the AS-C/EBPβ oligonucleotide and found that these were similar to the effects of AS-NFκB; AS-C/EBPβ reduced the constitutive activity of the K6b promoter and inhibited the induction by TNFα (Fig. 7). We note that antisense oligonucleotides have significant nonspecific effects; they can be toxic and inhibit the overall effect of TNFα, as evidenced in our control sample, AS-NCoR. In the presence of AS-NCoR (and other unrelated oligonucleotides, not shown) both the constitutive and the TNFα-induced activity of K6b promoter is reduced by approximately half. AS-NCoR oligonucleotide specifically blocked the effects of NCoR (79) in control experiments.3

IκB is a specific inhibitor of the NFκB transcription factor, and CHOP is a specific inhibitor of the C/EBP family proteins (80, 81). We therefore expected IκB to inhibit specifically the

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effects of NFκB and CHOP to inhibit specifically the effects of C/EBPβ. To our surprise, co-transfecting IκB abolished the activity of both NFκB and C/EBPβ, and similarly, co-transfecting CHOP abolished both the C/EBPβ and the NFκB effects (Fig. 8). The effects are specific, because neither IκB nor CHOP abolished the activity of AP1. IκB and CHOP did, however, remove the synergistic effect between AP1 and either NFκB or C/EBPβ. The simplest explanation of these results is that NFκB and C/EBPβ act in concert to activate the K6b promoter. Inhibiting either component abolishes the activation. Furthermore, both transcription factors are responsible for the induction by TNFα because co-transfection of either IκB or CHOP abolished the induction by TNFα (Fig. 8).

Importantly, the AP1 transcription factor acts independently. The AP1-responsive element can be separated physically from the C/EBPβ + NFκB-responsive one (60), and co-transfection of vectors expressing IκB or CHOP does not abolish the AP1 effect (Fig. 8). This means that AP1, which is responsive to EGF, and the C/EBPβ + NFκB, which are responsive to TNFα, independently regulate the K6b keratin gene promoter.

Mapping the TNFα-responsive DNA Element—In the promoter of the K6b keratin gene we found an NFκB site, two AP1 sites, and a cluster of three C/EBP sites (Fig. 9A). To determine whether these sites constitute the TNFα-responsive element, we prepared a series of deletion constructs leaving progressively shorter DNA sequences, and we transfected them into HeLa cells and into keratinocytes. The deletions that remove the NFκB site and one or both of the AP1 sites were fully responsive to TNFα (Fig. 9B). This means that the NFκB and AP1 sites do not play a role in the activation of the K6b promoter by TNFα. In contrast, the deletion D172, which removes the most distal C/EBP site of the cluster, showed a reduced responsiveness to both NFκB and C/EBPβ, whereas D139, which removes all C/EBP sites, was completely nonresponsive. This suggests that the C/EBP sites are essential for TNFα signaling. Virtually identical responses were obtained with co-transfected vector expressing C/EBPβ (Fig. 9B). This means that TNFα and C/EBPβ work through the same DNA elements. We have shown before similar responses of the deletion constructs to NFκB (60), which means that NFκB works through the same DNA elements. We prepared a series of deletion constructs leaving progressively shorter DNA sequences, and we transfected them into HeLa cells and into keratinocytes. The deletions that remove the NFκB site and one or both of the AP1 sites were fully responsive to TNFα (Fig. 9B). This means that the NFκB and AP1 sites do not play a role in the activation of the K6b promoter by TNFα. In contrast, the deletion D172, which removes the most distal C/EBP site of the cluster, showed a reduced responsiveness to both NFκB and C/EBPβ, whereas D139, which removes all C/EBP sites, was completely nonresponsive. This suggests that the C/EBP sites are essential for TNFα signaling. Virtually identical responses were obtained with co-transfected vector expressing C/EBPβ (Fig. 9B). This means that TNFα and C/EBPβ work through the same DNA elements. We have shown before similar responses of the deletion constructs to NFκB (60), which means that NFκB works through the same DNA elements. We prepared a series of deletion constructs leaving progressively shorter DNA sequences, and we transfected them into HeLa cells and into keratinocytes. The deletions that remove the NFκB site and one or both of the AP1 sites were fully responsive to TNFα (Fig. 9B). This means that the NFκB and AP1 sites do not play a role in the activation of the K6b promoter by TNFα. In contrast, the deletion D172, which removes the most distal C/EBP site of the cluster, showed a reduced responsiveness to both NFκB and C/EBPβ, whereas D139, which removes all C/EBP sites, was completely nonresponsive. This suggests that the C/EBP sites are essential for TNFα signaling. Virtually identical responses were obtained with co-transfected vector expressing C/EBPβ (Fig. 9B). This means that TNFα and C/EBPβ work through the same DNA elements. We have shown before similar responses of the deletion constructs to NFκB (60), which means that NFκB works through the same DNA elements.
responsive element of the K6b keratin gene is distinct from the EGF-responsive element.

To determine which transcription factors bind to the responsive element, we prepared NFκB- and C/EBPβ-tagged fusion proteins in E. coli, purified these proteins, and used them in gel shift assays. As the probe we used a synthetic 81-bp DNA oligonucleotide (K6 footprint long, Table I). We found robust binding of C/EBPβ to the probe, but NFκB did not bind (Fig. 10). The DNA binding of C/EBPβ is specific because it could be inhibited by a C/EBP consensus oligonucleotide but not by an NFκB-specific one (Fig. 10). Our current hypothesis is that NFκB acts via protein-protein interaction with C/EBPβ. We tested this hypothesis using bacterially expressed, His-tagged NFκB p65 protein in gel shift assays (63, 82). The purified His-p65 did not bind to the K6b sequence. In the presence of both NFκB and C/EBPβ we expected to see a “supershift” of the C/EBPβ-generated band by p65, indicating a direct interaction of the two proteins bound to DNA, but we were unable to demonstrate it. Under the same conditions p65 bound to its consensus element (not shown).

To identify the exact DNA sequences where C/EBPβ contacts keratin K6b promoter, we performed footprinting analysis. Addition of purified C/EBPβ protein protected the C/EBPβ sites from cleavage by DNase I in a dose-dependent manner (Fig. 11). Importantly, all C/EBPβ sites are protected, which is congruent with the result from mutation analysis. The mutations were designed to affect only one potential C/EBP site, leaving the other two intact. It was therefore of interest to examine whether these mutants are still competent to bind C/EBPβ. Indeed, when we prepared the corresponding oligonucleotides and used them in footprinting experiments, we found that the M1 mutant bound C/EBPβ in the downstream sequences, and M2 bound it in the upstream sequences. The binding to the double mutant was greatly reduced throughout the DNA (Fig. 11B).

In an attempt to correlate the in vitro DNA binding results with the in vivo effects of TNFα, we grew HeLa cultures, starved them for 16 h, and then treated them with TNFα, EGF, or serum. After 40 min extracts were prepared and used in footprinting experiments. This approach is significantly more difficult and less reproducible than the approach using purified proteins because the extracts contain many DNA-binding proteins, which may obscure the specific binding of C/EBPβ. However, we could show an increase of protein binding to the expected sites in the extracts prepared from TNFα-treated cells. The effects of EGF, if any, were much weaker, whereas the addition of serum was without effect (Fig. 11C).

**DISCUSSION**

Cutaneous response to injury results in the release of cytokines and growth factors that are proinflammatory and cause hyperproliferation. Cytokines and growth factors often use overlapping signal transducing pathways, which results in shared effects. Here we show that the proinflammatory cytokine TNFα directly induces K6b keratin expression in normal human skin, describe the mechanism of this induction, and define the TNFα-responsive regulatory element in the K6b gene promoter. The mechanism of TNFα-dependent induction is completely separate and independent from the EGF-dependent induction of K6b expression described previously (18). Thus, the proinflammatory signals induce the expression of the very same keratin, K6b, that the usually concomitant hyperproliferative signals induce.

We demonstrated the effects of TNFα, namely the induction of K6b expression and the activation of NFκB transcription factor, both in cultured keratinocytes and in explants of human skin, a new experimental system designed to emulate in vivo conditions (9, 71–74). We have also shown that TNFα induces K6b at the transcriptional level, and we identified NFκB and C/EBPβ as the responsible transcription factors. Deletions and point mutations that show TNFα, NFκB, and C/EBPβ all act at the same DNA site. The participation of both transcription factors is obligatory, neither C/EBPβ nor NFκB can act alone. This conclusion comes from experiments in which the specific inhibitors of NFκB and C/EBPβ, 1αB or CHOP, respectively, inhibited both transcription factors and from the use of antisense oligonucleotides, which by depleting one of the transcription factor also inhibited the other. Particularly informative are the antisense oligonucleotide studies because in these overexpression of regulatory proteins is avoided.

NFκB and C/EBPβ are known to interact in regulating gene expression; however, usually both transcription factors bind DNA (83, 84). The TNFα-responsive element in the K6b gene element binds exclusively C/EBPβ. The interaction of the two

**Fig. 10.** Gel shift of the TNFα-responsive element with purified NFκB and C/EBPβ proteins. NFκB and C/EBPβ, tagged with His6 and GST, respectively, were expressed in E. coli and column-purified. They were then used in gel shift assays with an 81-bp oligonucleotide containing the responsive element (see Table I). Whereas C/EBPβ binds the element, NFκB does not. Only the C/EBPβ consensus DNA competes for the binding, the NFκB consensus does not.
transcription factors seems to enhance the activity of promoters containing a C/EBP site and to suppress those with NFκB-binding sites (43). Correspondingly, in our case NFκB and C/EBP synergize. One possible mechanism for this regulation invokes a complex containing NFκB and C/EBP, a complex that has a different function from its individual components. Signals that activate only NFκB, but not C/EBP, do not induce K6b gene.4 NFκB plays a role in preventing keratinocyte apoptosis (85), C/EBP in keratinocyte differentiation, and both can be activated by proinflammatory signals. The strict reliance on both transcription factors ensures that only those proinflammatory signals that activate both NFκB and C/EBP, such as TNFα, induce K6b expression.

If NFκB and C/EBP act as a complex, we would expect synergistic induction when both are co-transfected, and indeed that is what we find. But when only one of the components is transfected we still see a very robust induction of the K6b promoter. We suggest that cells contain both the complex and the free partners and that their relative abundance is governed by the affinity coefficient and the mass action rules. We call this mechanism “abetting.” The excess of the co-transfected factor recruits the endogenous partner into the complex and activates the K6b promoter. It may thus deplete the partner from the cell and make it unavailable for other promoters, those responsive to the other partner alone. This depletion is one of the explanations for the “squelching” effects seen in other systems (86, 87). Abetting may apply to other interacting transcription factors and to processes other that transcriptional regulation, e.g. those affecting the signal transduction pathways.

The DNA element responsive to TNFα, NFκB, and C/EBP can be physically separated from the one responsive to EGF and AP1. Thus the inflammatory and the hyperproliferative signals that induce K6b expression are distinct and act independently. It is curious that the upstream consensus AP1 sites do not respond to AP1 and that the DNA sequences that do respond to AP1 have no resemblance to the consensus AP1-binding site. But then, neither does NFκB bind the NFκB-responsive element in the K6b promoter; AP1 may also acts by abetting another transcription factor. This will be a subject for future studies.

We also describe a novel system using explants to analyze gene regulation in human skin. The explants have several advantages over other available systems. They use human

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4 M. Blumenberg, manuscript in preparation.
The material described would be discarded otherwise. They are much easier and cheaper than grafts of human skin on nude mice (88-90). The tissue maintains its architecture and differentiation. Most important, we have demonstrated that the explants respond normally to external stimuli and can be used to study and test the effects of various physical, chemical, or biological agents on skin. The system could be scaled up and used as a model to test pharmaceutical and skin care products, alleviating the need for animal experiments. A major current drawback is the time limit; the experiments must be completed within a few days, before the tissue deteriorates.

In summary, our results show that the inflammatory and the proliferative signals separately regulate the expression of K6β keratin. The inflammatory signals depend on NF-κB and C/EBPβ, using the C/EBP-binding sites in the K6β promoter DNA. The proliferative signals implicate AP1, which interacts with K6β DNA through an unknown mechanism.

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